Natural transformation and DNA uptake in *Campylobacter jejuni* and *Campylobacter coli*

Inaugural-Dissertation

to obtain the academic degree

Doctor rerum naturalium (Dr. rer. nat.)

submitted to the Department of Biology, Chemistry, Pharmacy
of Freie Universität Berlin

by

Julia C. Golz

2022

This work was performed from November 2017 to February 2022 at German Federal Institute for Risk Assessment (BfR) in the National Reference Laboratory for *Campylobacter* and funded by Federal Ministry of Education and Research (BMBF) as a part of the consortium "Preventing and combating *Campylobacter* infections: On track towards a One Health approach" (PAC-CAMPY).

First Reviewer: Dr. Kerstin Stingl

National Reference Laboratory for *Campylobacter* –

German Federal Institute for Risk Assessment (BfR)

Diedersdorfer Weg 1, 12277 Berlin

Second Reviewer: Prof. Dr. Reinhard Kunze

Applied Genetics – Institute for Biology, Freie Universität Berlin

Albrecht-Thear-Weg 6, 14195 Berlin

Date of defense: 10th May 2022

1 Selbständigkeitserklärung

Hierdurch versichere ich, Julia C. Golz, dass ich meine Dissertation selbstständig verfasst und keine anderen als die von mir angegeben Quellen und Hilfsmittel verwendet habe.

_________________________ _________________________

Ort, Datum Julia C. Golz

Table of Contents

1 Selbständigkeitserklärung 3

2 Summary 6

3 Zusammenfassung 8

4 Introduction 10

4.1 The zoonotic disease campylobacteriosis 10

4.2 Prevalence and reservoir of thermophilic *Campylobacter* spp. 10

4.3 Characteristics of the thermophilic Epsilonproteobacteria *Campylobacter* spp. 11

4.3.1 Taxonomy and classification 11

4.3.2 Morphology and culture conditions 11

4.3.3 Typing methods and population structure 12

4.3.4 Survival strategies 13

4.4 Natural Transformation 14

4.4.1 Regulation of natural transformation 14

4.4.2 Process of natural transformation 15

4.4.3 Discrimination and recognition of DNA for uptake 17

4.4.4 Possible reasons for natural transformation 17

4.4.5 Methods for detection of natural transformation 18

4.5 Aim of the study 19

5 Publications 20

5.1 List of publications and own contribution 20

5.2 Publication 1: Curcumin mitigates immune-induced epithelial barrier dysfunction by *Campylobacter jejuni* 22

5.3 Publication 2: Comparison of different technologies for the decipherment of the whole genome sequence of *Campylobacter jejuni* BfR-CA-14430 41

5.4 Publication 3: Whole genome sequencing reveals extended natural transformation in *Campylobacter* impacting diagnostics and the pathogens adaptive potential. 49

5.5 Publication 4: Natural competence and horizontal gene transfer in *Campylobacter*. 61

5.6 Publication 5: "Take it or leave it"-Factors regulating competence development and DNA uptake in *Campylobacter jejuni*. 89

6 Discussion 103

6.1 Choice and characterization of a reference strain for the PAC-CAMPY consortium 103

6.2 Identification of parameters triggering competence development using a single cell assay 104

6.3 Curcumin plays neither a role in competence development nor in DNA uptake in *C. jejuni* 107

6.4 *C. coli* / *C. jejuni* hybrid strains with impact on species differentiation 107
6.5 Conclusions 109
7 References..................... 110
8 Appendix 118
8.1 List of Abbreviations 118
8.2 Supplementary material of own publications 119
8.2.1 Publication 1: Curcumin mitigates immune-induced epithelial barrier dysfunction by *Campylobacter jejuni*. 119
8.2.2 Publication 2: Comparison of different technologies for the decipherment of the whole genome sequence of *Campylobacter jejuni* BfR-CA-14430. 121
8.2.3 Publication 3: Whole genome sequencing reveals extended natural transformation in *Campylobacter* impacting diagnostics and the pathogens adaptive potential. 124
8.2.4 Publication 5: "Take it or leave it"-Factors regulating competence development and DNA uptake in *Campylobacter jejuni*. 157
8.3 Danksagung..................... 163

2 Summary

Thermophilic *Campylobacter* spp. are zoonotic bacteria leading to the most often reported gastroenteritis in the European Union. *Campylobacter jejuni* and *Campylobacter coli* are the major species relevant for human campylobacteriosis, which manifests with watery or bloody diarrhea, abdominal cramps, fever and in few cases leads to autoimmune sequelae, such as reactive arthritis, irritable bowel disease and Guillain-Barré syndrome. Even though the pathogens are fastidious *in vitro* and only grow at reduced oxygen levels, they are very successful in colonizing different warm-blooded hosts and in quickly spreading within a chicken flock.

This PhD theses was focused on genomic diversity of *C. jejuni* and *C. coli* and the underlying mechanisms, in particular, natural transformation, which is the capacity to take up free DNA from the environment and adapt to changing conditions.

We identified strains with ambiguous results in species differentiation by real-time PCR targeting genes, usually distinct for *C. coli* and *C. jejuni*. Using next generation sequencing and k-mer analysis the strains were identified as *C. coli*, harboring a substantial amount of recombined *C. jejuni* sequences, leading to gene variants. Interestingly, these so-called hybrid strains shared a common set of genes with *C. jejuni* sequence introgression, which may have a potential role in stress defense. Since the hybrid strains were preferentially isolated from egg shells, which is a dry and harmful environment for thermophilic *Campylobacter* spp., this may hint at selection for strains which survived harsh environmental conditions. It also demonstrated that *Campylobacter* is able to undergo extensive genetic exchange.

The question arose, under which conditions the pathogen acquires genetic material by natural transformation, leading to the introduction of such an amount of new genetic material. Factors regulating natural transformation and competence development are only poorly understood in *C. jejuni*. Therefore, we developed a single cell-based uptake assay to monitor competence development. As a fresh field isolate we used *C. jejuni* strain BfR-CA-14430 which had recently been isolated from chicken meat and belongs to clonal complex 21 (ST44), which is also commonly found in human isolates. For better comparability this strain was distributed for common use in the PAC-CAMPY consortium. Since no sequence data was available we sequenced this strain using short-read Illumina and single molecule real-time (SMRT) long-read sequence analysis. BfR-CA-14430 had a genome size of 1.6 Mb and harbored a 41 kb plasmid. For visualization of DNA uptake, we covalently labelled *C. jejuni* genomic DNA with a fluorophore and added this labelled DNA to *C. jejuni* cells under different growth conditions. The results obtained by the single cell assay correlated well with the results obtained by a classical assay approach based on the transformation and subsequent selection for a

resistance marker. This showed that the assay is a powerful tool to analyze competence development in *C. jejuni*. Increase from pH 6.5 to 7.5 resulted in a higher fraction of competent cells. Below pH 5 neither competence development nor DNA uptake was observed. Furthermore, aerobic conditions abolished competence development but not DNA uptake in already competent *C. jejuni*. These findings suggested a strong regulation of competence development in *C. jejuni*. We further showed that competence development in *C. jejuni* did not depend on growth temperatures or carbon dioxide concentrations. Since *C. jejuni* often resides in the slightly alkaline intestine of poultry, the results implicate extensive genetic exchange in the host. This might be beneficial to switch hosts and/or to survive in the environment.

In a cooperation study, the single cell assay was used to check the effect of an immune-stimulative substance, curcumin, which was suggested as a potential treatment option for campylobacteriosis, on natural transformation. The DNA uptake assay did not identify either stimulating or inhibiting effects of curcumin on natural transformation in *C. jejuni*, which corroborates its application during campylobacteriosis without triggering the adaptive potential of the pathogen.

In conclusion, extensive genetic exchange occurs in *Campylobacter*, potentially enlarging the adaptive potential of the food-borne pathogen. This might support the pathogen as successful colonizer and survivor. Besides, high genetic plasticity may hinder correct diagnostics and has to be taken into account for continuous monitoring of *Campylobacter* variants. It remains important to study natural transformation in detail in order to reduce the adaptability of thermophilic *Campylobacter* spp. in the future.

3 Zusammenfassung

Thermophile *Campylobacter* spp. sind zoonotische Bakterien, die zu der am häufigsten gemeldeten Gastroenteritis in der Europäischen Union führen. *Campylobacter jejuni* und *Campylobacter coli* sind die Spezies, die am häufigsten eine Campylobacteriose beim Menschen verursachen. Symptome sind wässriger oder blutiger Durchfall, abdominale Krämpfe und Fieber. In seltenen Fällen kommt es zu Autoimmunreaktionen und somit zu Langzeiterkrankungen wie reaktiver Arthritis, chronisch entzündlichen Darmerkrankungen oder dem Guillain-Barré Syndrom. Wenngleich die Pathogene *in vitro* anspruchsvoll sind und nur unter reduzierten Sauerstoffgehalten wachsen können, sind sie sehr erfolgreich in der Kolonisierung von verschiedenen warmblütigen Wirten sowie in der schnellen Verbreitung innerhalb einer Hühnerherde.

In dieser Doktorarbeit geht es um die genomische Diversität von *C. jejuni* und *C. coli* und die zugrundeliegenden Mechanismen, insbesondere die natürliche Transformation, welche die Fähigkeit beschreibt freie DNA aus der Umgebung aufzunehmen und eine Möglichkeit der Anpassung an sich ändernde Umweltbedingungen bietet.

Wir identifizierten Stämme mit uneindeutigen Ergebnissen in der Speziesdifferenzierung mittels Real-Time PCR. Diese basierte auf der Detektion von Zielgenen, die normalerweise distinkt für *C. coli* und *C. jejuni* sind. Mittels Sequenzierung und K-mer Analyse stellte sich heraus, dass es sich bei den Stämmen um *C. coli* handelte, welche einen erheblichen Anteil von *C. jejuni* Sequenzen enthielten, was zu genetischen Varianten führte. Interessanterweise teilten diese sogenannten Hybridstämme ein gemeinsames Set an Genen mit *C. jejuni* Sequenzeintrag, die möglicherweise eine potenzielle Rolle in der Stressantwort haben. Die Tatsache, dass die Hybridstämme präferenziell von Eierschalen isoliert wurden, welche eine trockene und schädliche Umgebung für thermophile *Campylobacter* spp. darstellen, könnte dies auf eine Selektion von Stämmen hindeuten, die fähig sind harsche Umweltbedingungen zu überleben. Es zeigt außerdem, dass *Campylobacter* zu einem erheblichen genetischen Austausch fähig ist.

Es stellte sich die Frage, unter welchen Bedingungen das Pathogen genetisches Material durch natürliche Transformation akquiriert, was zur Integration dieser Mengen an genetischem Material führte. Faktoren, die natürliche Transformation und Kompetenzentwicklung regulieren, sind in *C. jejuni* nur unzureichend bekannt. Deshalb entwickelten wir einen Einzelzell-basierten Aufnahme-Assay, um die Kompetenzentwicklung zu untersuchen. Als neues Feldisolat verwendeten wir den *C. jejuni* Stamm BfR-CA-14430, welcher kürzlich von Hühnerfleisch isoliert worden war und zum klonalen Komplex 21 (ST44) gehörte, welcher ebenfalls häufig in Humanisolaten gefunden wird. Für eine bessere Vergleichbarkeit, wurde

dieser Stamm im PAC-CAMPY Konsortium verteilt und gemeinsam verwendet. Da keine Sequenzdaten dieses Stammes vorlagen, sequenzierten wir diesen mit *short-read* Illumina und Einzelmolekül Echtzeit *long-read* Sequenzierung.

BfR-CA-14430 hatte eine Genomgröße von 1,6 Mb und trug ein 41 kb großes Plasmid. Zur Visualisierung der DNA-Aufnahme, markierten wir genomische *C. jejuni* DNA kovalent mit einem Fluorophor und gaben diese markierte DNA zu *C. jejuni* Zellen, die unter verschiedenen Bedingungen gewachsen waren. Die Ergebnisse, die mit dem Einzelzell-Assay beobachtet wurden, korrelierten gut mit jenen, die mittels klassischem Assay beobachtet wurden, der auf der Transformation und anschließenden Selektion mit einem Resistenzmarker basierte. Dies zeigte, dass der Assay sehr gut geeignet ist, um Kompetenzentwicklung in *C. jejuni* zu analysieren. Eine Erhöhung des pH-Werts von pH 6,5 auf 7,5 führte zu einer Erhöhung des Anteils an kompetenten Zellen. Unter pH 5 wurden weder Kompetenzentwicklung noch DNA-Aufnahme beobachtet. Aerobe Bedingungen verhinderten Kompetenzentwicklung jedoch nicht die DNA-Aufnahme in bereits kompetenten *C. jejuni*. Dies spricht für eine starke Regulierung der Kompetenzentwicklung in *C. jejuni*. Darüber hinaus zeigten wir, dass Kompetenzentwicklung in *C. jejuni* nicht von der Wachstumstemperatur oder von Kohlendioxidkonzentrationen abhing. Da *C. jejuni* häufig im alkalinen Darm von Geflügel vorkommt, implizieren die Ergebnisse, dass erblicher genetischer Austausch während des Aufenthalts im Wirt stattfindet. Das könnte Vorteile im Falle eines Wirtswechsels haben oder um in der Umwelt zu überleben.

Im Rahmen einer Kooperation, wurde der Einzelzell-Assay verwendet, um den Effekt der immunstimulierenden Substanz Curcumin, welche als potenzielle Behandlungsoption für eine Campylobacteriose vorgeschlagen wurden, auf die natürliche Transformation zu untersuchen. Es wurden keine stimulierenden oder inhibierenden Effekte von Curcumin auf die natürliche Transformation von *C. jejuni* mittels Aufnahme-Assay beobachtet. Dies unterstützt die Anwendung während einer Campylobacteriose, ohne dass das adaptive Potenzial dieser Pathogene erhöht wird.

Zusammenfassend lässt sich sagen, dass erheblicher genetischer Austausch in *Campylobacter* stattfindet. Dies erhöht möglicherweise das adaptive Potenzial dieses lebensmittel-assoziierten Keims und könnte das Pathogen in der erfolgreichen Kolonisierung und im Überleben unterstützen. Außerdem kann genetische Plastizität die korrekte Diagnostik behindern und muss berücksichtigt werden, um kontinuierlich *Campylobacter*-Varianten erkennen zu können. Es bleibt wichtig, natürliche Transformation gründlicher zu untersuchen, um die Adaptationsfähigkeit von thermophilen *Campylobacter* spp. in der Zukunft zu reduzieren.

4 Introduction

4.1 The zoonotic disease campylobacteriosis

Campylobacteriosis is a zoonotic disease, which is caused by thermophilic *Campylobacter* spp.. In humans this leads to a gastroenteritis with symptoms like watery and/or bloody diarrhea, abdominal pain, fever and nausea. In most cases this disease is self-limiting, but in some cases, especially for immune-compromised people the illness can have a severe course. Furthermore, severe long-term complications can occur, including degenerative autoimmune disorders, such as Guillain-Barré syndrome, irritable bowel disease, or reactive arthritis (Wijdicks and Klein 2017; Facciolà et al. 2017). A Swedish cohort study suggested that the risk for Guillain-Barré syndrome in the two months after campylobacteriosis is 100-fold higher than in the general population in which the incidence was 0.3 per 100,000 per two months (McCarthy and Giesecke 2001). An antibody cross-reaction between a lipopolysaccharide structure of *Campylobacter* and a ganglioside structure of the peripheral nerves is responsible for the Guillain-Barré syndrome (Yuki et al. 1993). Lipopolysaccharide structures vary among strains, therefore the specific *Campylobacter* surface structure determines if the cross-reaction of antibodies occur. It has been shown that *Campylobacter* serotype Penner 19 was associated with Guillain-Barré syndrome. For reactive arthritis after campylobacteriosis incidences of 4.3 per 100,000 per year have been reported (Hannu et al. 2002; Pope et al. 2007). That means 1 - 5% of campylobacteriosis patients suffer from the long-term complication reactive arthritis. In rare cases of *Campylobacter* infections and sequela mortality has been reported (Ternhag et al. 2005).

Since 2005 campylobacteriosis is the most frequently reported gastrointestinal infection with 220,682 reported cases in the European Union in 2019 (EFSA 2021a). Interestingly, *Campylobacter* infections show a seasonality peak in summer months. It is tempting to speculate that handling of chicken meat during barbeque could be a reason for that. Additionally, there is a peak in winter suggesting infections during Christmas and New Year's Eve (Rosner et al. 2021). The majority of confirmed cases in the European Union was caused by *Campylobacter jejuni* followed by *Campylobacter coli*, but also *Campylobacter lari*, *Campylobacter fetus* and *Campylobacter upsaliensis* have been reported (EFSA 2019).

4.2 Prevalence and reservoir of thermophilic *Campylobacter* spp.

Campylobacter is wildly distributed and resides in many animals like poultry, wild birds, pigs, cattle, sheep as well as other animals and has been isolated from different environments like water or sand (Waldenström et al. 2010; Mughini Gras et al. 2012).

The main transmission route of *Campylobacter* is via contaminated food. Mostly this is due to cross-contamination from poultry and other raw meat to ready-to-eat products or consumption

of undercooked poultry or raw milk (Rosner et al. 2017; Wilson et al. 2008). *C. jejuni* infecting humans mostly originated from chickens, whereas *C. coli* mostly originated from chickens or pigs (Rosner et al. 2017). Additionally, campylobacteriosis can also be acquired by contaminated water or through direct contact with animals, including farm as well as companion animals (Igwaran and Okoh 2019).

4.3 Characteristics of the thermophilic Epsilonproteobacteria *Campylobacter* spp.

4.3.1 Taxonomy and classification

The genus *Campylobacter* belongs to the family Campylobacteraceae, which also includes *Arcobacter*, and *Sulfurospirillum* (Garrity et al. 2005; Euzéby 1997; Parte et al. 2020). Campylobacteraceae belong to the order Campylobacterales, which additionally comprises of the families Helicobacteraceae, including the human pathogen *Helicobacter pylori*, and Nautiliaceae. They all belong to the class of Epsilonproteobacteria and the phylum Proteobacteria in the domain Bacteria. There are currently 32 identified species in the genus *Campylobacter* including 9 subspecies (Costa and Iraola 2019). Since the first description of *Campylobacter* in 1886 by Theodor Escherich there has been some reclassification (Debruyne et al. 2008; Vandamme and On 2001). For example, *Vibrio fetus* was renamed to *C. fetus*, or *Campylobacter pylori* was changed to the new genus *Helicobacter* (Sebald and Veron 1963; Goodwin et al. 1989).

4.3.2 Morphology and culture conditions

Campylobacter are gram-negative bacteria mostly with a length of 0.5 - 5 µm and a width of 0.2 - 0.8 µm (Debruyne and Gevers 2008; Garrity et al. 2005). The word *Campylobacter* originates from the Greek words “campylo” which means “curved” and “bacter” which is translated as “rod”. The name was chosen to describe the typical spirally curved rods. Most of the species show corkscrew-like motility due to the flagella at one or both poles. Flagella are post-translationally modified. Recently a mutant in the gene cj1388 was generated showing a “cell-train” morphotype meaning that several bacteria stuck together by their flagella and moved like a “train” (Reuter et al. 2015). *Campylobacter* are non-spore forming but under unfavorable conditions e.g. during starvation the spiral-shaped *C. jejuni* can change into a coccoid form which is thought to be a survival strategy (Frirdich et al. 2019).

C. jejuni, C. coli, C. lari and *C. upsaliensis* are considered as thermophilic bacteria but the term thermotolerant is more precise since they grow at temperatures of up to around 44°C (Corry et al. 2003). Commonly they are cultured at 37°C or 42°C and are not able to grow below 30°C (Corry et al. 2003; Kim et al. 2020). Since the bacteria are microaerophile, reduced oxygen levels compared to aerobic conditions are necessary for proper growth. Routinely

Campylobacter isolates are cultured in an atmosphere consisting of 5 % oxygen, 10 % carbon dioxide, and 85 % nitrogen on Columbia blood agar plates.

4.3.3 **Typing methods and population structure**

To determine the relativeness of strains and to gain knowledge about the population structure of the species it is important to classify isolates. In former times classification of *Campylobacter* was performed for example by pulse-field gel electrophoresis, fingerprinting like randomly amplified polymorphic DNA (RAPD), serotyping, or *flaA* typing (Yan et al. 1991; Mazurier et al. 1992; Penner et al. 1983; Meinersmann et al. 1997). Since 2001 classification by multilocus sequence typing (MLST) became more and more popular. This method was developed for the differentiation of *C. jejuni* and *C. coli* (Dingle et al. 2001). In that study, 194 C*. jejuni* strains were characterized based on the 7 housekeeping genes *aspA*, *glnA*, *gltA*, *glyA*, *pgm*, *uncA*, *tkt*. Out of this, 155 sequence types were observed and attributed to 62 clonal complexes, showing the diverse population structure of *C. jejuni*. Today there are 11,615 sequence types and 45 clonal complexes defined (https://pubmlst.org, accession on 26.12.2021). In contrast, *C. coli* can be divided into three clades which have separate niches. Clade I is mostly found in the agricultural niche, whereas clade II and clade III seem to reside in the environment (Sheppard et al. 2013; Skarp-de Haan et al. 2014). Even though *C. jejuni* and *C. coli* only share ~85% at the nucleotide sequence level, they are both characterized by the same MLST scheme, which has the advantage that their relativeness can be evaluated (Sheppard et al. 2013). To increase the discriminatory power of the MLST scheme it was extended for the characterization of the short variable region of the flagella genes *flaA* and *flaB* as well as the gene for the outer membrane pore PorA (Dingle et al. 2008). Since nowadays whole genome sequencing is wildly available, core genome MLST (cgMLST) was established to further enlarge the discriminatory power. This allows for better resolution of relativeness and therefore of the population structure. This has not only advantages in understanding the population dynamics and relativeness, but also to elucidate *Campylobacter* outbreak scenarios, answer epidemiological questions, and trance transmission routes of infections (Epping et al. 2021). There are different cgMLST and whole genome MLST (wgMLST) methods published. They are based on different reference datasets and include different loci for discrimination. A commonly used cgMLST scheme was suggested by Cody and colleagues (Cody et al. 2017). This scheme compares 1,343 loci resulting in a much higher resolution than MLST. Today, there are 35,383 profiles defined in this scheme (https://pubmlst.org, accession on 26.12.2021). Another typing method for characterization is the cgMLST INNUENDO scheme. It is based on 678 loci which were present in at least 99.9 % of 6.526 *C. jejuni* genomes initially uploaded to the European Nucleotide Archive or NCBI Sequence Read Archive passing the INNUca v3.1 pipeline as well as 835 additionally published datasets (Rossi et al. 2018). A wgMLST schema from INNUENDO platform includes 2.795 loci (Llarena et al. 2018). This

scheme uses three different levels of strain nomenclatures developed for different resolution requirements concerning outbreak analysis and surveillance. A proprietary scheme by Ridom GmbH uses 637 loci from the software SeqSphere+ (Nennig et al. 2020) (https://cgmlst.org). For overall comparability it is necessary to harmonize the different typing schemes, which is still pending.

A faster method than whole genome sequencing and consequently wgMLST or cgMLST is used to differentiate the most human cases related *Campylobacter* species *C. jejuni*, *C. coli* and *C. lari*. This is important for surveillance of *Campylobacter* in the food chain. This faster method is the qPCR-based detection of *mapA*, *ceuE*, and *gyrA* and often performed by routine laboratories (Best et al. 2003; Mayr et al. 2010). For this species differentiation by qPCR primers were designed based on known gene variants in a way that they theoretically can only anneal in *mapA* of *C. jejuni*, in *ceuE* of *C. coli* and *gyrA* of *C. lari*. Therefore, a positive qPCR signal in the appropriate gene is thought to indicate the species.

4.3.4 **Survival strategies**

Campylobacter is a fastidious organism standing in the need of reduced oxygen levels and cannot grow below 30°C (Kim et al. 2020). It has been shown that *Campylobacter* loses its cultivability after incubation at 4°C (Baffone et al. 2006). Nevertheless, *Campylobacter* is very successful in colonizing farm animals and spreading in a flock (Natsos et al. 2020; Rawson et al. 2020). This hints to powerful survival strategies of the pathogen. In addition to a coccoid form to endure unfavorable conditions, *Campylobacter* can undergo a so called viable but non-culturable (VBNC) status. It has been shown that different types of environmental stress are able to induce the VBNC status. These include starvation and cold-stress (Magajna and Schraft 2015; Chaisowwong et al. 2012), osmotic stress (Lv et al. 2019) and probably aerobic stress (Oh et al. 2015). Since the VBNC status is thought to be a persistence status to survive harsh environmental conditions it is highly likely that *Campylobacter* can resuscitate from VBNC status under favorable conditions. So far, the exact parameters for resuscitation are not known. Wulsten and colleagues suggested that aerobic stress might play a role. Therefore, they used raw milk which was tested to be free from *Campylobacter*. Then they spiked raw milk with *Campylobacter* to an initial concentration of 6 log_{10} cells/ml milk. The suspension was incubated until *Campylobacter* was non-cultivable according to ISO 10272-2:2017 (Wulsten et al. 2020; ISO_10272-2:2017 2019). In an atmosphere with reduced oxygen levels and elevated hydrogen levels it was possible to re-cultivate *Campylobacter* on agar plates. This could be a hint that the absence or at least a drastic reduction of aerobic stress plays a role in resuscitation from VBNC status.

Another more long-term strategy to outlast and survive unfavorable conditions might be the acquisition of suitable genes or gene variants to overcome and cope with the stress.

Campylobacter is known to have a high adaptive potential because of its high genetic diversity (Sheppard et al. 2011; Parker et al. 2006). Genetic exchange is very frequent and enlarges the diversity (Sheppard et al. 2008; Wilson et al. 2009). The high adaptive potential taken together with the ability to undergo the VBNC status could be the reason for the long and widespread persistence of this fastidious pathogen.

4.4 Natural Transformation

4.4.1 **Regulation of natural transformation**

Natural transformation describes the uptake of free DNA from the environment. Depending on homology DNA will be incorporated into the genome by recombination. This has first been observed by Frederick Griffith in 1928 (Griffith 1928). He investigated the exchange of information between virulent and non-virulent *Streptococcus pneumoniae*. In the 1940th Avery and colleagues found out that the transformation agent was DNA (Avery et al. 1944). DNA can be acquired from dead cells or actively secreted from living cells (Hamilton et al. 2005). Additionally, it has been observed that bacteria, for example *S. pneumoniae* can produce a bacteriocin killing neighboring organisms, resulting in the release of DNA due to cell lysis which was called neighbor predation (Wholey et al. 2016). The acquisition of new genetic material offers the chance for a cell to obtain new genes or gene variants. These could be beneficial for survival, for example by acquisition of antibiotic resistances or virulence factors (Hull et al. 2021). But extensive genetic exchange can be harmful for genome integrity (Sheppard et al. 2008). Therefore, natural transformation, even though it is widespread among bacteria, is regulated in various bacteria (Dubnau and Blokesch 2019). A possible strategy for the species to minimize harmful aspects of genetic exchange is, that only a subpopulation becomes competent (Hamoen et al. 2003). One example for this is *Bacillus subtilis* (Albano et al. 1987; Hamoen et al. 2003; Gamba et al. 2015). In *B. subtilis* competence development is regulated by the transcription factor ComK leading to competence in stationary phase. Even under optimal conditions for competence development only a minority of 10-20% expresses ComK and therefore is competent. So, based on stochastic events in every cell it will be determined if a cell develops competence. In most bacteria competence is regulated by internal and external factors. In *S. pneumoniae* competence develops in exponential phase of most of the cells and is inhibited in stationary phase (Claverys et al. 2006). *Haemophilus influenzae* is competent in stationary phase and competence can also be triggered by transfer from rich medium to nutrient depletion (Johnston et al. 2014; Herriott et al. 1970). This in fact mirrors the change of growth condition from exponential phase, where nutrients are available and unrestricted growth is possible to stationary phase where nutrients become restricted. In *Vibrio cholerae* competence development was induced for example by the presence of chitin (Matthey and Blokesch 2016; Meibom et al. 2005). In other organisms, competence development is thought to be constitutive, like in *Neisseria gonorrhoeae* (Sparling 1966;

Biswas et al. 1977; Obergfell and Seifert 2016). In *Helicobacter pylori*, which is closely related to *Campylobacter*, it has been shown that above pH 6.5 a window for competence development opens up. In that window competence development increased with rising pH and was further triggered by oxidative stress (Krüger et al. 2016).

There is only little known about the factors triggering competence development in *Campylobacter*. Nevertheless, it was suggested that natural transformation occurred best under optimal growth conditions (Vegge et al. 2012). But it was also shown that a higher pH than optimal for growth still supported natural transformation. However, the assay by Vegge and colleagues did not discriminate between competence development, meaning the expression and assembly of the DNA uptake apparatus and the DNA uptake process. So, it is possible that already assembled DNA uptake complexes were still able to take up free DNA from the environment but that no additional complexes could be built under the tested conditions. This could mean that no competence developed under the tested conditions but consequent steps of natural transformation still occurred. Wilson and colleagues suggested that the CO_2 concentration played a role in competence development but pH effects cannot be ruled out since higher CO_2 levels resulted in a lower pH (Wilson et al. 2003).

4.4.2 **Process of natural transformation**

As a first step of natural transformation free DNA from the environment is bound by the cell surface (Dubnau and Blokesch 2019). In gram-positive bacteria DNA is taken up over the cell wall into the cytoplasm and reaches a DNase-resistant state. In gram-negative bacteria DNA is taken up over the outer membrane into the periplasm (Johnston et al. 2014). Most bacteria harbor a type II secretion/type IV pili system for uptake of the macromolecule DNA. One exception is *H. pylori* which uses a type IV secretion system (Hofreuter et al. 2001). Once in the periplasm, a single strand of DNA is translocated over the inner membrane into the cytoplasm. In most bacteria this is achieved by ComEC which serves as a membrane channel. Once in the cytoplasm, depending on homology chromosomal integration of the DNA and subsequent expression might occur.

There are different mechanisms known how DNA is bound and transported into the cell. For *B. subtilis*, *V. cholera* and *N. gonorrhea* it has been shown that DNA uptake occurs via a type IV pilus (Hahn et al. 2021; Ellison et al. 2018; Gangel et al. 2014). The exact mechanisms and structures essential for uptake vary. In *V. cholerae* it has been shown that an essential step in DNA uptake is the binding of DNA by the tip of the pili (Ellison et al. 2018). By pilus retraction DNA is delivered to the cell surface which is associated with DNA uptake over the membrane. In *N. gonorrhoeae* not the full-length pilus consisting of the major pilin subunit PilE is necessary for uptake (Obergfell and Seifert 2016). This indicates that competence could occur during

antigenic variation and hints that the way of DNA binding and pilus retraction is different from *V. cholerae*.

Generally, genes involved in DNA uptake of *Campylobacter* are only poorly understood. Nevertheless, using random transposon mutagenesis Wiesner and colleagues identified 11 genes with a potential role in DNA uptake and transformation (Wiesner et al. 2003). These include cj1028c (*ctsW*), cj1076 (*proC*), cj1077 (*ctsT*), cj1343c (*ctsG*), cj1352 (*ceuB*), cj1470c (*ctsF*), cj1471c (*ctsE*), cj1473c (*ctsP*), cj1474c (*ctsD*), cj1475c (*ctsR*) and the 3' region of *ansA*. The nomenclature "cts" is an acronym of "*Campylobacter* transformation system". Four of these genes have homologies or are partly homologous to genes with known role in type II secretion systems. These include *ctsD*, *ctsE*, *ctsF*, *ctsG*. There is a weak homology between CtsD and PilQ (e value = 0.038). In *N. gonorrhoeae* PilQ is thought to be the outer membrane pore which let the macromolecule DNA pass through the outer membrane into the periplasm (Drake and Koomey 1995). CtsE and CtsP are located in an operon putatively encoding nucleoside triphosphatases or binding proteins of nucleoside triphosphate (Beauchamp et al. 2015). CtsE which was shown to be soluble and cytosolic has homology to ComGA of *B. subtilis* and pilT of *N. gonorrhoeae* (Wiesner et al. 2003). CtsP is associated with the membrane. CtsF has homology to PilG of *N. gonorrhoeae* which has been shown to play a role in transformation. At its N-terminus CtsG has some similarity to type II system G orthologues. These proteins show similarity to type IV pili subunits as well as pseudopilins and might play a role in the secretion apparatus. Furthermore, CtsX which is an integral membrane protein was found to play a role in transformation (Beauchamp et al. 2015).

In *N. gonorrhoeae* ComE is essential for DNA uptake. It generates the force for pulling DNA over the membrane (Hepp and Maier 2016). The speed of DNA uptake is based on ComE concentration. ComE is also conserved in *V. cholerae* where it is also located in the periplasm, binds DNA in an unspecific manner and supports DNA uptake (Seitz and Blokesch 2013). The gene Cj0011c from *Campylobacter* shows some homology to *comE*. ComE binds dsDNA as well as single-stranded DNA (ssDNA) in a nonspecific manner and is putatively exported to the periplasm as signal sequence suggests (Jeon and Zhang 2007). Also immunoblotting showed mainly a periplasmic localization. Knock out of the one existing copy of Cj0011c led to a 10 to 50-fold reduced transformation activity. In contrast to *B. subtilis*, *C. jejuni* only has one copy of the *comE* homolog Cj0011c. In *C. coli* and *C. lari comE* is absent. This could also hint that the role of ComE in transformation of *C. jejuni* is not essential as in *B. subtilis*. Like all known competent bacteria *Campylobacter* harbors a *comEC* homolog, which is Cj1211 and was shown to be essential for natural transformation but not for binding and DNA uptake (Jeon et al. 2008). ComEC is known as the inner membrane channel which translocates ssDNA over the inner membrane into the cytoplasm (Dubnau and Blokesch 2019).

4.4.3 **Discrimination and recognition of DNA for uptake**

Since unrestricted DNA uptake and recombination could have a negative effect on genome integrity competence development is often regulated (Claverys et al. 2006; Johnston et al. 2014; Herriott et al. 1970; Meibom et al. 2005; Krüger et al. 2016). Additionally, there is a selection of DNA for uptake in many bacteria. For example, *N. gonorrhoeae* uses an uptake sequence with the motive GCCGTCTGAA (Goodman and Scocca 1988; Elkins et al. 1991). This means that DNA containing this sequence is recognized by *Neisseria* for proper uptake, whereas binding of DNA to PilQ occurs in an unspecific manner (Assalkhou et al. 2007). Like *Neisseria*, *H. influenzae* selects DNA for uptake using the uptake sequence AAGTGCGGT and two T-rich tracts but only the four bases GCGG are essential for uptake (Danner et al. 1980; Mell et al. 2012). Early on it was observed that *Campylobacter* discriminates DNA for uptake as Wang and Taylor showed that DNA from *E. coli* was only a poor substrate for natural transformation, whereas DNA from siblings was readily taken up (Wang and Taylor 1990). It has long been an enigma how *Campylobacter* recognizes DNA for uptake. This has long been a problem for genetic manipulation of the pathogen since cloning is often performed in *E. coli*. Beauchamp and colleagues found out that in *C. jejuni* DNA discrimination and recognition for uptake depends on a methylation motive rather than an uptake sequence (Beauchamp et al. 2017). Only DNA which is methylated at the N6 residue of adenine in the RAATTY motif will be recognized for uptake. This motive has already been identified by Zautner and colleagues to be the only methylated motive shared by *C. coli* strain BfR-CA-09557 and in *C. jejuni* strains, like NCTC11168 or 81-176 (Zautner et al. 2015). The methylase which methylates *Campylobacter* DNA is CtsM (Beauchamp et al. 2017). Since the commercially available *EcoR*I-methylase recognizes GAATTC sites and methylates the N6 residue of adenine DNA can be mobilized for uptake by *in vitro* methylation. This largely simplifies genetic manipulation in *Campylobacter*. Nevertheless, there are bacteria which do not seem to discriminate DNA depending on an uptake sequence and readily take up free DNA from the environment. One example is *H. pylori*, for which a DNA uptake selection mechanism could not be identified so far (Zhang and Blaser 2012; Krüger et al. 2016).

4.4.4 **Possible reasons for natural transformation**

There have been many reasons discussed why natural transformation occurs. One possible reason for the uptake of exogenous DNA is to use it as a nutrient source (Finkel and Kolter 2001). Nucleotides can be recycled, used for DNA replication or can be used as a sole carbon source for growth as reported for *E. coli*. So, DNA uptake can serve as a proper nutrient source, since it is easily available in the environment. But tight regulation of DNA uptake and the selection for specific DNA, for example from siblings carrying the uptake signal as for example seen in *Campylobacter* (Beauchamp et al. 2017) suggests that DNA uptake at least has an additional function and is not exclusively a nutrient supply.

DNA can serve as a template for repair of non-functional genes or damaged chromosomes in general (Dubnau and Blokesch 2019). Since for most genes there is no second copy in the genome, once a harmful mutation occurs, the organism often has no functional version of the gene. Therefore, it could appear that DNA which is taken up serves as a repair template. Furthermore, there is also the chance for recombination of gene variants which could enhance fitness and therefore gives an advantage in survival (de Boer et al. 2002; Johnston et al. 2013). So, genetic exchange and the incorporation into the genome leads to genetic diversity in a population and enhances the adaptive potential. Advantages can be the acquisition of new gene variants or even new genes resulting in new functions, e.g. for better host adaptation or general survival outside of the host.

External DNA has been shown to be part of nearly all biofilms (Campoccia et al. 2021). Also, in *Campylobacter* extracellular DNA can serve as a matrix for biofilm formation (Feng et al. 2018; Svensson et al. 2014). In *C. jejuni* it has been reported that the formation of biofilms occurred in the presence of extracellular DNA as a response to aerobic as well as starvation stress (Feng et al. 2018). As expected extracellular DNA was found in the biofilm. Interestingly, biofilm formation was also associated with cell lysis probably resulting in even more extracellular DNA supporting the formation and maintenance of biofilms. Digestion by DNaseI disrupted the biofilm whereas external DNA allocated bacteria at distinct locations and supported biofilm formation. All this shows that external DNA plays a role in biofilm formation.

4.4.5 **Methods for detection of natural transformation**

In a classical approach to analyze natural transformation bacterial cells were cultivated, incubated with free DNA carrying an antibiotic resistance marker, and subsequently selected for this resistance (Vegge et al. 2012; Jeon and Zhang 2007). The number of total colony forming units without selection agent as well as colony forming units with selection agent are determined. This allows the calculation of the transformation frequency. Disadvantages of this classical assay are that there is no resolution of individual steps of transformation. The final result of transformation including recombination and expression of the selection marker is analyzed. Especially for fastidious bacteria like *Campylobacter* where determination of colony forming units can be challenging depending on the physiological status, for example when bacteria undergo the VBNC status the results can be distorted.

Therefore, another approach is a single cell based DNA uptake assay. For this assay bacteria cells are cultured and then incubated for a defined time-period with fluorescently labelled DNA (Stingl et al. 2010). One advantage of this assay is that factors can be tested for their effect on competence development. This includes the expression of genes necessary for assembly of the uptake machinery and the assembly of the machinery itself. Furthermore, when already competent cells are challenged with DNA the influence on the DNA uptake process can be

monitored. Therefore, it can be distinguished if parameters like temperature have an influence on competence development or DNA uptake. The single cell assay allows for resolution of single cells and makes it possible to quantify the amount of imported DNA as has been shown for *H. pylori* (Krüger et al. 2016). Based on findings in this organism it is assumed that due to steric hindrance of fluorescent dyes like fluorescein labelled DNA will be trapped in the periplasm and is not further transported into the cytoplasm (Stingl et al. 2010). Using intercalating dyes like YOYO-1, which labels only double-stranded DNA (dsDNA), it was possible to indirectly monitor uptake into the cytoplasm by loss of signal since only ssDNA will be taken up into the periplasm.

4.5 Aim of the study

C. jejuni has a diverse population structure and natural transformation seems to play a huge role in this pathogen. Nevertheless, parameters stimulating or inhibiting competence development are only poorly understood. Therefore, we developed a single cell based assay to monitor competence development and DNA uptake in *C. jejuni*. We wanted to test different parameters like temperature, pH, and growth phase on the ability to stimulate or inhibit competence development. Since external factors can trigger competence development we further used this assay to test if curcumin, maybe a potential therapeutic and protective agent against campylobacteriosis, plays a role in competence development or DNA uptake of *C. jejuni*. To analyze which factors influence competence development we used a field strain recently isolated from chicken meat. To characterize the strain, we performed next generation sequencing and used different sequencing technologies including short read as well as long read technologies and different assembly tools. Since genetic exchange seems to be very common among *Campylobacter*, we performed next generation sequencing on isolates with ambiguous species differentiation results to find out if extensive genetic exchange occurred between *C. coli* and *C. jejuni* strains.

5 Publications

5.1 List of publications and own contribution

Publication 1: Curcumin mitigates immune-induced epithelial barrier dysfunction by *Campylobacter jejuni*.

Lobo de Sá, F. D., E. Butkevych, P. K. Nattramilarasu, A. Fromm, S. Mousavi, V. Moos, J. C. Golz, K. Stingl, S. Kittler, D. Seinige, C. Kehrenberg, M. M. Heimesaat, S. Bereswill, J. D. Schulzke, and R. Bücker.

2019 Int J Mol Sci **20**(19) https://doi.org/10.3390/ijms20194830

I took part in the conceptualization of the single cell-based assay to monitor competence development and DNA uptake. I performed the experiment in the laboratory and analyzed the results. Additionally, I contributed in re-writing and editing of the manuscript.

Publication 2: Comparison of different technologies for the decipherment of the whole genome sequence of *Campylobacter jejuni* BfR-CA-14430.

Epping, L., J. C. Golz, M. T. Knüver, C. Huber, A. Thürmer, L. H. Wieler, K. Stingl, and T. Semmler.

2019 Gut Pathog **11**: 59 https://doi.org/10.1186/s13099-019-0340-7

I took part in the laboratory work, including cultivation of *C. jejuni* BfR-CA-14430 and DNA extraction as well as quality control.

Publication 3: Whole genome sequencing reveals extended natural transformation in *Campylobacter* impacting diagnostics and the pathogens adaptive potential.

Golz, J. C., L. Epping, M. T. Knüver, M. Borowiak, F. Hartkopf, C. Deneke, B. Malorny, T. Semmler, and K. Stingl.

2020 Sci Rep **10**(1): 3686 https://doi.org/10.1038/s41598-020-60320-y

I contributed in the experimental, including MALDI-TOF analysis. I took part in the interpretation of the results, analyzed sequencing data and co-wrote the manuscript.

Publication 4: Natural competence and horizontal gene transfer in *Campylobacter*.

Golz, J. C., and K. Stingl.

2021 In: Backert S. (eds) Fighting *Campylobacter* Infections. Current Topics in Microbiology and Immunology, vol 431. Springer, Cham. https://doi.org/10.1007/978-3-030-65481-8_10

I took major part in literature research and writing of the book chapter.

Publication 5: "Take it or leave it"-Factors regulating competence development and DNA uptake in *Campylobacter jejuni*.

Golz, J. C., and K. Stingl.

2021 Int J Mol Sci **22**(18) https://doi.org/10.3390/ijms221810169

I contributed in the conceptualization of the study. I performed the experiments and contributed most of the data analysis, interpretation and writing of the manuscript.

5.2 Publication 1: Curcumin mitigates immune-induced epithelial barrier dysfunction by *Campylobacter jejuni*.

International Journal of
Molecular Sciences

Article

Curcumin Mitigates Immune-Induced Epithelial Barrier Dysfunction by *Campylobacter jejuni*

Fábia Daniela Lobo de Sá [1], Eduard Butkevych [1], Praveen Kumar Nattramilarasu [1], Anja Fromm [1], Soraya Mousavi [2], Verena Moos [3], Julia C. Golz [4], Kerstin Stingl [4], Sophie Kittler [5], Diana Seinige [5], Corinna Kehrenberg [6], Markus M. Heimesaat [2], Stefan Bereswill [2], Jörg-Dieter Schulzke [1,*] and Roland Bücker [1]

[1] Institute of Clinical Physiology/Nutritional Medicine, Medical Department, Division of Gastroenterology, Infectiology, Rheumatology, Charité–Universitätsmedizin Berlin, 12203 Berlin, Germany
[2] Institute of Microbiology, Infectious Diseases and Immunology, Charité–Universitätsmedizin Berlin, Campus Benjamin Franklin, 14195 Berlin, Germany
[3] Medical Department, Division of Gastroenterology, Infectiology and Rheumatology, Charité–Universitätsmedizin Berlin, 12203 Berlin, Germany
[4] German Federal Institute for Risk Assessment (BfR), Department of Biological Safety, National Reference Laboratory for Campylobacter, 12277 Berlin, Germany
[5] University of Veterinary Medicine Hannover, Research Center for Emerging Infections and Zoonoses, 30559 Hannover, Germany
[6] Institute for Veterinary Food Science, Justus-Liebig-University, 35392 Giessen, Germany
* Correspondence: joerg.schulzke@charite.de

Received: 5 August 2019; Accepted: 26 September 2019; Published: 28 September 2019

Abstract: *Campylobacter jejuni* (*C. jejuni*) is the most common cause of foodborne gastroenteritis worldwide. The bacteria induce diarrhea and inflammation by invading the intestinal epithelium. Curcumin is a natural polyphenol from turmeric rhizome of *Curcuma longa*, a medical plant, and is commonly used in curry powder. The aim of this study was the investigation of the protective effects of curcumin against immune-induced epithelial barrier dysfunction in *C. jejuni* infection. The indirect *C. jejuni*-induced barrier defects and its protection by curcumin were analyzed in co-cultures with HT-29/B6-GR/MR epithelial cells together with differentiated THP-1 immune cells. Electrophysiological measurements revealed a reduction in transepithelial electrical resistance (TER) in infected co-cultures. An increase in fluorescein (332 Da) permeability in co-cultures as well as in the germ-free IL-$10^{-/-}$ mouse model after *C. jejuni* infection was shown. Curcumin treatment attenuated the *C. jejuni*-induced increase in fluorescein permeability in both models. Moreover, apoptosis induction, tight junction redistribution, and an increased inflammatory response—represented by TNF-α, IL-1β, and IL-6 secretion—was observed in co-cultures after infection and reversed by curcumin. In conclusion, curcumin protects against indirect *C. jejuni*-triggered immune-induced barrier defects and might be a therapeutic and protective agent in patients.

Keywords: *Campylobacter jejuni*; curcumin; tight junction; claudin; apoptosis; co-culture; mouse colon; cytokines; TNF; NFκB

---

1. Introduction

Campylobacter jejuni is the most prevalent pathogenic bacterium of zoonotic gastroenteritis [1]. Typical symptoms provoked by *C. jejuni* are watery to bloody diarrhea, abdominal pain, fever, and nausea [2]. This human pathogen is present in the intestinal microbiota of farm animals, especially poultry, which is the main source of infection for humans by ingestion of contaminated or undercooked food [1]. The bacteria adhere to the mucus and the surface of intestinal epithelial cells, invade the intestinal

epithelium, while they pass the cells via the transcellular or paracellular route [3,4]. Consequently, direct epithelial barrier defects such as dysregulation of tight junction (TJ) proteins, induction of epithelial lesions or also indirect effects by inflammatory responses of epithelial or immune cells occur [5,6].

Epithelial lesions can go along with single-cell lesions by increased apoptosis, mid-sized leaks like focal leaks, as well as erosions or even ulcerations. These pathological findings could explain the type of diarrhea for the *Campylobacter* infection as leak flux pathomechanism, which is characterized by a loss of water and solutes from the organism into the intestinal lumen through a leaky epithelium [6,7]. For a physiological intestinal integrity, an intact epithelium is essential. The main components of intestinal epithelial barrier are the TJs. They are the apical components of the intercellular seal and divide the epithelial cell membrane into an apical and basolateral compartment (fence function) [8]. Tight junctions form a barrier for water, ions, and solutes (barrier function) [9] and consist of various molecular transmembrane proteins like claudins and occludin, junction adhesion molecules (JAM), tricellulin, and cytoplasmatic scaffolding proteins as zonula occludens protein-1 (ZO-1) [8,9].

Recently, the number of *C. jejuni* infections surpasses *Salmonella* infections as the leading cause of zoonotic bacterial gastroenteritis in the US and Europe. Moreover, high levels of fluoroquinolone resistance have been reported [10] and levels of multi drug resistances (MDR) are increasing [11]. In consequence, new therapeutic targets as well as innovative strategies to reduce the amount of *C. jejuni* in farm animals and environment are urgently required. A potent natural substance thought to interfere with *Campylobacter* infection is curcumin.

Curcumin is a polyphenolic compound found in the turmeric root of the *Curcuma longa* plant (commonly known as turmeric), a member of the ginger family [12–14]. It is used as coloring agent, cosmetics, and it is the principal ingredient in oriental food spice like curry powder [12,13]. For centuries, curcumin has been used in Asian traditional medicine as a common nontoxic and active agent against different gastrointestinal and digestive disorders [12,14,15]. In addition, curcumin is known to possess anti-inflammatory, antioxidative, antibacterial, anticarcinogenic [16], antiviral [15,17], antiapoptotic, and antiproliferative properties [12]. The antibacterial effect was shown against *Salmonella* spp. [18], *Helicobacter pylori* [16,19,20], methicillin-resistant *Staphylococcus aureus*, *Bacillus subtilis*, and *Escherichia coli* [21]. Various studies have shown that curcumin modulates inflammatory cytokine pathways like IL-1β, TNF-α, IL-6, IL-8, IL-10 [12,13,22,23], and inhibit signaling pathways like NFκB [12,17,19].

The aim of the presented study was to evaluate the barrier-protective and anti-inflammatory properties of curcumin on intestinal epithelial barrier function, which is compromised by the *C. jejuni*-mediated immune response. Therefore, a novel in vitro co-culture model consisting of intestinal epithelial HT-29/B6-GR/MR and immune THP-1 cells was established. This co-culture model enables the screening of potential barrier-protective and anti-inflammatory compounds in *C. jejuni* infection, as a result of which curcumin was identified as being effective.

2. Results

2.1. Establishment of a Co-Culture with Colon Epithelial Cells HT-29/B6-GR/MR and Immune THP-1 Cells

To analyze the impact of the immune response during a *Campylobacter* infection on epithelial barrier function, human colon epithelial cells and immune cells were co-cultured. Colonic epithelial cells (HT-29/B6-GR/MR) were seeded on cell culture filter supports (0.4 µm pore size) and represented the epithelial compartment. Under the membrane of the epithelial cell filter, THP-1 immune cells were seeded on the bottom of the cell culture well and represented the subepithelial compartment. Basal bacterial infection with *C. jejuni* decreased transepithelial electrical resistance (TER) when colonic epithelial and immune cells (differentiated THP-1 cells) were co-cultured (Figure 1). From the apical supernatant, no bacteria could be cultured after the experiment, indicating that the bacteria could not pass the filter membrane with 0.4 µm pore size. As a result of this, and in contrast to the results in the co-culture model, *C. jejuni* infection at the basal membrane had no effect on TER in HT-29/B6-GR/MR mono-culture (Figure 1).

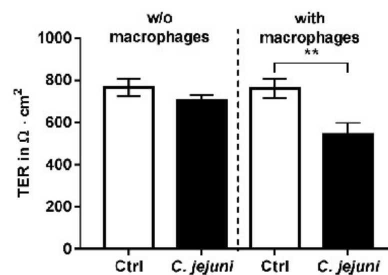


Figure 1. *C. jejuni* infection in human intestinal epithelial cells (HT-29/B6-GR/MR) or in co-culture together with human immune cells (differentiated THP-1 cells). Transepithelial electrical resistance (TER) in co-culture after *C. jejuni* infection from the basal side decreased 48 h post infection ($n = 7$–9, ** $p < 0.01$, unpaired Student's *t*-test).

2.2. Curcumin Improves Disturbed Intestinal Barrier Function in the Co-Culture System

Using mRNA sequencing and subsequent ingenuity pathway analysis (IPA) of human mucosa biopsies from *C. jejuni*-infected patients, inhibition of curcumin-dependent pathways indicated that the presence of curcumin might prevent the *C. jejuni*-induced changes in gene expression. Based on this bioinformatic prediction of curcumin's effect on host gene expression, the addition of curcumin should activate downstream pathways and might reduce the effects of *Campylobacter* infections (Supplementary Table S1). In order to test the barrier protective and anti-inflammatory properties of curcumin in *Campylobacter*-induced barrier dysfunction, a curcumin solution was added to the infected co-culture model. As shown in Figure 2, *C. jejuni* reduced TER significantly 48 h after infection, whereas this change was inhibited by the presence of curcumin.

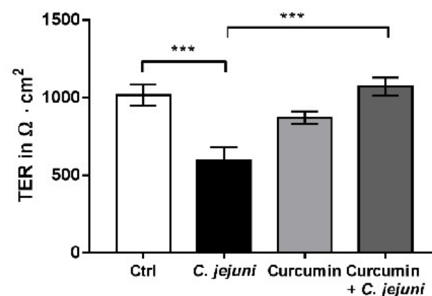


Figure 2. Curcumin protects against *C. jejuni*-induced barrier disruption in co-culture. Cells were treated with 50 µM curcumin and infected with *C. jejuni* for 48 h ($n = 13$, *** $p < 0.001$, one-way ANOVA with Bonferroni correction for multiple comparisons).

Permeability studies were performed with the paracellular flux marker fluorescein (332 Da). Compared to controls, *C. jejuni* infection caused an increase in permeability to fluorescein. Application of curcumin not only ameliorated the permeability increase for fluorescein, but even resulted in an improved barrier function for paracellular passage of macromolecules when exceeded the control level (Figure 3).

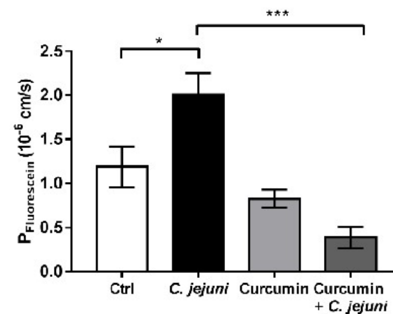


Figure 3. Curcumin improves the *C. jejuni*-induced increase in permeability to fluorescein (332 Da) in co-cultures with colon epithelial HT-29/B6-GR/MR and immune THP-1 cells. The co-cultures were infected with *C. jejuni* after incubation with 50 µM curcumin. Fluorescein permeabilities were measured 48 h post infection ($n = 7–8$, * $p < 0.05$, *** $p < 0.001$, one-way ANOVA with Bonferroni correction for multiple comparisons).

2.3. Effect of Curcumin In Vivo on Intestinal Barrier Regulation in IL-10$^{-/-}$ Mice

Secondary abiotic IL-10$^{-/-}$ mice were infected with *C. jejuni* and treated with 0.5 mg/mL of curcumin, starting 4 days prior infection until the end of the observation period (i.e., day 6 post infection). Epithelial barrier function of distal colon specimens from IL-10$^{-/-}$ mice was analyzed in Ussing chambers. Conductance (G, Figure 4A) and permeability to fluorescein (Figure 4B) was significant higher in *C. jejuni*-infected mice than in untreated infected mice. Mice receiving curcumin showed a significantly lower conductance and permeability to fluorescein than untreated infected mice. The electrophysiological results obtained in vivo (Figure 4) resemble the results from the in vitro co-culture system (Figure 3).

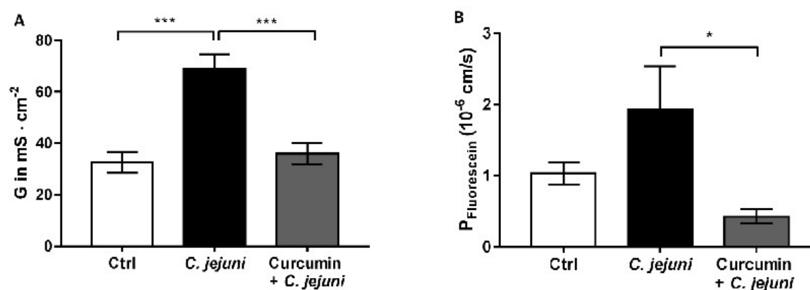


Figure 4. Curcumin ameliorates the *C. jejuni*-induced barrier dysfunction in colon of IL-10$^{-/-}$ mice. Colon specimens of IL-10$^{-/-}$ mice were mounted in Ussing chambers designed for impedance spectroscopy. (**A**) Epithelial conductance (G) ($n = 5–18$, *** $p < 0{,}001$, Mann–Whitney *U*-test) and (**B**) fluorescein (332 Da) permeability ($n = 5–14$, * $p < 0.05$, Mann–Whitney *U*-test) of IL-10$^{-/-}$ mouse colon were measured in Ussing chambers. IL-10$^{-/-}$ mice were either infected with *C. jejuni* (per oral gavage with 10^{8} CFU in 0.3 mL) or infected and treated for 6 days with Curcumin (0.5 mg/mL).

2.4. Effect of Curcumin on Bacterial Integrity

To determine whether curcumin had a direct effect on the viability of *C. jejuni* in the concentration used in our experiments, the minimal inhibitory concentration (MIC) for curcumin was determined. The MIC of curcumin in *C. jejuni* 81–176 amounted to 87 µM under physiological conditions at a pH of 7.4, and is thus above our experimental curcumin concentrations in the co-culture. Moreover,

if curcumin is used as treatment, it is necessary to know whether the substance exerts any stress to the bacterium, leading to enhanced adaption by, e.g., stimulation of natural transformation capacity. Therefore, competence development and DNA uptake of *C. jejuni* was investigated under the treatment of curcumin using a single cell assay. After curcumin treatment, no change in the ratio of competent bacteria was registered (control 26% ± 2% versus treated 25.5% ± 0.5% with 50 μM curcumin, $n = 3$, n.s., unpaired Student's *t*-test). In addition, also the DNA uptake processes in competent *C. jejuni* was unaffected after curcumin treatment (control 100% versus treated 86.9% ± 6.7%, $n = 3$, n.s., unpaired Student´s *t*-test).

2.5. Cytokine Secretion in C. jejuni-Infected Co-Cultures

To identify which cytokines were released by the immune cells and affected epithelial cells function, cytometric bead arrays (CBA) from supernatants of treated co-cultures were performed. TNF-α, IL-1β, and IL-6 were increased in *C. jejuni*-infected co-cultures, while this changes were prevented by curcumin treatment (Figure 5).

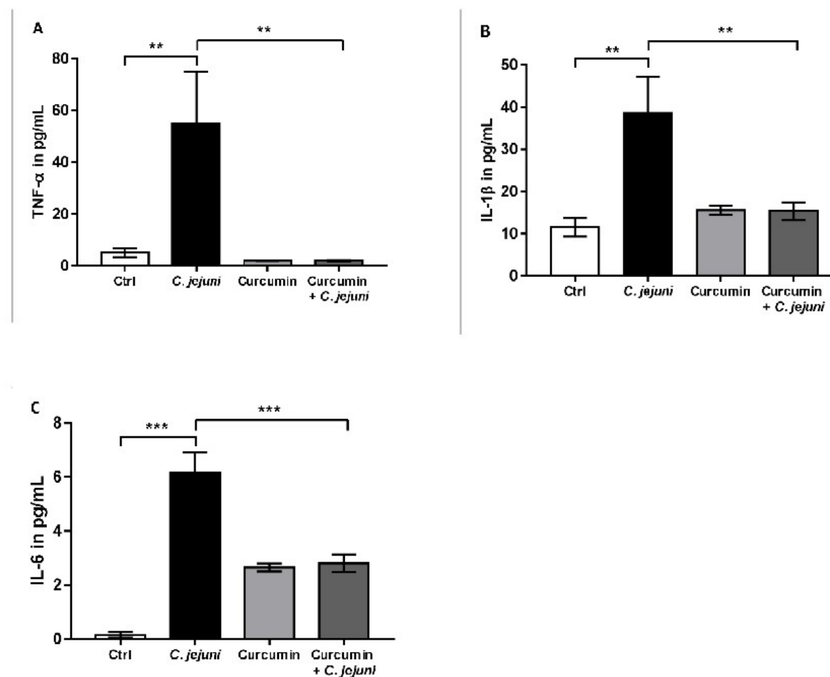


Figure 5. Cytokine release in co-cultures after *C. jejuni* infection. (**A**) Tumor necrosis factor-α (TNF-α), (**B**) Interleukin-1β (IL-1β), and (**C**) Interleukin-6 (IL-6) secretion after treatment with curcumin in *C. jejuni*-infected co-cultures ($n = 5$–6, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, one-way ANOVA with Bonferroni correction for multiple comparisons).

2.6. NFκB Signaling Pathway is Involved in C. jejuni-Induced Barrier Dysfunction

Furthermore, we evaluated the possible relevance of the NFκB pathway for barrier function in our cell model using the NFκB inhibitor BAY 11-7082 (BAY). BAY inhibited the *C. jejuni*-induced reduction in TER, similar to the effect of curcumin (Figure 6A). In addition, the increase in permeability to fluorescein could be reversed after BAY inhibition and almost reached control values (Figure 6B).

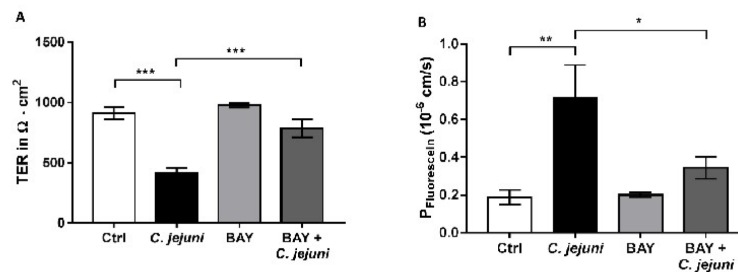


Figure 6. The NFκB pathway is barrier-relevant. Inhibitory effect of BAY 11-7082 in *C. jejuni*-infected co-cultures. (**A**) Transepithelial electrical resistance (TER) and (**B**) permeability to fluorescein (332 Da) after incubation with 10 µM BAY 11-7082 and infection with *C. jejuni* for 48 h ($n = 5$, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, one-way ANOVA with Bonferroni correction for multiple comparisons).

2.7. Epithelial Apoptosis is Blocked by Curcumin in the Co-Culture Model

Given that the induction of epithelial apoptosis constitutes a barrier-relevant pathomechanism, and *Campylobacter* infections have been shown to induce epithelial apoptosis, the impact of curcumin on apoptosis was analyzed using cleaved caspase-3 staining and visualized by confocal laser-scanning microscopy (CLSM) (Figure 7).

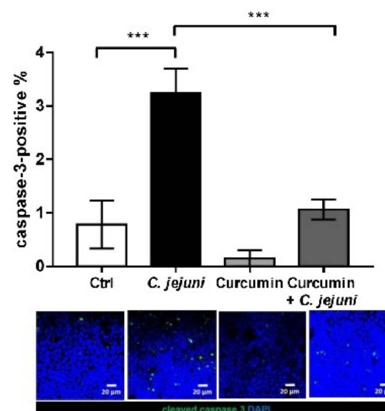


Figure 7. Apoptosis induction in *C. jejuni*-infected co-cultures. Cleaved caspase-3 staining indicates the number of apoptotic cells and is visualized by confocal microscopy. Monolayers were stained with antibodies against cleaved caspase-3 (green) and nuclei were colored with 4′-d-diamidino-2-phenylidole dihydrochloride (DAPI, blue). Signals of cleaved caspase-3-positive cells in CLSM were counted in the top view pictures and related to the number of DAPI stained nuclei (~300 nuclei/frame) to calculate the percentage of apoptotic cells in the monolayers ($n = 6$, *** $p < 0.001$, one-way ANOVA with Bonferroni correction for multiple comparisons). Representative pictures are shown for HT-29/B6-GR/MR cells, obtained from co-culture after treatment with 50 µM curcumin and infection with *C. jejuni*.

After infection with *C. jejuni*, the apoptotic ratio was increased 3-fold in comparison to control. Incubation with curcumin during the infection with *C. jejuni* reduced the apoptotic ratio to that of controls (Figure 7) (control 0.7% ± 0.4% versus infected 3.3% ± 0.4% versus treated 1.1% ± 0.2%, $n = 6$, ** $p < 0.01$, *** $p < 0.001$, one-way ANOVA with Bonferroni correction for multiple comparisons).

2.8. Influence of C. jejuni and Curcumin on Tight Junction Protein Expression in the Co-Culture Model

To define the *C. jejuni*-induced tight junction (TJ) expression changes, protein lysates were generated from colon epithelial cells of co-cultures after treatment with curcumin and infection with *C. jejuni* (Figure 8). The expression change was quantified by densitometric analysis with β-actin as loading control, which showed an increase in protein expression of claudin-1 protein expression after *C. jejuni* infection. Claudin-1 expression seemed to decrease again after curcumin treatment in the *C. jejuni* infected group. However, this did not reach statistical significance. No further expression changes of the tested TJ proteins could be observed. A reduction by trend after *C. jejuni* infection in occludin and claudin-8 was detectable, but without reaching statistical significance after multiple test correction (claudin-8 infected versus curcumin-treated $p = 0.046$, uncorrected Student´s *t*-test).

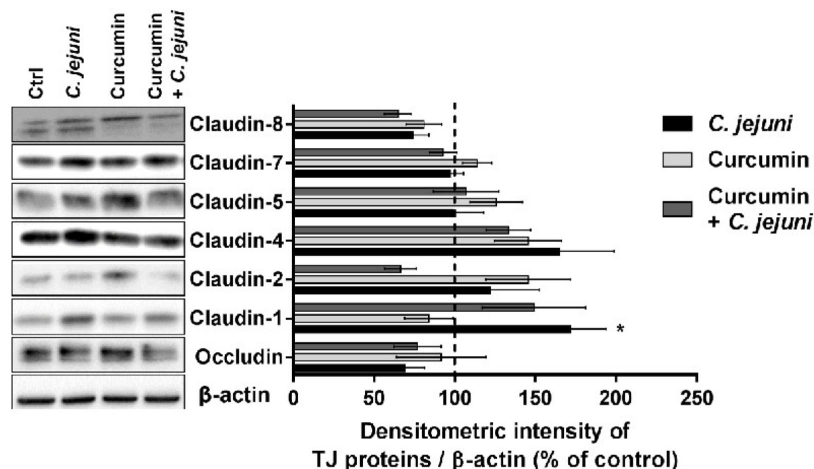


Figure 8. Tight junction (TJ) protein expression in western blot. Representative western blots and densitometry of TJ protein expression from HT-29/B6-GR/MR cells obtained from co-culture after incubation with 50 µM curcumin and infection with *C. jejuni*. TJ proteins were normalized to the level of β-actin ($n = 6$–9, * $p < 0.05$, two-way ANOVA with Bonferroni correction for multiple comparisons. Control value is marked with a dashed line.

2.9. Influence of C. jejuni and Curcumin on Subcellular TJ Protein Distribution in Co-Cultures in Confocal Laser-Scanning Microscopy

Since the composition and presence of TJ proteins in the TJ strands influence barrier function, the subcellular distribution of the TJs was analyzed with confocal laser-scanning microscopy (CLSM). Z-stacks were generated from different TJ proteins of the immunostained epithelial cell monolayers, and intensity-distance plots were generated. The TJ strands after *C. jejuni* infection indicated a redistribution of claudin-4 (Figure 9A) and claudin-8 (Figure 9B). Claudin-4 and claudin-8 signals were retracted from the TJ strands. Moreover, claudin-4 and claudin-8 were no longer co-localized with zonula occludens protein-1 (ZO-1) after *C. jejuni* infection. Intensity–distance plots with high background intensities indicate re-localization into the intracellular regions. After treatment with curcumin, the appearance of claudin-4 and claudin-8 improved. As a consequence, the infected and curcumin treated group showed co-localization (merging), with high peak intensities in the areas of TJ contact, comparable to the control. Curcumin control groups showed no difference compared to the untreated controls (Supplementary Figure S2).

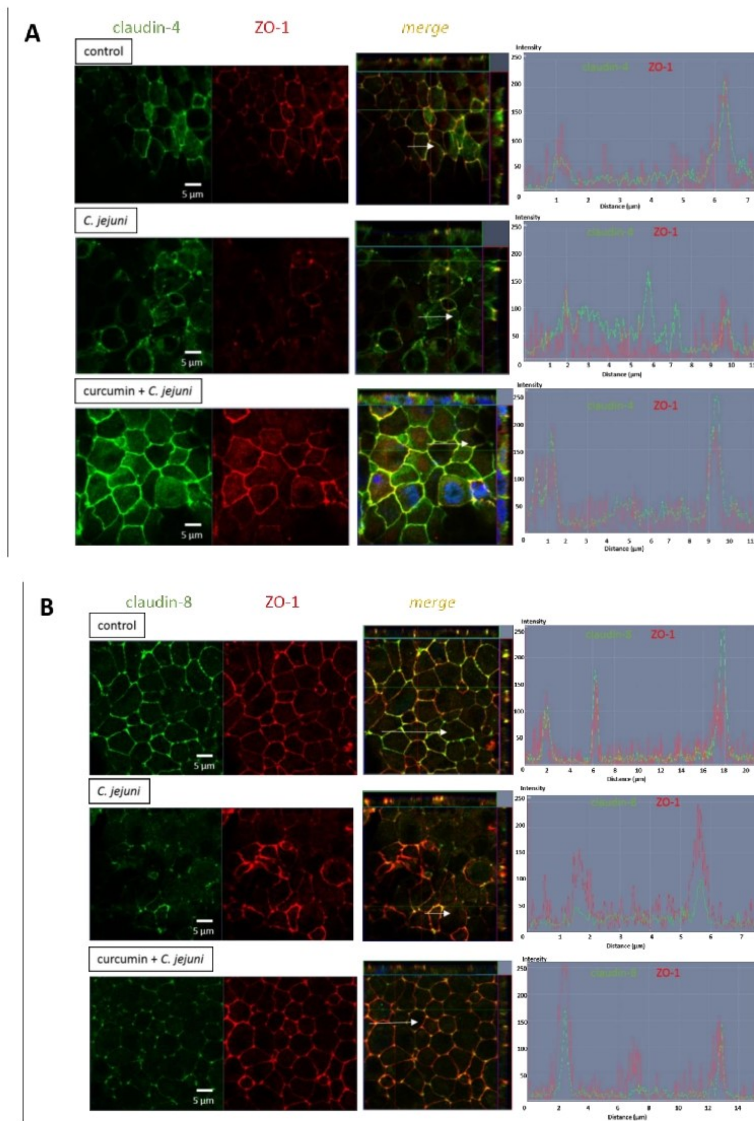


Figure 9. Tight junction distribution in *C. jejuni* infection after treatment with curcumin. Representative confocal laser-scanning microscopy pictures of HT-29/B6-GR/MR after co-culturing together with immune cells. (**A**) Claudin-4 (green) and zonula occludens protein-1 (ZO-1, red), and (**B**) claudin-8 (green) and ZO-1 (red). Nuclei are stained in blue with 4´-6-diamidino-2-phenylindole dihydrochloride (DAPI).

3. Discussion

It is important to contain the *Campylobacter jejuni* infection because of its impact on gastroenteritis worldwide and resulting sequelae, such as irritable bowel syndrome, reactive arthritis, or Guillian–Barré syndrome. Therefore, we aimed to study the underlying pathomechanisms for barrier dysfunction

and the influence of the immune system in more detail. For this purpose, we used a new in vitro co-culture model and an in vivo mouse model to study the protective properties of the natural polyphenol curcumin.

Curcumin is a vitamin D receptor (VDR) ligand [24], and we found the downstream signaling pathway of curcumin to be inhibited in the mucosa of acutely infected *C. jejuni* patients by means of the bioinformatic prediction in Qiagen ingenuity pathway analysis (IPA) from RNA-sequencing (Supplementary Table S1). This prediction indicated that curcumin could be a potential therapeutic or preventive target against *C. jejuni* infections. Based on this bioinformatics prediction, we tested the effects of curcumin on *C. jejuni* infection in vitro and in vivo. The inhibitory effect of another top hit upstream regulator in IPA analysis on the mucosa of *C. jejuni*-infected patients was calcitriol (active form of vitamin D). Calcitriol exhibited a high significance value comparable to curcumin (Appendix A, Table S1). Annulment of *C. jejuni*-induced cytotoxicity after calcitriol treatment has been demonstrated in HT-29/B6 cells previously, supporting the view that the bacterium affects intestinal barrier function via a VDR-dependent pathway [6].

As predicted by the pathway analysis in acute human campylobacteriosis, curcumin was effective in our experiments. It could prevent the decrease in TER as well as the increase in fluorescein permeability in our co-culture model, indicating improved barrier function by curcumin in immune-mediated epithelial barrier dysfunction by *C. jejuni*.

Because we expected an essential contribution of the immune system, we established a co-culture model with basal infection. In this model, the *C. jejuni*-triggered immune response reduced the TER after 48 h. The apical bathing medium did not contain bacteria even after 48 h, which suggests that the effect on resistance was caused indirectly by the *C. jejuni*-induced immune response. The decrease in TER may therefore reflected an increase in paracellular permeability secondary to epithelial barrier defects, which would increase permeability to small molecules and toxins. Furthermore, this change in barrier function may increase the access of bacteria to the underlying tissue and potentiate the immune response, causing additional barrier disruption, with loss of cellular integrity [6,25]. Concomitantly, a leak-flux type of diarrhea is caused that represents a main diarrheal mechanism.

Depending on the cell type and the experimental infection conditions, *C. jejuni* was more or less able to modulate TER in previous studies. No effects on TER have been found in MKN-28 cells [26] or Caco-2 monolayers [4]. In contrast, exposure to *C. jejuni* has been reported to reduce TER in T84 and MDCK-I cells [27]. In our HT-29/B6-GR/MR cells, TER was also not different from control even after 48 h basal infection, although a decrease in TER after basolateral infection has been reported to be more pronounced than after apical infection in T84 cells [28]. That this was not seen in our HT-29/B6-GR/MR cells may reflect differences in cell type and/or the 0.4 μm filter pore size of our filters, limiting the bacterial access to the epithelial cells.

Within 6 days following peroral *C. jejuni* infection, secondary abiotic IL-10$^{-/-}$ mice develop acute ulcerative enterocolitis mimicking key features of severe campylobacteriosis in humans, with a specific cytokine response [29]. We were able to show for the first time, using impedance spectroscopy in Ussing chambers, compromised barrier integrity with an increased epithelial conductance and permeability to macromolecules in this mouse model. These data reflect the results of our co-culture model, in which the barrier defects could be similarly prevented by curcumin. Bücker et al. [6] incubated colonic mucosal biopsies from *C. jejuni*-infected patients in culture medium and quantified cytokine release into the supernatant. Secretion of TNF-α, IFN-γ, IL-1β, and IL-13 was enhanced in comparison to controls [6]. It was concluded that this mucosal cytokine storm led to disruption of intestinal TJs and the induction of epithelial apoptosis. In our infected co-cultures, release of cytokines was also mainly responsible for the barrier defects, even though THP-1 cells produce and secrete a lower amount and spectrum of cytokines in comparison to fresh isolated peripheral blood mononuclear cells (PBMC). After *C. jejuni* infection, levels of the pro-inflammatory cytokine TNF-α, IL-1β, and IL-6 increased remarkably, and these changes were largely prevented by curcumin. Curcumin not only reduced local inflammation in the intestine, it also reduced systemic inflammation triggered by LPS [23]. In another

study, curcumin nanoparticles suppressed the expression of mRNAs encoding pro-inflammatory mediators, including TNF-α, IL-1β, and IL-6 [14].

C. jejuni was found to activate the NFκB signaling pathway [28], which was suppressed by curcumin [12,17,19]. To determine the role of the NFκB pathway in maintaining barrier function, inhibition studies with the NFκB-inhibitor BAY 11-7082 were performed in *C. jejuni*-infected co-cultures. BAY 11-7082 treatment inhibited both the *C. jejuni*-induced decrease in TER and the increase in fluorescein permeability. This points to the involvement of NFκB in the regulation of epithelial barrier function. Similar results were obtained by our group previously with the bioactive ginger ingredient 6-shogaol after TNF-α stimulation in Caco-2 and HT-29/B6 cells [30].

In our co-culture, *C. jejuni* provoked paradoxical upregulation of claudin-1 (Claudin-1 paradox); that is, upregulation of the barrier-forming TJ protein claudin-1 while epithelial resistance was reduced, which we explained by the re-distribution of claudin-1 off the tight junction domain [6]. Claudin-1 induction has also been observed before in human colon biopsies after *C. jejuni* infection [6], *C. fetus* or *C. coli* infection in HT-29/B6 cells [31], and pro-inflammatory cytokine stimulation in HT-29/B6 cells [32,33]. A correlation between increased claudin-1 protein expression and apoptosis induction also occurs in HT-29/B6 cells [34]. In addition, curcumin induced claudin-4 mRNA expression in Caco-2 cells [8]. However, in our co-culture model, curcumin did not induce claudin-4 protein expression. Cell type-dependent differences in TJ expression were also found for quercetin, another plant-derived polyphenol with barrier-improving properties, which increased claudin-4 protein expression in Caco-2, but not in HT-29/B6 cells [35,36].

In order to exclude antibacterial effects of curcumin in our study, the MIC was determined with 87 µM (at pH 7.4). This suggests that the protective effects of 50 µM curcumin were not based on its antibacterial properties. Thus, we conclude that curcumin had direct barrier-protective and anti-inflammatory properties in the host cells.

To further exclude interfering properties of curcumin with bacterial fitness and adaptive response, we investigated if curcumin changes natural transformation capacity. Therefore, we used a single cell DNA uptake assay and challenged the bacteria with 50 µM curcumin. *C. jejuni* is naturally competent for DNA uptake from the environment. Generally, this leads to genetic recombinations between bacterial strains, and as a consequence, to more diversity [37]. Curcumin did not influence the competence development and horizontal DNA uptake of *C. jejuni* in our experimental concentrations.

One of the major challenges to the development of therapeutic approaches is the remarkable diversity between different *Campylobacter* strains in animals, the environment, and food [2]. Consequently, the efficacy of curcumin should also be tested using other *C. jejuni* field strains and, subsequently, in other *Campylobacter* species such as *C. coli* or *C. concisus*. Curcumin is a natural agent, and should be more appropriate than synthetic drugs [12]. Curcumin has been shown to be beneficial in maintaining remission in ulcerative colitis patients when used as a complementary therapeutic substance together with the antiphlogistic compound mesalazine in several randomized clinical studies [38–40]. Since the immune response plays an important role in pathogenesis in IBD as well as in *C. jejuni* infection, curcumin may be equally effective in immune-induced barrier dysfunctions by *C. jejuni* as an add-on to other anti-inflammatory drugs. Indeed, multimodal therapies may have an even greater protective effect, and synergism has been reported between curcumin and quercetin [41], genistein [42], epigallocatechin-3-gallate [15,43], and antioxidants [12,44]. Thus, curcumin alone or in combination with other agents may be useful in preventing, treating, and combating *Campylobacter* infection in humans, *C. jejuni*-associated sequelae as well as colonization of farm animals and especially poultry.

Taken together, our results suggest that a significant part of the *C. jejuni*-induced barrier defect is mediated indirectly by the immune cells. Curcumin abolishes functional epithelial disorders as well as pro-inflammatory cytokine secretion. Further barrier-protective or anti-inflammatory compounds, as well as curcumin combinations with other therapies, should be tested in the co-culture model.

4. Materials and Methods

4.1. Epithelial Cell Culture and Differentiation of THP-1 Cells

The human colon carcinoma cell line HT-29/B6-GR/MR [45], a subclone of the HT-29/B6 line [46] was cultured in 25 cm^2 culture flasks in RPMI 1640 culture medium (Sigma Aldrich, St. Louis, MO, USA). HT-29/B6-GR/MR cells expressing the human glucocorticoid receptor α and the human mineralocorticoid receptor, were established earlier by stable transfection [45,47]. The culture medium was supplemented with 10% fetal calf serum (FCS; Gibco, Carlsbad, CA, USA), 1% penicillin/streptomycin (Corning, Wiesbaden, Germany), G418-BC (300 µg/mL; Invitrogen, Carlsbad, CA, USA) and hygromycin B (200 µg/mL; Biochrom GmbH, Berlin, Germany), and cells were passaged every week. Cells were seeded on Millicell PCF filters membranes (Merck Millipore, Billerica, MA, USA; 0.4 µm pore size and an effective growth area of 0.6 cm^2). The cell medium in the culture flask, as well as in the filters, were changed every other day. Experiments were performed between 7 and 9 days after seeding, when polarized cells formed confluent monolayers and the transepithelial electrical resistance was 600–900 $\Omega \cdot cm^2$. These cells were chosen because of their stable growth behavior and the high resistance. The human monocyte leukemia cell line THP-1 (ATCC TIB-202) was provided by Verena Moos. THP-1 cells were cultured in RPMI 1640 supplemented with 10% heat-inactivated FCS and 1% penicillin/streptomycin. Cultures were maintained by the addition of fresh medium and were subcultured before reaching a density of 8×10^5 cells/mL once a week. THP-1 cells were re-suspended in antibiotic-free medium supplemented with phorbol 12-myristate 13-acetate (PMA; Sigma Aldrich, St. Louis, MO, USA; solved in DMSO) at a final concentration of 100 nM, seeded with a density of 1.8×10^5 in 12-well plates, and incubated for 24 h for differentiation to macrophages [48]. After incubation, PMA-containing medium was removed and the differentiated and adherent macrophages-like cells were cultured together with epithelial monolayers grown on filters in co-culture (Figure 10). All cells were cultured at 37 °C in a humidified 5% CO_2 atmosphere.

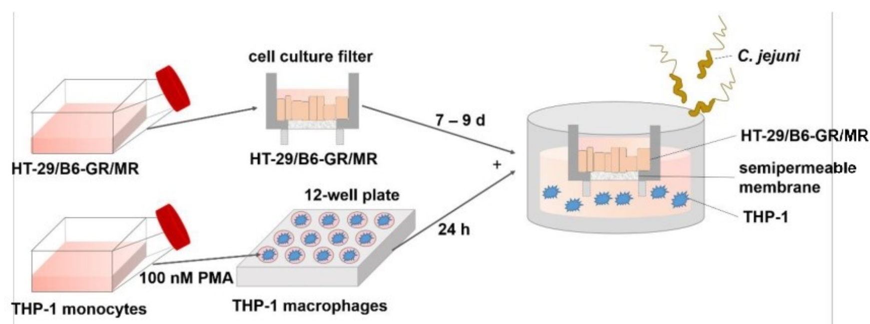


Figure 10. Experimental setting for co-culture of colon epithelial HT-29/B6-GR/MR and THP-1 immune cells. For the continuous culture of epithelial and immune cells, cells were cultured in culture flask. Epithelial cells were then seeded on filter membranes and differentiated over 7–9 days to a polarized monolayer with tight junctions. 24 h before co-culture started, immune THP-1 cells were stimulated with phorbol 12-myristate 13-acetate (PMA) to allow a differentiation to adherent macrophage-like immune cells. After the differentiation of epithelial and immune cells, filters were placed into the 12-well plate with epithelial cells in the apical and immune cells in the basal compartment. Infection with *C. jejuni* was performed in the basal compartment.

4.2. Growth Conditions of *C. jejuni*, Treatment and Infection Procedure In Vitro

Campylobater jejuni wildtype (wt) 81-176 reference strain was pre-cultured on blood agar plates (Oxoid, Thermo Scientific, Waltham, MA, USA) under microaerobic conditions (5–10% O_2, 10% CO_2, 85% N_2) at 37 °C and re-cultured a second time before infection experiments. Microaerobic conditions

were generated in a plastic jar with CampyGen gaspacks from Oxoid (Oxoid, Thermo Scientific, Waltham, MA, USA). Bacteria were then cultured in Mueller–Hinton broth for at least 2.5 h at 37 °C under microaerobic conditions, centrifuged (2 min, 5000 *g*, 10 °C), and re-suspended in the cell culture medium. The optical density at OD_{600} was adjusted to 1 and the cells where infected from basal side with a multiplicity of infection (MOI) of 100. At least 1.5 h before the infection, cells were washed three times with antibiotic-free culture medium supplemented with 10% heat-inactivated FCS. To analyze the barrier-protective and anti-inflammatory properties of curcumin (Sigma Aldrich, St. Louis, MO, USA; final concentration 50 µM), different conditions were tested: control, *C. jejuni* infected, curcumin control (50 µM), and curcumin in combination with *C. jejuni*. Curcumin control showed no adverse effects on the cell viability of HT-29/B6-GR/MR monolayers at the concentration tested. Cells were pre-incubated with curcumin on both sides for 2 h, and infected with *C. jejuni* from the basal side for a direct immune cell infection and cytokine-induced barrier effect. Curcumin was added to the apical and basal side, since functional measurements depend on identical solutions on both the sides of the monolayer to avoid, e.g., potential osmotic effects. Moreover, curcumin readily accessed the basolateral compartment via *C. jejuni*-induced leaks. After infection, cells were incubated at 37 °C under microaerobic conditions favorable to the bacteria for 48 h.

4.3. Generation of Secondary Abiotic IL10$^{-/-}$ Mice, Treatment Infection

Permeability to fluorescein (332 Da; Sigma Aldrich, St. Louis, MO, USA) measurements were performed in Ussing chambers with colon of secondary abiotic IL-10$^{-/-}$ mice suffering from acute enterocolitis within 6 days following peroral *C. jejuni* infection [49]. IL-10$^{-/-}$ mice (in C57BL/6j background) were held under specific pathogen free (SPF) conditions in the animal facilities of the Forschungseinrichtung für Experimentelle Medizin (Charité–Universitätsmedizin Berlin). To remove the commensal gut microbiota, mice were transferred to sterile cages and treated for 8 weeks with an antibiotic cocktail in the drinking water ad libitum containing ampicillin/sulbactam (1.5 g/L), ciprofloxacin (200 mg/L), imipenem/cilastatin (250 mg/L), metronidazole (1 g/L), and vancomycin (500 mg/L) as described previously [50]. Four days before infection, the antibiotic cocktail was replaced by curcumin (Sigma Aldrich, St. Louis, MO, USA; 0.5 mg/mL; solved in 2% Carboxymethyl cellulose (Sigma Aldrich, St. Louis, MO, USA) in phosphate-buffered saline (PBS; pH7.4; Sigma Aldrich, St. Louis, MO, USA)) in autoclaved drinking water. Mice were then infected via oral gavage with 10^8 colony forming units (CFU) of *C. jejuni* strain 81-176 in a volume of 0.3 mL PBS. Six days after infection mice were sacrificed by isoflurane inhalation and colon samples were removed for tracer flux measurements analysis.

4.4. Ethics Statement

The animal experiments were carried out in our animal facility according to the German animal protection law (LaGeSo Berlin; approval number G0172/16, 13th Oct. 2016).

4.5. Electrophysiological Studies

Transepithelial electrical resistance (TER) was measured in vitro with a chopstick electrode pair under sterile conditions at 37 °C. The TER-values were corrected with the resistance of an empty cell filter and the bath solution. Colon samples from mice were mounted in modified miniaturized Ussing chambers (Institute of Clinical Physiology, Charité, Berlin, with an effective area of 0.049 cm^2) in modified Ringer´s solution. Ringer´s solution for the Ussing experiments contained (in mM): NaCl (113.6), $NaHCO_3$ (21), KCL (5.4), Na_2HPO_4 (2.4), $MgCl_2$ (1.2), $CaCl_2$ (1.2), NaH_2PO_4 (0.6), D(+)-glucose (10), D(+)-mannose (10) beta-hydroxybutyric acid (0.5), L-glutamine (2.5), and the antibiotics piperacillin (50 mg/L) and imipenem (4 mg/L), and was equilibrated for 15 min with carbogen gas to a pH of 7.4. One-path impedance spectroscopy measurements were performed as described previously [51] to delineate between epithelial and subepithelial conductance (G).

4.6. Epithelial Permeability

For permeability measurements, unidirectional flux studies were conducted with the paracellular marker fluorescein (332 Da; 100 μM) from the mucosal to the serosal compartment, either directly in 12-well plates or under short circuit current (I_{SC}) conditions in Ussing chambers, as described earlier [7]. I_{SC} was recorded under voltage clamp conditions by an automatic clamp device (CVC6, Fiebig Hard & Software, Berlin, Germany) at 37 °C over 1.5 h. Fluorescein was dissolved either in Ringer´s solution or in media. Samples were taken every 15 min for one hour from the basolateral side, and fluorescence was measured in a spectrophotometer (Tecan GmbH, Maennedorf, Switzerland). Permeability was calculated from flux over concentration difference.

4.7. Cytometric Bead Array

Supernatants of co-cultures were collected 48 h after infection and analyzed using the Cytometric Bead Assay (CBA; BD Biosciences, Franklin Lakes, NJ, USA) according to manufacturer´s instructions (human Th1, Th2, Th17 Kit, Flex Set IL-1β) to determine the secretion of cytokines TNF-α, IL-1β, IL-6. Flow cytometric measurement were performed with FACS CantoII (BD Biosciences; Franklin Lakes, NJ, USA) and analyzed with FACP Array^{TM} software v3.0 (BD Biosciences, Franklin Lakes, NJ, USA).

4.8. Western Blot Analysis

For protein quantification, epithelial cells were washed twice with ice-cold PBS. Whole cell lysates were extracted with ice-cold lysis buffer. Whole cell lysis buffer was prepared with 150 mM NaCl, 10 mM Tris buffer pH of 7.5, 0.5% Triton *X*-100, and 1% SDS. A volume of 10 mL lysis buffer was supplemented with one Complete Protease Inhibitor Cocktail tablet (Roche AG, Basel, Switzerland). After lysis, cells were scraped from the filters, incubated for 60 min on ice, and vortexed every 10 min. After centrifugation (30 min, 15,000× *g* at 4 °C), the supernatant was collected. Sonification of the lysate followed by further centrifugation. Total protein quantification was performed by Pierce BCA assay (Thermo Scientific, Waltham, MA, USA) according to the product instructions using a Tecan plate reader (Tecan GmbH, Maennedorf, Switzerland) at an absorbance of 562 nm. Protein samples (10–20 μg) were mixed with 5xLaemmli buffer and loaded on a SDS polyacrylamide gel (for claudins 12.5%, for occludin 10% polyacrylamide). After electrophoretic separation of the proteins and transfer to a nitrocellulose membrane, membranes were blocked for 2 h at room temperature with 1% PVP-40 (Polyvinylpyrrolidone; Sigma Aldrich, St. Louis, MO, USA) in TBST/0.05%Tween-20 buffer. Primary antibodies anti-occludin (1:100; Sigma Aldrich, St. Louis, MO, USA), anti-claudin-1, -2, -4, -5, -7, -8 (1:100; Invitrogen, Carlsbad, CA, USA), and anti-β-actin (1:5000; Sigma Aldrich, St. Louis, MO, USA) as internal loading control were incubated overnight at 4 °C. Peroxidase conjugated secondary antibodies goat anti-rabbit IgG or goat anti-mouse IgG (Jackson ImmunoResearch, Ely, UK) were incubated for 2 h at room temperature. For protein detection, SuperSignal West Pico PLUS Stable Peroxide Solution (Thermo Scientific, Waltham, MA, USA) was used and signals were detected with Fusion FX7 imaging system (Vilber Lourmat Deutschland GmbH, Eberhardzell, Germany). Densitometric quantification was performed using ImageJ software 1.48v/Java 1.6.0_20 (Rasband, W. S., ImageJ, NIH, Bethesda, MD, USA), and the values were normalized to β-actin.

4.9. Immunofluorescence Staining

Cells grown on filters were rinsed twice with PBS and fixed for 30 min in 2% paraformaldehyde (PFA; Electron Microscopy Sciences, Hatfield, PA, USA) for immunostaining and microscopic analysis. Afterwards, the cells were washed twice with PBS, permeabilized with 0.5% Triton *X*-100 (Sigma Aldrich, St. Louis, MO, USA) for 7 min, and blocked for 10 min with 1% goat serum (Gibco, Carlsbad, CA, USA). The cells were incubated with the primary antibodies anti-claudin-4 and -8 (1:100; Invitrogen, Carlsbad, CA, USA), anti-ZO-1 (1:100; BD Biosciences, Franklin Lakes, NJ, USA) or cleaved caspase-3 (1:100; Cell Signaling Technology, Cambridge, UK) for 1 h at room temperature, followed by the secondary

anti-rabbit or anti-mouse antibody for 1 h (1:500; Invitrogen, Carlsbad, CA, USA). Secondary antibodies were conjugated to Alexa-Fluor 488 or 594. The nuclei were stained with 4´-6-diamidino-2-phenylindole dihydrochloride (DAPI, 1:1000, Roche AG, Basel, Switzerland). Subsequently, the cells on the filters were washed with water and ethanol, then embedded in ProTaq Mount Fluor (Biocyc, Luckenwalde, Germany). The subcellular distribution of the tight junctions was analyzed by confocal laser-scanning microscopy (CLSM, Zeiss LSM780, Jena, Germany). Stained apoptoses with cleaved caspase-3 were counted microscopically.

4.10. Determination of Minimal Inhibitory Concentration Values

Determinations of minimal inhibitory concentration values (MICs) of *Campylobacter* strain 81-176 were performed in a broth microdilution assay. The wells of microtiter plates contained 2-fold serial dilutions of curcumin with a test range of 1–1024 µg/mL. For MIC determinations, inoculum level, growth medium, incubation time, and conditions were performed in accordance with the recommendations given in the Clinical and Laboratory Standards Institute (CLSI) document VET01-A4. In brief, *Campylobacter* colonies were taken from a blood agar plate and transferred into a tube containing 5 mL of 0.85% saline. The suspension was adjusted to the turbidity equivalent of 0.5 McFarland standard, and diluted 1:100 with growth medium. Subsequently, 50 µL of the diluted inoculum was added to each test well (50 µL) in the dilution series (containing substances at double the desired final concentration) and mixed. The microtiter plates were incubated for 24 to 48 h at 42 °C under microaerobic conditions to obtain sufficient growth. They were analyzed visually, and the lowest concentration preventing visible growth of bacteria was defined as the MIC. All susceptibility tests were performed in growth medium adjusted to pH of 7.4. *Campylobacter jejuni* reference strain DSM 4688 was used for quality control purposes. The MIC values of the quality control strain were determined in advance in three independent experiments using the broth microdilution and macrodilution method.

4.11. DNA-Uptake Assay

C. jejuni strain 81-176 was streaked out from a −80 °C stock and subcultured on Columbia blood agar containing 5% sheep blood (Oxoid, Thermo Scientific, Waltham, MA, USA). Plates were incubated overnight under an atmosphere containing 3.5% H_2, 6% O_2, 7% CO_2, rest N_2 at 37 °C. Cells were inoculated at OD_{ini} ~0.3 in sterile-filtered brain heart infusion broth (BHI; Oxoid, Thermo Scientific, Waltham, MA, USA), and incubated under the same atmosphere at 37 °C for 6–9 h at 140 rpm. Liquid cultures were subcultured under the same conditions in 5 mL BHI with or without 50 µM curcumin. A suitable OD_{ini} was chosen to reach optical densities of 0.1–0.6 after 17 h (± 3 h). 500 µL (± 300 µL) of the cell suspension was harvested by centrifugation (~16000× *g* for 5 min). The pellet was resuspended in 100 µL BHI with or without 50 µM curcumin. DNA labeling and uptake analysis were performed as previously described for *H. pylori* [52]. In short, *C. jejuni* BfR-CA-14430 genomic DNA was extracted using the PureLink Kit (Life Technologies, Thermo Scientific, Waltham, MA, USA), and labeled with fluorescein in a 1:1 (volume:weight) ratio of Label IT reagent to nucleic acid according to the manufacturer's protocol (Mirus Label IT Fluorescein, Mirus Bio LLC, Madison, WI, USA). One µL of labeled DNA (100 ng/µL) was added to the cell suspension and incubated at 5% O_2, 10% CO_2, rest N_2 for 30 min at 37 °C. Subsequently, cells were centrifuged (~16000× *g* for 5 min) and resuspended in 15 µL BHI supplemented with at least 3 U DNaseI (Roche AG, Basel, Switzerland). DNaseI digestion was performed at 37 °C for 5–10 min. The fluorescence microscope Axio Observer ZI (Zeiss, Jena, Germany) with a plan apochromatic 63*x*/1.4 objective and differential interference contrast (DIC) was used for the analysis of *Campylobacter* cells immobilized on 1.5% agarose pads. A metal halide light source (HXP120C) and a filter set with excitation at 470 ± 20 nm and emission at 525 ± 25 nm were used to visualize fluorescein. Exposing times varied between 250 ms and 750 ms. Images were taken by the 12-bit monochromatic AxioCam MRm camera. Cells with at least one fluorescent focus were considered active for DNA uptake, i.e., competent for natural transformation. For each condition, the fraction of competent cells was calculated and data are presented from three independent experiments.

4.12. Cytotoxicity

The CCK-8 assay (Cell Counting Kit-8, Thermo Scientific, Waltham, MA, USA) was performed in 96-well plates with colon epithelial cells HT-29/B6-GR/MR 1 day after seeding in accordance with the manufactures' instructions. The cells were treated with different curcumin concentrations 2 h before infection. The absorption at 450 nm was measured by a spectrophotometer (Tecan GmbH, Maennedorf, Switzerland), and the percentage of viable cells calculated. In HT-29/B6-GR/MR cells, *C. jejuni* induced no significant loss in viability in a pre-test (control 100 ± 18% and DMSO control 81 ± 7% versus *C. jejuni*-infected 81 ± 4%, $n = 3$–4, *n*.s., one-way ANOVA with Bonferroni´s multiple comparison). No effect on cell viability was also seen at curcumin concentrations 30 µM, 40 µM, and 50 µM (85 ± 8%, 64 ± 4%, and 66 ± 6%, $n = 3$–4, *n*.s., one-way ANOVA with Bonferroni´s multiple comparison).

4.13. Ingenuity Pathways Analysis

Ingenuity Pathways Analysis (IPA, Qiagen Silicon Valley, Reswood, CA, USA) was used to evaluate curcumin-dependent expression data from a dataset from human colon biopsies, generated, and analyzed previously [6].

4.14. Statistical Analysis

All data are expressed as mean values ± standard error of the mean (SEM). Statistical analyses were performed with GraphPad Prism (version 7.0, GraphPad Software, Inc., San Diego, CA, USA) using one-way ANOVA with Bonferroni adjustment for multiple comparison or unpaired Student´s *t*-test. For data that were not normally distributed, the nonparametric Mann–Whitney *U*-test was used. $p < 0.05$ was considered to be statistically significant.

Supplementary Materials: Supplementary materials are available online at http://www.mdpi.com/1422-0067/20/19/4830/s1.

Author Contributions: Conceptualization, F.D.L.d.S., J.-D.S., and R.B.; Data curation, F.D.L.d.S., E.B., P.K.N., A.F., S.M., V.M., J.C.G., D.S., M.M.H., and S.B.; Formal analysis, F.D.L.d.S., E.B., P.K.N., A.F., S.M., V.M., J.C.G., K.S., S.K., D.S., C.K., M.M.H., and S.B.; Funding acquisition, J.-D.S. and R.B.; Investigation, F.D.L.d.S., J.-D.S., R.B.; Methodology, J.-D.S. and R.B.; Project administration, J.-D.S. and R.B.; Resources, V.M.; Supervision, J.-D.S. and R.B.; Writing—original draft, F.D.L.d.S.; Writing—review and editing, E.B., P.K.N., A.F., S.M., V.M., J.C.G., K.S., S.K., D.S., C.K., M.M.H., S.B., J.-D.S., and R.B.

Funding: The German Federal Ministries of Education and Research (BMBF) funded the study in connection with the zoonoses research consortium PAC-CAMPY to R.B. and J.D.S. (IP8/01KI1725D), S.B. and M.M.H. (IP7/01KI1725D), K.S. (IP3/01KI1725B), and S.K. (IP3/01KI1725C). The funders had no influence in the study design, data collection, analysis, decision to publish, or preparation of the manuscript. We acknowledge support from the German Research Foundation (DFG) and the Open Access Publication Fund of the Charité–Universitätsmedizin Berlin.

Acknowledgments: In-Fah Maria Lee is gratefully acknowledged for her excellent technical support. We thank Geoffrey I. Sandle, (University of Leeds, Leeds, UK) for his critical discussion and support of the study.

Conflicts of Interest: The authors declare no conflict of interest.

Abbreviations

BHI	Brain heart infusion broth
CBA	Cytometric bead array
CFU	Colony forming units
CLSM	Confocal laser-scanning microscopy
DAPI	4´-6-diamidino-2-phenylindole dihydrochloride
IFN	Interferon
IL	Interleukin
LPS	Lipopolysaccharide
MIC	Minimal inhibitory concentration
PBS	Phosphate buffered saline
PMA	Phorbol 12-myristate 13-acetate

TER	Transepithelial electrical resistance
TJ	Tight junction
TNF	Tumor necrosis factor
ZO	Zonula occludens

Appendix A

Acute Campylobacteriosis Contains Activated Signaling Pathways for which Curcumin Has Counter-Regulatory Properties in IPA Analysis

From acute infected *C. jejuni* patients a RNA-Sequencing (RNA-Seq) analysis with concomitant ingenuity pathway analysis (IPA, Qiagen Silicon Valley) was performed. Once gene expression modifications were revealed, a bioinformatic prediction about possible inhibitors of the changed gene regulation could be carried out [6]. The hypothesis is, that upstream regulators, which have an inhibited activation pattern, may re-activate in *Campylobacter* infection, when the substance is applied during infection. Consequently, inhibited upstream regulators could be protective or therapeutic approaches in *Campylobacter* infection by activation of the corresponding downstream pathways. Different potential candidates were screened for barrier-protective and anti-inflammatory properties in *C. jejuni* infection. One promising predicted regulator candidate that might counter-regulate the *C. jejuni*-induced downstream pathways was curcumin. Curcumin showed a significant effect on downstream target genes, with a p-value of $2.06E^{-5}$ and an activation ^{z}-score of −3.489, and might therefore be another promising barrier-protecting or potential therapeutic substance in campylobacteriosis (Table S1). The *C. jejuni*-induced target genes in the dataset that could be counter-regulated by curcumin belong mainly to pro-inflammatory pathways, such as TNF-α or IL-1β. Another promising and studied candidate against *C. jejuni* infections is calcitriol (active vitamin D). Vitamin D shows in the RNA-Seq analysis with concomitant IPA analysis from patients in contrast to curcumin an even higher significance value (overlap p-value of $8.97E^{-25}$, z-score −6.25; with negative expression direction) [6].

References

1. Kaakoush, N.O.; Castaño-Rodríguez, N.; Mitchell, H.M.; Man, S.M. Global Epidemiology of *Campylobacter* Infection. *Clin. Microbiol. Rev.* **2015**, *28*, 687–720. [CrossRef] [PubMed]
2. Burnham, P.M.; Hendrixson, D.R. *Campylobacter jejuni*: Collective components promoting a successful enteric lifestyle. *Nat. Rev. Microbiol.* **2018**, *16*, 551–565. [CrossRef] [PubMed]
3. Konkel, M.E.; Mead, D.J.; Hayes, S.F.; Cieplak, W., Jr. Translocation of *Campylobacter jejuni* across human polarized epithelial cell monolayer cultures. *J. Infect. Dis.* **1992**, *166*, 308–315. [CrossRef] [PubMed]
4. Brás, A.M.; Ketley, J.M. Transcellular translocation of *Campylobacter jejuni* across polarised epithelial monolayers. *FEMS Microbiol. Lett.* **1999**, *179*, 209–215. [CrossRef] [PubMed]
5. Ketley, J.M. Pathogenesis of enteric infection by *Campylobacter*. *Microbiology* **1997**, *143*, 5–21. [CrossRef] [PubMed]
6. Bücker, R.; Krug, S.M.; Moss, V.; Bojarski, C.; Schweiger, M.R.; Kerick, M.; Fromm, A.; Janßen, S.; Fromm, M.; Hering, N.A.; et al. *Campylobacter jejuni* impairs sodium transport and epithelial barrier function via cytokine release in human colon. *Mucosal Immunol.* **2018**, *11*, 575–577. [CrossRef] [PubMed]
7. Bücker, R.; Schulz, E.; Günzel, D.; Bojarski, C.; Lee, I.F.; John, L.J.; Wiegand, S.; Janßen, T.; Wieler, L.H.; Dobrindt, U.; et al. α-Haemolysin of *Escherichia coli* in IBD: A potentiator of inflammatory activity in the colon. *Gut* **2014**, *63*, 1893–1901. [CrossRef] [PubMed]
8. Watari, A.; Yagi, K.; Kondoh, M. A simple reporter assay for screening Claudin-4 modulators. *Biochem. Biophys. Res. Commun.* **2012**, *426*, 454–460. [CrossRef]
9. Chiba, H.; Osanai, M.; Murata, M.; Kojima, T.; Sawada, N. Transmembrane proteins of tight junctions. *Biochim. Biophys. Acta* **2008**, *1778*, 588–600. [CrossRef]
10. Hakanen, A.; Jousimies-Somer, H.; Siitonen, A.; Huovinen, P.; Kotilainen, P. Fluoroquinolone resistance in *Campylobacter jejuni* isolates in travelers returning to Finland: Association of ciprofloxacin resistance to travel destination. *Emerg. Infect. Dis.* **2003**, *9*, 267–270. [CrossRef]
11. Hakanen, A.J.; Lehtopolku, M.; Siitonen, A.; Huovinen, P.; Kotilainen, P. Multidrug resistance in *Campylobacter jejuni* strains collected from Finnish patients during 1995–2000. *J. Antimicrob. Chemother.* **2003**, *52*, 1035–1039. [CrossRef] [PubMed]
12. Goel, A.; Kunnumakkara, A.B.; Aggarwal, B.B. Curcumin a “Curecumin”: From kitchen to clinic. *Biochem. Pharmacol.* **2008**, *75*, 787–809. [CrossRef] [PubMed]

13. Cho, J.A.; Park, E. Curcumin utilizes the anti-inflammatory response pathway to protect the intestine against bacterial invasion. *Nutr. Res. Pract.* **2015**, *9*, 117–122. [CrossRef] [PubMed]
14. Masahi, O.; Nishida, A.; Sugitani, Y.; Nishino, K.; Inatomi, O.; Sugimoto, M.; Kawahara, M.; Andoh, A. Nanoparticle curcumin ameliorated experimental colitis via modulation of gut microbiota and induction of regulatory T cells. *PLoS ONE* **2017**, *12*, e0185999. [CrossRef]
15. Anggakusuma; Colpitts, C.C.; Schang, L.M.; Rachmawati, H.; Frentzen, A.; Pfaender, S.; Behrendt, P.; Brown, R.J.; Bankwitz, D.; Steinmann, J.; et al. Tumeric curcumin inhibits entry of all hepatitis C virus genotypes into human liver cells. *Gut* **2014**, *63*, 1137–1149. [CrossRef]
16. Mahady, G.B.; Pendland, S.L.; Yun, G.; Lu, Z.Z. Turmeric (*Curcuma longa*) and curcumin inhibit the growth of *Helicobacter pylori*, a group 1 carcinogen. *Anticancer Res.* **2002**, *22*, 4179–4181.
17. Chen, D.-Y.; Shien, J.-H.; Tiley, L.; Chiou, S.S.; Wang, S.Y.; Chang, T.J.; Lee, Y.J.; Chan, K.W.; Hsu, W.L. Curcumin inhibits influenza virus infection and haemagglutination activity. *Food Chem.* **2010**, *119*, 1346–1351. [CrossRef]
18. Marathe, S.A.; Balakrishnan, A.; Negi, V.D.; Sakorey, D.; Chandra, N.; Chakravortty, D. Curcumin reduces the motility of *Salmonella enterica* serovar typhymurium by binding to the flagella, thereby leading to flagellar fragility and shedding. *J. Bacteriol.* **2016**, *198*, 1798–1811. [CrossRef]
19. Moghadamtousi, S.Z.; Kadir, H.A.; Hassandarvish, P.; Tajik, H.; Abubakar, S.; Zandi, K. A review on antibacterial, antiviral, and antifungal activity of curcumin. *Biomed. Res. Int.* **2014**, *2014*, 186864. [CrossRef]
20. Santos, A.M.; Lopes, T.; Oleastro, M.; Gato, I.V.; Floch, P.; Benejat, L.; Chaves, P.; Pereira, T.; Seixas, E.; Machado, J.; et al. Curcumin inhibits gastric inflammation induced by *Helicobacter pylori* infection in a mouse model. *Nutrients* **2015**, *7*, 306–320. [CrossRef]
21. Gunes, H.; Gulen, D.; Mutlu, R.; Gumus, A.; Tas, T.; Topkaya, A.E. Antibacterial effects of curcumin: An in vitro minimum inhibitory concentration study. *Toxicol. Ind. Health* **2016**, *32*, 246–250. [CrossRef] [PubMed]
22. Sharma, R.A.; Geschner, A.J.; Steward, W.P. Curcumin: The story so far. *Eur J. Cancer* **2005**, *41*, 1955–1968. [CrossRef] [PubMed]
23. Wang, J.; Ghosh, S.S.; Ghosh, S. Curcumin improves intestinal barrier function: Modulation of intracellular signaling, and organization of tight junctions. *Am. J. Physiol.-Cell. Physiol.* **2017**, *312*, C438–C445. [CrossRef] [PubMed]
24. Bartik, L.; Whitfield, G.K.; Kaczmarska, M.; Lowmiller, C.L.; Moffet, E.W.; Furmick, J.K.; Hernandez, Z.; Haussler, C.A.; Haussler, M.R.; Jurutka, P.W. Curcumin: A novel nutritionally derived ligand of the vitamin D receptor with implications for colon cancer chemoprevention. *J. Nutr. Biochem.* **2010**, *21*, 1153–1161. [CrossRef] [PubMed]
25. Backert, S.; Boehm, M.; Wessler, S.; Tegtmeyer, N. Transmigration route of *Campylobacter jejuni* across polarized intestinal epithelial cells: Paracellular, transcellular or both? *Cell Commun. Signal.* **2013**, *11*, 72. [CrossRef] [PubMed]
26. Boehm, M.; Hoy, B.; Rohde, M.; Tegtmeyer, N.; Bæk, K.T.; Oyarzabal, O.A.; Brøndsted, L.; Wessler, S.; Backert, S. Rapid paracellular transmigration of *Campylobacter jejuni* across polarized epithelial cells without affecting TER: Role of proteolytic-active HtrA cleaving E-cadherin but not fibronectin. *Gut Pathog.* **2012**, *4*, 3. [CrossRef] [PubMed]
27. Wine, E.; Chan, V.L.; Sherman, P.M. *Campylobacter jejuni* Mediated Disruption of Polarized Epithelial Monolayers is Cell-Type Specific, Time Dependent, and Correlates with Bacterial Invasion. *Pediatric Res.* **2008**, *64*, 599–604. [CrossRef] [PubMed]
28. Chen, M.L.; Ge, Z.; Fox, J.G.; Schauer, D.B. Disruption of tight junctions and induction of proinflammatory cytokine responses in colonic epithelial cells by *Campylobacter jejuni*. *Infect. Immun.* **2006**, *74*, 6581–6589. [CrossRef]
29. Heimesaat, M.M.; Grundmann, U.; Alutis, M.E.; Fischer, A.; Bereswill, S. Small Intestinal Pro-Inflammatory Immune Responses Following *Campylobacter jejuni* Infection of Secondary Abiotic IL-10–/– Mice Lacking Nucleotide-Oligomerization-Domain-2. *Eur. J. Microbiol. Immunol.* **2017**, *7*, 138–145. [CrossRef]
30. Luettig, J.; Rosenthal, R.; Lee, I.-F.M.; Krug, S.M.; Schulzke, J.D. The ginger component 6-shogaol prevents TNF-α-induced barrier loss via inhibition of PI3K/Akt and NF-κB signaling. *Mol. Nutr. Food Res.* **2016**, *60*, 2576–2586. [CrossRef]

31. Bücker, R.; Krug, S.M.; Fromm, A.; Nielsen, H.L.; Fromm, M.; Nielsen, H.; Schulzke, J.-D. *Campylobacter fetus* impairs barrier function in HT-29/B6 cells through focal tight junction alterations and leaks. *Ann. N. Y. Acad. Sci.* **2017**, *1405*, 189–201. [CrossRef] [PubMed]
32. Amasheh, M.; Fromm, A.; Krug, S.M.; Amasheh, S.; Andres, S.; Zeitz, M.; Fromm, M.; Schulzke, J.-D. TNFalpha-induced and berberine-antagonized tight junction barrier impairment via tyrosine kinase, Akt and NFkappaB signaling. *J. Cell Sci.* **2010**, *123*, 4145–4155. [CrossRef]
33. Rosenthal, R.; Luettig, J.; Hering, N.A.; Krug, S.M.; Albrecht, U.; Fromm, M.; Schulzke, J.D. Myrrh exerts barrier-stabilising and –protective effects in HT-29/B6 and Caco-2 intestinal epithelial cells. *Int. J. Colorectal Dis.* **2017**, *32*, 623–634. [CrossRef]
34. Bojarski, C.; Weiske, J.; Schöneberg, T.; Schröder, W.; Mankertz, J.; Schulzke, J.-D.; Florian, P.; Fromm, M.; Tauber, R.; Huber, O. The specific fates of tight junction proteins in apoptotic epithelial cells. *J. Cell Sci.* **2004**, *117*, 2097–2107. [CrossRef]
35. Amasheh, M.; Schlichter, S.; Amasheh, S.; Mankertz, J.; Zeitz, M.; Fromm, M.; Schulzke, J.D. Quercetin enhances epithelial barrier function and increases claudin-4 expression in Caco-2 cells. *J. Nutr.* **2008**, *138*, 1067–1073. [CrossRef] [PubMed]
36. Amasheh, M.; Luettig, J.; Amasheh, S.; Zeitz, M.; Fromm, M.; Schulzke, J.-D. Effects of quercetin studied in colonic HT-29/B6 cells and rat intestine in vitro. *Ann. N. Y. Acad. Sci.* **2012**, *1258*, 100–107. [CrossRef] [PubMed]
37. Young, K.T.; Davis, L.M.; Dirita, V.J. *Campylobacter jejuni*: Molecular biology and pathogenesis. *Nat. Rev. Microbiol.* **2007**, *5*, 665–679. [CrossRef] [PubMed]
38. Hanai, H.; Iida, T.; Takeuchi, K.; Watanabe, F.; Maruyama, Y.; Andoh, A.; Tsujikawa, T.; Fujiyama, Y.; Mitsuyama, K.; Sata, M.; et al. Curcumin maintenance therapy for ulcerative colitis: Randomized, multicenter, double-blind, placebo-controlled trial. *Clin. Gastroenterol. Hepatol.* **2006**, *4*, 1502–1506. [CrossRef] [PubMed]
39. Singla, V.; Pratap Mouli, V.; Garg, S.K.; Rai, T.; Choudhury, B.N.; Verma, P.; Deb, R.; Tiwari, V.; Rohatgi, S.; Dhingra, R.; et al. Induction with NCB-02 (curcumin) enema for mild-to-moderate distal ulcerative colitis—A randomized, placebo-controlled, pilot study. *J. Crohn's Colitis* **2014**, *8*, 208–214. [CrossRef] [PubMed]
40. Lang, A.; Salomon, N.; Wu, J.C.; Kopylov, U.; Lahat, A.; Har-Noy, O.; Ching, J.Y.; Cheong, P.K.; Avidan, B.; Gamus, D.; et al. Curcumin in Combination With Mesalamine Induces Remission in Patients With Mild-to-Moderate Ulcerative Colitis in a Randomized Controlled Trial. *Clin. Gastroenterol. Hepatol.* **2015**, *13*, 1444–1449. [CrossRef] [PubMed]
41. Cruz-Correa, M.; Shoskes, D.A.; Sanchez, P.; Zhao, R.; Hylind, L.M.; Wexner, S.D.; Giardiello, F.M. Combination treatment with curcumin and quercetin of adenomas in familial adenomatous polyposis. *Clin. Gastroenterol. Hepatol.* **2006**, *4*, 1035–1038. [CrossRef] [PubMed]
42. Verma, S.P.; Salamone, E.; Goldin, B. Curcumin and genistein, plant natural products, show synergistic inhibitory effects on the growth of human breast cancer MCF-7 cells induced by estrogenic pesticides. *Biochem. Biophys. Res. Commun.* **1997**, *233*, 692–696. [CrossRef] [PubMed]
43. Balasubramanian, S.; Eckert, R.L. Green tea polyphenol and curcumin inversely regulate human involucrin promoter activity via opposing effects on CCAAT/enhancer-binding protein function. *J. Biol. Chem.* **2004**, *279*, 24007–24014. [CrossRef] [PubMed]
44. Wang, Y.J.; Pan, M.H.; Cheng, A.-L.; Lin, L.-I.; Ho, Y.-S.; Hsieh, C.-Y.; Lin, J.-K. Stability of curcumin in buffer solutions and characterization of its degradation products. *J. Pharm. Biomed. Anal.* **1997**, *15*, 1867–1876. [CrossRef]
45. Bergann, T.; Plöger, S.; Fromm, A.; Zeissig, S.; Borden, S.A.; Fromm, M.; Schulzke, J.D. A colonic mineralocorticoid receptor cell model expressing epithelial Na+ channels. *Biochem. Biophys. Res. Comm.* **2009**, *381*, 280–285. [CrossRef] [PubMed]
46. Kreusel, K.M.; Fromm, M.; Schulzke, J.D.; Hegel, U. Cl- secretion in epithelial monolayers of mucus-forming human colon cells (HT-29/B6). *Am. J. Physiol.* **1991**, *261*, C574–C582. [CrossRef] [PubMed]
47. Zeissig, S.; Bürgel, N.; Günzel, D.; Richter, J.; Mankertz, J.; Wahnschaffe, U.; Kroesen, A.J.; Zeitz, M.; Fromm, M.; Schulzke, J.D. Changes in expression and distribution of claudin 2, 5 and 8 lead to discontinuous tight junctions and barrier dysfunction in active Crohn's disease. *Gut* **2007**, *56*, 61–72. [CrossRef] [PubMed]
48. Kämpfer, A.A.M.; Urbán, P.; Gioria, S.; Kanase, N.; Stone, V.; Kinsner-Ovaskainen, A. Development of an in vitro co-culture model to mimic the human intestine in healthy and diseased state. *Toxicol. In Vitro* **2017**, *45*, 31–43. [CrossRef]

49. Haag, L.M.; Fischer, A.; Otto, B.; Plickert, R.; Kühl, A.A.; Göbel, U.B.; Bereswill, S.; Heimesaat, M.M. Intestinal microbiota shifts towards elevated commensal *Escherichia coli* loads abrogate colonization resistance against Campylobacter jejuni in mice. *PLoS ONE* **2012**, *7*, e35988. [CrossRef]
50. Bereswill, S.; Plickert, R.; Fischer, A.; Kühl, A.A.; Loddenkemper, C.; Batra, A.; Siegmund, B.; Gobel, U.B.; Heimesaat, M.M. What you eat is what you get: Novel *Campylobacter* models in the quadrangle relationship between nutrition, obesity, microbiota and susceptibility to infection. *Eur. J. Microbiol. Immunol.* **2011**, *1*, 237–248. [CrossRef]
51. Gitter, A.H.; Schulzke, J.-D.; Sorgenfrei, D.; Fromm, M. Ussing chamber for high-frequency transmural impedance analysis of epithelial tissues. *J. Biochem. Biophys. Methods* **1997**, *35*, 81–88. [CrossRef]
52. Krüger, N.J.; Knüver, M.T.; Zawilak-Pawlik, A.; Appel, B.; Stingl, K. Genetic Diversity as Consequence of a Microaerobic and Neutrophilic Lifestyle. *PLoS Pathog.* **2016**, *12*, e1005626. [CrossRef] [PubMed]

© 2019 by the authors. Licensee MDPI, Basel, Switzerland. This article is an open access article distributed under the terms and conditions of the Creative Commons Attribution (CC BY) license (http://creativecommons.org/licenses/by/4.0/).

5.3 Publication 2: Comparison of different technologies for the decipherment of the whole genome sequence of *Campylobacter jejuni* BfR-CA-14430.

Epping *et al. Gut Pathog* (2019) 11:59
https://doi.org/10.1186/s13099-019-0340-7

Gut Pathogens

RESEARCH Open Access

Comparison of different technologies for the decipherment of the whole genome sequence of *Campylobacter jejuni* BfR-CA-14430

Lennard Epping[1], Julia C. Golz[2], Marie-Theres Knüver[2], Charlotte Huber[3], Andrea Thürmer[4], Lothar H. Wieler[4], Kerstin Stingl[2] and Torsten Semmler[1*]

Abstract

Background: *Campylobacter jejuni* is a zoonotic pathogen that infects the human gut through the food chain mainly by consumption of undercooked chicken meat, raw chicken cross-contaminated ready-to-eat food or by raw milk. In the last decades, *C. jejuni* has increasingly become the most common bacterial cause for food-born infections in high income countries, costing public health systems billions of euros each year. Currently, different whole genome sequencing techniques such as short-read bridge amplification and long-read single molecule real-time sequencing techniques are applied for in-depth analysis of bacterial species, in particular, Illumina MiSeq, PacBio and MinION.

Results: In this study, we analyzed a recently isolated *C. jejuni* strain from chicken meat by short- and long-read data from Illumina, PacBio and MinION sequencing technologies. For comparability, this strain is used in the German PAC-CAMPY research consortium in several studies, including phenotypic analysis of biofilm formation, natural transformation and in vivo colonization models. The complete assembled genome sequence most likely consists of a chromosome of 1,645,980 bp covering 1665 coding sequences as well as a plasmid sequence with 41,772 bp that encodes for 46 genes. Multilocus sequence typing revealed that the strain belongs to the clonal complex CC-21 (ST-44) which is known to be involved in *C. jejuni* human infections, including outbreaks. Furthermore, we discovered resistance determinants and a point mutation in the DNA gyrase (*gyrA*) that render the bacterium resistant against ampicillin, tetracycline and (fluoro-)quinolones.

Conclusion: The comparison of Illumina MiSeq, PacBio and MinION sequencing and analyses with different assembly tools enabled us to reconstruct a complete chromosome as well as a circular plasmid sequence of the *C. jejuni* strain BfR-CA-14430. Illumina short-read sequencing in combination with either PacBio or MinION can substantially improve the quality of the complete chromosome and epichromosomal elements on the level of mismatches and insertions/deletions, depending on the assembly program used.

Keywords: *Campylobacter jejuni*, Long read sequencing, Hybrid assemblies, Assembler comparison, Antibiotic resistance

Background

Campylobacter jejuni is a Gram-negative bacterium that colonizes a wide range of hosts as part of the natural gut microbiota [1]. It is frequently found in farm animals such as chicken and cattle or in wild birds. While consuming undercooked poultry meat, unpasteurized milk or cross-contaminated ready-to-eat food it can colonize the human gut and cause an infectious gastroenteritis together with diarrhea, fever and cramps [2].

Over the past two decades the incidence of *Campylobacter* infections has continued to increase worldwide and has become a dangerous threat to public health. To

*Correspondence: SemmlerT@rki.de
[1] NG1-Microbial Genomics, Robert Koch Institute, Nordufer 20, 13353 Berlin, Germany
Full list of author information is available at the end of the article

© The Author(s) 2019. This article is licensed under a Creative Commons Attribution 4.0 International License, which permits use, sharing, adaptation, distribution and reproduction in any medium or format, as long as you give appropriate credit to the original author(s) and the source, provide a link to the Creative Commons licence, and indicate if changes were made. The images or other third party material in this article are included in the article's Creative Commons licence, unless indicated otherwise in a credit line to the material. If material is not included in the article's Creative Commons licence and your intended use is not permitted by statutory regulation or exceeds the permitted use, you will need to obtain permission directly from the copyright holder. To view a copy of this licence, visit http://creativecommons.org/licenses/by/4.0/. The Creative Commons Public Domain Dedication waiver (http://creativecommons.org/publicdomain/zero/1.0/) applies to the data made available in this article, unless otherwise stated in a credit line to the data.

date, campylobacteriosis is the most common bacterial cause of food-born infections in high income countries, with costs amounting to 2.4 billion euros each year for the public health system and lost productivity in the European Union [3].

The BfR-CA-14430 strain was first isolated during the zoonosis monitoring program, in which distinct matrix–pathogen combinations were collected by federal state laboratories. The strain was isolated from a German chicken meat sample in August 2016 using ISO 10272-1:2006 [4]. Since this strain was chosen to serve as a fresh field strain for the German research consortium PAC-CAMPY, we analyzed characteristics of BfR-CA-14430, like antibiotic resistance and virulence factors. In addition, we gained a deeper insight into whole genome sequencing and the impact of various assembly programs, including different hybrid assemblers on various combinations of long and short read sequencing technologies. This revealed a complete chromosomal sequence as well as one closed plasmid sequence.

Methods

Bacterial isolation and initial characterization

BfR-CA-14430 was isolated in the framework of the zoonosis monitoring program 2016 from chicken meat according to ISO 10272-1:2006. Species identification was performed by Real-time PCR according to Mayr et al. [5]. The multi locus sequence type was determined by Sanger sequencing (PubMLST) and confirmed by whole-genome sequencing (WGS). The flagellin subunit A (*flaA)* type was Sanger sequenced [6], typing was done according to PubMLST (pubmlst.org) and compared with the outcome of the WGS analysis. BfR-CA-14430 was cultured either on Columbia blood agar (Oxoid) or in brain heart infusion (Oxoid) at 42 °C under microaerobic conditions (5% O_2, 10% CO_2) and cells were harvested by centrifugation.

Antimicrobial resistance determination by microdilution

BfR-CA-14430 was pre-cultured on Columbia blood agar for 24 h at 42 °C under microaerobic atmosphere. Broth microdilution susceptibility testing was performed according to VET06 and M45-A [7]. $2–8 \times 10^5$ CfU/ml were inoculated into cation-supplemented Mueller Hinton broth (TREK Diagnostic Systems, UK) supplemented with 5% fetal calf serum (PAN-Biotech, Germany), into the European standardized microtiter EUCAMP2 or EUVSEC plate formats (TREK Diagnostic Systems). Samples were incubated for 48 h at 37 °C under microaerobic conditions. Minimal inhibitory concentrations (MIC; [mg/l]) were semi-automatically analyzed using the Sensititre Vizion system and the SWIN-Software (TREK Diagnostic Systems). Epidemiological cut-off values for resistance determination were based on the European Committee on Antimicrobial Susceptibility Testing (EUCAST.org), if already defined for *C. jejuni* or, alternatively, for *Salmonella* (EUVSEC plate format).

Genomic DNA extraction and sequencing

DNA extraction for Sanger MLST analyses was performed with GeneJET Genomic DNA Purification Kit (Thermo Fisher Scientific). DNA for WGS was prepared using the MagAttract HMW Genomic Extraction Kit (Qiagen) (for PacBio and Illumina sequencing) and QIAamp DNA Mini Kit (Qiagen) for MinION sequencing and further concentrated by precipitation with 0.3 M sodium acetate pH 5 and 0.7 volume isopropanol at room temperature for 30 min. After centrifugation and washing of the precipitate with 70% ice-cold ethanol, the DNA was dissolved in Tris buffer pH 7.5. The quality of the DNA was evaluated by spectral analysis (NanoDrop Spectrophotometer, Thermo Fisher Scientific, USA) and the concentration was fluorimetrically quantified to be 110 ng/µl by Qubit 3.0 Fluorometer (dsDNA BR Assay Kit; Invitrogen, USA). DNA was additionally controlled for lack of sheering products < 20 kb on a 0.8% agarose gel. Sequencing was performed on a MiSeq sequencer (MiSeq Reagent Kit v.3; Illumina Inc., San Diego, CA, USA), using the Library Preparation kit Nextera XT (Illumina Inc., San Diego, CA, USA) resulting in 300-bp paired-end reads and an average coverage of around 100-fold. Furthermore, size selection was performed using 10 K Blue Pippin and DNA was sequenced with Single Molecule Real-Time (SMRT) Sequencing Technology on a PacBio RS II by GATC Biotech AG (Konstanz, Germany) as well as with long read sequencing on Oxford Nanopore MinION (Oxford, UK) (Library-Kit: Rapid Barcoding Kit (SQK-RBK004), Flowcell: 1D R9.4, without size selection, base calling with albacore v2.1.0) in order to compare these three techniques for establishing a complete genome with epichromosomal elements. Total amounts of extracted DNA of 1 ng, 5 µg and 400 ng was used as starting material for sequencing by MiSeq, PacBio or MinION, respectively. A general overview of the raw data from the different sequencing machines can be found in Table 1.

Genome assembly and annotation

Sequencing reads obtained from the MiSeq sequencer were (i) assembled by the SPAdes v3.12 [8] and plasmid-SPAdes [9] assembler or (ii) used to correct long read data. Furthermore we used the CLC Genomics Workbench v12.0.1 as well as an assembly from the PacBio in-house pipeline HGAP v3.0 [10] and Flye v2.5 [11] for

Table 1 Summary of the raw output from Illumina, MinION, and PacBio sequencing technologies

Technology	Number of reads	Total number of bases	Median read length	Calculated mean genome coverage
Illumina MiSeq	658,314	165,840,055	285	98×
PacBio RS II	88,482	802,118,168	9065	475×
MinION	61,960	737,318,830	8073	436×

the PacBio long read assemblies. The assembly based on MinION raw reads was only performed by Flye v2.5. All assemblers were run with default settings. To generate an optimal assembly and derive a closed genome sequence we tested various de novo hybrid assembly tools on different combinations of short and long reads (Unicycler v0.4.7 [12] and wtdbg2 v2.1 [13]). Unicycler first creates a draft genome assembly with SPAdes v3.12 and connects the contigs only afterwards by using the long reads from PacBio or MinION. Wtdbg2, on the other hand, first assembles the long reads and corrects the assembly afterwards by mapping the short reads against the genome. Long reads were mapped to the genomes by minimap2 v2.14 [14]. The different combinations of short and long reads used for each tool are shown in Table 2. In order to annotate the genomes, a custom-made database of 137 complete genomes of *C. jejuni* downloaded from NCBI (Additional file 1: Table S1) was built and used as a Genus-specific BLAST database for Prokka v1.13 [15].

Assembly comparison and in silico analysis

The assembled genomes were compared by the progressive Mauve algorithm [16] to detect major structural differences. Single nucleotide polymorphisms (SNPs) were detected by mapping the Illumina paired-end reads against the assemblies by bowtie2 v4.8.2 [17] with the end-to-end sensitive mode. SNPs, insertions and deletions were counted within an allele frequency of at least 75% at positions with a minimum of 10 reads by freebayes v.1.2.0 [18] according to Illumina short reads. The multi locus sequence typing (MLST) was performed by a BLAST based pipeline (https://github.com/tseemann/mlst) to identify the allele variants of the seven housekeeping genes (*aspA*, *glnA*, *gltA*, *glyA*, *pgm*, *tkt* and *uncA*). Point mutations conferring antibiotic resistance or individual antibiotic resistance genes were revealed by ResFinder 3.0 [19] (CGE, DTU, Lyngby, DK; https://cge.cbs.dtu.dk/services/ResFinder/).

Table 2 Summary of the assembler performance based on different sequencing technologies

Index	Data	Assembler	#Contigs	#bp total length	#Chromosomal contigs; #bp	#plasmid; #bp	Insertions, deletions and SNPs	Covered by illumina reads	Sequence identity of *flaA*
A	Illumina	SPAdes	30	1,666,0451	30; 77,674 (N50)[a]	Cannot be directly detected	0[b]	99.9[c]	100
B	PacBio	HGAP	2	1,733,585	1; 1,668,827	1; 64,758	155	99.46	100
C	PacBio	Flye	2	1,687,377	1; 1,645,611	1; 41,766	255	99.99	100
D	PacBio	CLC	2	1,688,161	1; 1,646,367	1; 41,794	253	99.97	100
E	PacBio + Illumina	Unicycler	3	1,684,748	2; 1,631,764/ 11,212	1; 41,772	0	99.9	100
F	PacBio + Illumina	wtdbg2	3	1,693,078	2; 1,644,895/ 6,442	1; 41,741	47	99.65	100
G	MinION	Flye	2	1,720,675	1; 1,678,003	1; 42,673	24,439	99.36	99.6
H	MinION + Illumina	Unicycler	2	1,687,752	1; 1,645,980	1; 41,722	20	99.94	100
I	MinION + Illumina	wtdbg2	5	1,672,121	4; 1,648,160/ 15,620/12,211/6,130	1/41,957	169	98.15	100

[a] Quality of draft genomes can be measured by the N50 value

[b] Illumina paired-end data is taken as ground truth for identification of SNPs, insertions and deletions

[c] As result of the scaffolding process, performed by the SPAdes assembler, contigs with known distance, but unknown sequence content, are connected by "N"s. Thus, the SPAdes assembly is not covered by Illumina data by 100%

Quality assurance

In order to perform an in-silico control for contamination within the sequenced DNA, Illumina short reads were adapter trimmed with Flexbar [20] and all reads were taxonomically classified as *C. jejuni* by Kraken v2.0.6 [21]. Taxonomic classification of the long reads could identify 3.71% of Human related DNA within the PacBio read, which has been removed. Assembly completeness and contamination was controlled with checkM v. 1.0.18 [22].

Results

Antimicrobial resistance profile of BfR-CA-14430

The minimal inhibitory concentration (MIC) of different antibiotics was determined using the broth microdilution susceptibility approach (CLSI). Using the standard EUCAMP2 plate format, which is used for screening of *C. jejuni* resistance during zoonosis monitoring, the strain showed resistance against ciprofloxacin, nalidixic acid and tetracycline but was sensitive towards erythromycin, gentamicin and streptomycin. We extended the antimicrobial substances and applied the EUVSEC plate format, usually tested with *Salmonella* and *Escherichia coli* isolates. As *C. jejuni* is intrinsically resistant against most of the cephalosporine antibiotics, it was expected that strain BfR-CA-14430 was also resistant against cefotaxime, cefoxitime, cefepime, ceftazidime. The cephalosporine cefoperazone is used as a selective supplement in ISO 10272:2017 in mCCDA (modified charcoal-cefoperazone agar) and Bolton broth. Besides, the strain revealed natural resistance against trimethroprim due to the absence of the target dihydrofolate reductase (FolA). However, MIC values for sulfamethoxazole were 16 mg/l, rendering the strain sensitive, on the basis of a cut-off value used for *Salmonella* of 64 mg/l. Furthermore, resistance against ampicillin was also seen with MIC values >64 mg/l, while MIC values for meropeneme, ertapeneme and colistin were 0.25 and 0.5 and 2 mg/l, respectively. BfR-CA-14430 was fully susceptible to chloramphenicol, tigecycline, azithromycin and imipeneme, with MIC values below the lowest test concentration.

Genomic features of the strain BfR-CA-14430

Using multilocus sequence typing, the strain BfR-CA-14430 was identified as sequence type ST-44 which belongs to the clonal complex CC-21 that is frequently found in human infections and well known to cause *C.*

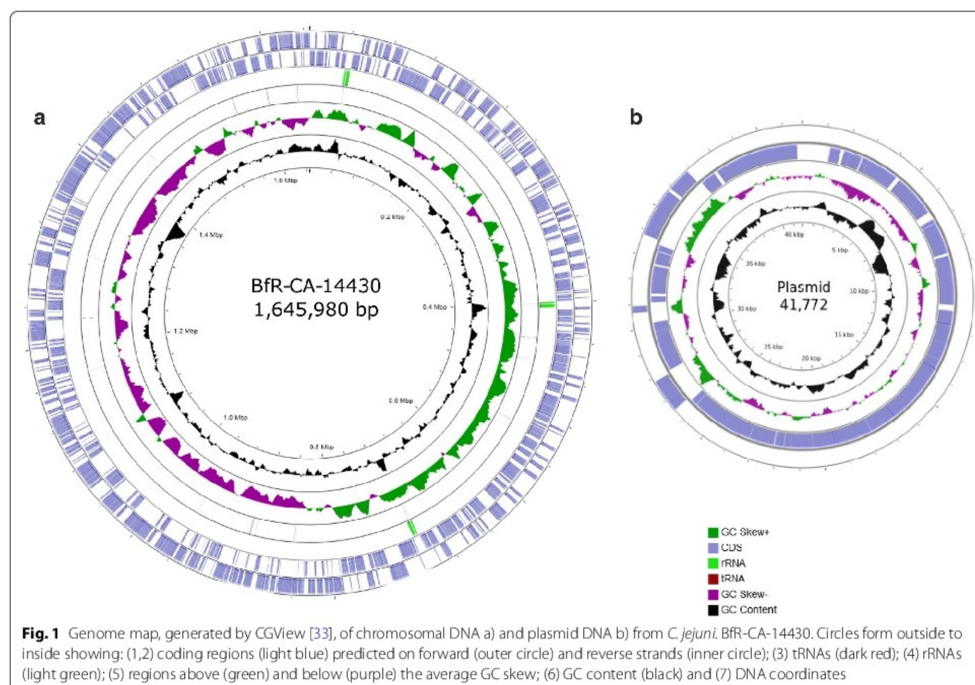


Fig. 1 Genome map, generated by CGView [33], of chromosomal DNA a) and plasmid DNA b) from *C. jejuni*. BfR-CA-14430. Circles form outside to inside showing: (1,2) coding regions (light blue) predicted on forward (outer circle) and reverse strands (inner circle); (3) tRNAs (dark red); (4) rRNAs (light green); (5) regions above (green) and below (purple) the average GC skew; (6) GC content (black) and (7) DNA coordinates

jejuni outbreaks [23]. The complete genome sequence, assembled from MinION and Illumina reads by Unicycler, consists of one chromosome of 1,645,980 bp covering 1,665 coding sequences (CDSs), including bla_{OXA-61} (Cj0299 in NCTC 11168) that encodes for a beta-lactam resistance gene [24] and a point mutation in the gyrase subunit A (gyrA) (T86I) [25], conferring resistance against (fluoro-)quinolones. All AMR genes or AMR associated SNPs could be detected within the hybrid assembly as well as in the Illumina paired-end reads. Additionally, the genome has 44 transfer RNA (tRNA) genes, 9 ribosomal RNA (rRNA) genes forming three identical operons consisting of 16S, 23S and 5S subunits and an overall GC content of 30.4%. The chromosome harbors the virulence factors *cdtA*, *cdtB*, *cdtC*, coding for the cytolethal distending toxin, the gene encoding the fibronectin-binding protein CadF and the *Campylobacter* invasion antigens CiaB and CiaC. Genes encoding the monofunctional α 2,3-sialyltransferase CstIII and the *N*-acetylneuraminic acid biosynthesis proteins NeuA1, NeuB1 and NeuC1 are present for lipooligosacharide (LOS) sialylation, which was shown to be linked to Guillain–Barré syndrome onset [26, 27]. The conserved capsule biosynthesis *kpsC* and *kpsF* genes flank the variable capsule locus of approximately 26 kb, belonging to the Penner type HS1 complex [28]. Besides, the *pseA-I* genes involved in flagellar protein glycosylation [29] were detected on the chromosome. Furthermore, the strain carries a single circular plasmid of 41,772 bp including 46 CDSs. Among these genes the plasmid carries a *tetO* gene for tetracycline resistance as well as *virB2-11* and *virD4* genes encoding for a putative type IV secretion system (T4SS), for conjugative DNA transfer between *Campylobacter* strains [30]. The plasmid showed 93% identity and 98% coverage with plasmid pTet from *C. jejuni* strain 81–176 (45,025 bp) (CP000549) and 98% identity and 97% coverage with plasmid pMTVDSCj16-1 (42,686 bp) from *C. jejuni* strain MTVDSCj16 (NZ_CP017033.1) that carry type IV secretion systems and *tetO* genes as well [31]. By mapping of Illumina paired-end reads, plasmid pMTVDSCj16-1 was covered by 97% with 99% identity and 611 SNPs. Two regions of 600 bp and 1113 bp were not covered by the Illumina reads. However, read mapping was not able to detect a region 927 bp containing a CDS that can also be found in pTet-M129 (NZ_CP007750.1) [32] of *C. jejuni* strain M129 (NZ_CP007749.1) and pRM5611 (NZ_CP007180.1) from C. coli strain RM5611 (NZ_CP007179.1).

Fig. 2 Progressive Mauve Alignment of chromosomal genomes generated by different assemblers. The Misassembly made by SPAdes is marked by the red square. Assemblies are index by alphabetic letters as shown in Table 2. Color coded blocks indicating homology between the genomes

The genomic structure and annotation of the chromosome and plasmid are visualized in Fig. 1 and can be accessed at the National Center for Biotechnology Information (NCBI) database with the accession numbers CP043763 and CP043764.

Assembly comparison

Whole genome comparison of all assemblies showed that each assembler created one chromosome of around 1.6 Mb as well as one plasmid of around 42 kb while using PacBio or MinION long reads in combinations with Illumina short reads (Table 2). Gel electrophoresis of extracted DNA from BfR-CA-14430 suggested the occurrence of chromosomal and plasmid DNA. All long read assembler reconstructed the chromosomal genome in one single contig without large structural variations (Fig. 2). Reads from MinION and Illumina that were assembled by Unicycler resulted in a circular genome. However, some tools generated small extra contigs (Table 2): The combination of Illumina and PacBio data as well as MinION with Illumina data as input to the wtdgb2 assembler generated contigs that were later identified by BLAST to be part of the chromosomal sequence of the strain. With the advantage of using long reads, one misassembly inside a repeat region in the SPAdes assembly based on the Illumina short reads was discovered (Fig. 2). Additionally, we were able to identify the Sanger sequenced *flaA* gene with a sequence

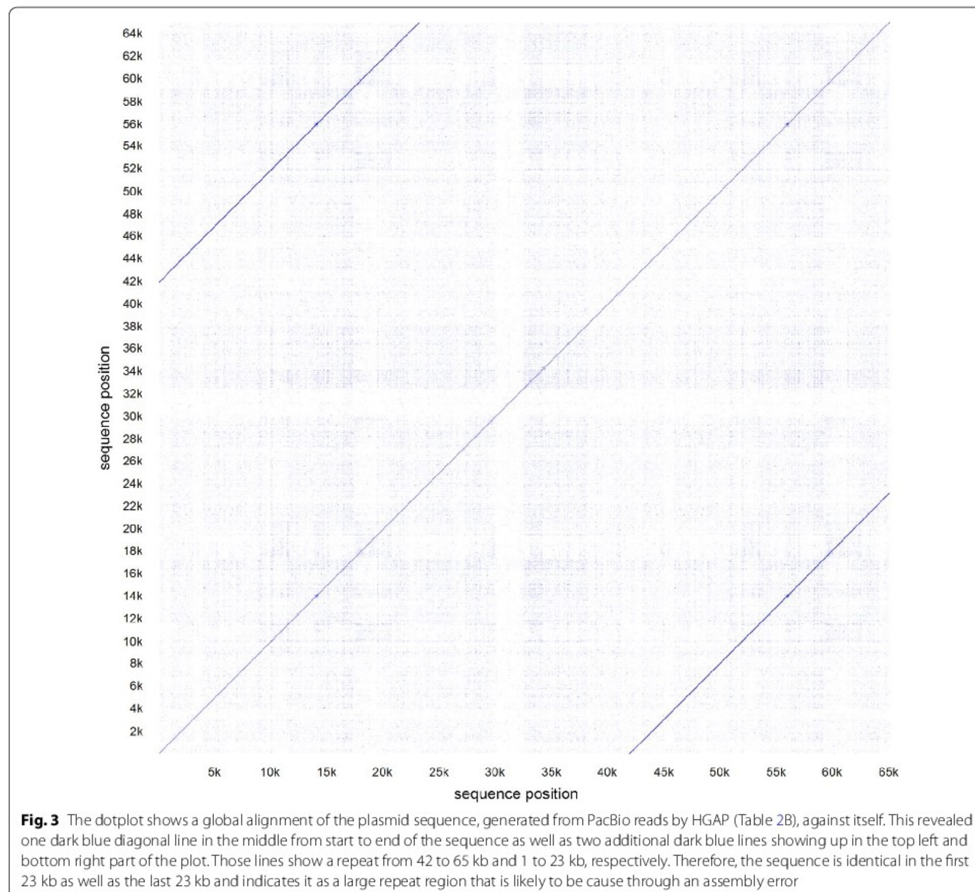


Fig. 3 The dotplot shows a global alignment of the plasmid sequence, generated from PacBio reads by HGAP (Table 2B), against itself. This revealed one dark blue diagonal line in the middle from start to end of the sequence as well as two additional dark blue lines showing up in the top left and bottom right part of the plot. Those lines show a repeat from 42 to 65 kb and 1 to 23 kb, respectively. Therefore, the sequence is identical in the first 23 kb as well as the last 23 kb and indicates it as a large repeat region that is likely to be cause through an assembly error

identity of 100% in most of the cases (Table 2). The MinION assembly generated with Flye did not reach 100% sequence identity, due to the high number of SNPs within this assembly.

Furthermore, all tools assembled a plasmid with a size of around 42 kb, except from the PacBio in-house pipeline that created a 64 kb plasmid. By performing a global alignment against itself and generating a dotplot we could show a large repeat region between the first and the last 20 kb in the circular sequence that obviously originates from an assembly error (Fig. 3). Plasmid assemblies produced by Unicycler were found to be circularized, while using PacBio as well as MinION data. Identification of plasmid sequences by plasmidSPAdes, revealed 9 from 3 components. Besides the ca. 42 kb plasmid described earlier, the 8 other sequences could be identified as part of the chromosomal DNA by BLAST from strain BfR-CA-11430 as well as in several closed genomes from Additional file 1: Table S1. Those assembled DNA fragments mainly have their origin in low coverage or repeat regions, which cannot be resolved by short reads and is known to lead to misassemblies in plasmidSPAdes [9].

Standalone assemblies of long read data from MinION generated the overall correct structure of the genome and the plasmid, but a lot of small insertions, deletions and SNPs were additionally created (Table 2). The assembly of MinION raw reads contains more than 25,000 SNPs, which is around 100 times more compared to assemblies of PacBio reads with HGAP and Flye. However, by combining MinION with Illumina data the SNP count decreased to only 20 SNPs. The assembly from HGAP or Flye based on PacBio raw reads contains 155 SNPs and 255 SNPs respectively whereas the combination of PacBio and Illumina contains 0 SNPs.

The final chromosomal assembly of MinION and Illumina reads is covered by 95×, 424× and 375×, whereas the plasmid sequence is covered by 204×, 291× and 3021× from Illumina, PacBio and MinION reads. Genome completeness was calculated to be at 99.36% and contamination was predicted to be 0.15%.

Conclusion

Here, we describe the *C. jejuni* strain BfR-CA-14430 that carries a beta lactamase and tetracycline resistance gene as well as potential virulence factors that might play a role in human gut infection. Furthermore we compared multiple hybrid assembly methods based on different sequencing technologies. This revealed that the combination of long reads with short reads decreases the SNP rate in de novo assemblies to a large extent. In general, using a combination of long and short reads as input to the Unicycler assembler resulted in accurate and closed chromosomal and plasmidal sequences for our data. However, assemblies based only on PacBio reads, seem to be highly accurate and can also be used without being polished by Illumina data.

Supplementary information

Supplementary information accompanies this paper at https://doi.org/10.1186/s13099-019-0340-7.

Additional file 1: Table S1. *Campylobacter jejuni* Genomes used to build the Prokka Database

Acknowledgements
Not applicable.

Authors' contributions
LE, KS, LHW and TS designed the study. JCG, MTK CH and AT performed the laboratory work. LE and TS did the bioinformatics analysis. All authors read and approved the final manuscript.

Funding
Funding was received by the German Federal Ministry of Education and Research (BMBF) and by the German Federal Ministry of Health (BMG) for the PAC-CAMPY and MinION-Zoo research project and by BfR. L.E. and T.S. were financed in PAC-CAMPY IP10/01KI1725F and MinION-Zoo ZMVI1-2519NIK704 and J.G. and K.S. in PAC-CAMPY IP3/01KI1725B and BfR-SFB-1332-646. The funders had no influence in the study design, data analysis or publication.

Availability of data and materials
The completed genome sequence of *BfR-CA-14430* has been deposited into GenBank database with accession number CP043763 (chromosome) and CP043764 (plasmid), respectively. Raw read data from Illumina Miseq, PacBio RS II and Oxford Nanopore MinION is available at NCBI with SRA accession number PRJNA562653.

Ethics approval and consent to participate
Not applicable.

Competing interests
The authors declare that they have no competing interests.

Author details
[1] NG1-Microbial Genomics, Robert Koch Institute, Nordufer 20, 13353 Berlin, Germany. [2] National Reference Laboratory for Campylobacter, Department of Biological Safety, Federal Institute for Risk Assessment, Berlin, Germany. [3] Advanced Light and Electron Microscopy, Robert Koch Institute, Berlin, Germany. [4] Methodology and Research Infrastructure, Robert Koch Institute, Berlin, Germany.

Received: 18 September 2019 Accepted: 5 December 2019
Published online: 16 December 2019

References

1. Young KT, Davis LM, DiRita VJ. *Campylobacter jejuni*: molecular biology and pathogenesis. Nat Rev Microbiol. 2007;5:665–79.
2. Humphrey T, O'Brien S, Madsen M. *Campylobacters* as zoonotic pathogens: a food production perspective. Int J Food Microbiol. 2007;117(3):237–57.
3. Food Safety Authority E, Boelaert F, Van der Stede Y, Nagy K, Rizzi V, Garcia Fierro R, et al. The European Union summary report on trends and sources of zoonoses, zoonotic agents and food-borne outbreaks in 2016 Acknowledgements: EFSA and the ECDC wish to thank the members of

the Scientific Network for Zoonoses Monitoring Data and the Food and Wat. EFSA J. 2017;15(12):5077.
4. Jacobs-Reitsma WF, Jongenburger I, de Boer E, Biesta-Peters EG. Validation by interlaboratory trials of EN ISO 10272—microbiology of the food chain—horizontal method for detection and enumeration of *Campylobacter* spp.—part 2: Colony-count technique. Int J Food Microbiol. 2019;288:32–8.
5. Mayr AM, Lick S, Bauer J, Thärigen D, Busch U, Huber I. Rapid detection and differentiation of *Campylobacter jejuni*, *Campylobacter coli*, and *Campylobacter lari* in food, using multiplex real-time PCR. J Food Protect. 2010;73(2):241–50.
6. Wassenaar TM, Newell DG. Genotyping of *Campylobacter* spp. Appl Environ Microbiol. 2000;66:1–9.
7. Clinical and Laboratory Standards Institute. VET06: methods for antimicrobial susceptibility testing of infrequently isolated or fastidious bacteria isolated from animals. 1st ed. Wayne, PA: Clinical and Laboratory Standards Institute; 2017.
8. Bankevich A, Nurk S, Antipov D, Gurevich AA, Dvorkin M, Kulikov AS, et al. {SPAdes}: a new genome assembly algorithm and its applications to single-cell sequencing. J Comput Biol. 2012;19(5):455–77.
9. Antipov D, Hartwick N, Shen M, Raiko M, Lapidus A, Pevzner PA. PlasmidSPAdes: assembling plasmids from whole genome sequencing data. Bioinformatics. 2016;32(22):3380–7.
10. Chin CS, Alexander DH, Marks P, Klammer AA, Drake J, Heiner C, et al. Nonhybrid, finished microbial genome assemblies from long-read SMRT sequencing data. Nat Methods. 2013;10(6):563–9.
11. Kolmogorov M, Yuan J, Lin Y, Pevzner PA. Assembly of long, error-prone reads using repeat graphs. Nat Biotechnol. 2019;37(5):540–6.
12. Wick RR, Judd LM, Gorrie CL, Holt KE. Unicycler: Resolving bacterial genome assemblies from short and long sequencing reads. PLoS Comput Biol. 2017;13(6):e1005595.
13. Ruan J, Li H. Fast and accurate long-read assembly with wtdbg2. bioRxiv. 2019. https://doi.org/10.1101/530972.
14. Li H. Minimap2: pairwise alignment for nucleotide sequences. Bioinformatics. 2018;34(18):3094–100.
15. Seemann T. Prokka: rapid prokaryotic genome annotation. Bioinformatics. 2014;30(14):2068–9.
16. Darling AE, Mau B, Perna NT. progressiveMauve: multiple genome alignment with gene gain, loss and rearrangement. PLoS ONE ONE. 2010;5(6):e11147.
17. Langmead B, Salzberg SL. Fast gapped-read alignment with Bowtie 2. Nat Methods. 2012;9(4):357–9.
18. Garrison E, Marth G. Haplotype-based variant detection from short-read sequencing. arXiv preprint. 2012. arXiv:1207.3907.
19. Zankari E, Hasman H, Cosentino S, Vestergaard M, Rasmussen S, Lund O, et al. Identification of acquired antimicrobial resistance genes. J Antimicrob Chemother. 2012;67(11):2640–4.
20. Roehr JT, Dieterich C, Reinert K. Flexbar 3.0—{SIMD} and multicore parallelization. Bioinformatics. 2017;33(18):2941–2.
21. Wood DE, Salzberg SL. Kraken: Ultrafast metagenomic sequence classification using exact alignments. Genome Biol. 2014;15(3):R46.
22. Parks DH, Imelfort M, Skennerton CT, Hugenholtz P, Tyson GW. CheckM: assessing the quality of microbial genomes recovered from isolates, single cells, and metagenomes. Genome Res. 2015;25:1043–55.
23. Sails AD, Swaminathan B, Fields PI. Utility of multilocus sequence typing as an epidemiological tool for investigation of outbreaks of gastroenteritis caused by *Campylobacter jejuni*. J Clin Microbiol. 2003;41(10):4733–9.
24. Griggs DJ, Peake L, Johnson MM, Ghori S, Mott A, Piddock LJV. β-lactamase-mediated β-lactam resistance in *Campylobacter* species: prevalence of Cj0299 (blaOXA-61) and evidence for a novel β-lactamase in *C. jejuni*. Antimicrob Agents Chemother. 2009;53(8):3357–64.
25. Luo N, Sahin O, Lin J, Michel LO, Zhang Q. In vivo selection of *Campylobacter* isolates with high levels of fluoroquinolone resistance associated with gyrA mutations and the function of the CmeABC efflux pump. Antimicrob Agents Chemother. 2003;47(1):390–4.
26. Guerry P, Ewing CP, Hickey TE, Prendergast MM, Moran AP. Sialylation of lipooligosaccharide cores affects immunogenicity and serum resistance of *Campylobacter jejuni*. Infect Immun. 2000;68(12):6656–62.
27. Godschalk PCR, Heikema AP, Gilbert M, Komagamine T, Wim Ang C, Glerum J, et al. The crucial role of *Campylobacter jejuni* genes in anti-ganglioside antibody induction in Guillain-Barré syndrome. J Clin Investig. 2004;114(11):1659–65.
28. Poly F, Serichatalergs O, Schulman M, Ju J, Cates CN, Kanipes M, et al. Discrimination of major capsular types of *Campylobacter jejuni* by multiplex PCR. J Clin Microbiol. 2011;49(5):1750–7.
29. Guerry P, Ewing CP, Schirm M, Lorenzo M, Kelly J, Pattarini D, et al. Changes in flagellin glycosylation affect *Campylobacter autoagglutination* and virulence. Mol Microbiol. 2006;60(2):299–311.
30. Cao TB, Saier J. Conjugal type IV macromolecular transfer systems of Gram-negative bacteria: Organismal distribution, structural constraints and evolutionary conclusions. Microbiol Microbiol Soc. 2001;147:3201–14.
31. Taveirne ME, Dunham DT, Perault A, Beauchamp JM, Huynh S, Parker CT, et al. Complete annotated genome sequences of three *Campylobacter jejuni* strains isolated from naturally colonized Farm-Raised chickens. Genome Announc. 2017;5(4):e01407-16.
32. Day WA, Pepper IL, Joens LA. Use of an arbitrarily primed PCR product in the development of a *Campylobacter jejuni*-specific PCR. Appl Environ Microbiol. 1997;63:1019–23.
33. Grant JR, Stothard P. The CGView Server: a comparative genomics tool for circular genomes. Nucleic Acids Res. 2008;36(Web Server issue):W181–W184184.

Publisher's Note

Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

Ready to submit your research? Choose BMC and benefit from:

- fast, convenient online submission
- thorough peer review by experienced researchers in your field
- rapid publication on acceptance
- support for research data, including large and complex data types
- gold Open Access which fosters wider collaboration and increased citations
- maximum visibility for your research: over 100M website views per year

At BMC, research is always in progress.

Learn more biomedcentral.com/submissions

5.4 Publication 3: Whole genome sequencing reveals extended natural transformation in *Campylobacter* impacting diagnostics and the pathogens adaptive potential.

www.nature.com/scientificreports

SCIENTIFIC REPORTS
natureresearch

OPEN

Whole genome sequencing reveals extended natural transformation in *Campylobacter* impacting diagnostics and the pathogens adaptive potential

Julia C. Golz[1,4], Lennard Epping[3,4], Marie-Theres Knüver[1], Maria Borowiak[2], Felix Hartkopf[3], Carlus Deneke[2], Burkhard Malorny[2], Torsten Semmler[3] & Kerstin Stingl[1*]

***Campylobacter* is the major bacterial agent of human gastroenteritis worldwide and represents a crucial global public health burden. Species differentiation of *C. jejuni* and *C. coli* and phylogenetic analysis is challenged by inter-species horizontal gene transfer. Routine real-time PCR on more than 4000 *C. jejuni* and *C. coli* field strains identified isolates with ambiguous PCR results for species differentiation, in particular, from the isolation source eggs. K-mer analysis of whole genome sequencing data indicated the presence of *C. coli* hybrid strains with huge amounts of *C. jejuni* introgression. Recombination events were distributed over the whole chromosome. MLST typing was impaired, since *C. jejuni* sequences were also found in six of the seven housekeeping genes. cgMLST suggested that the strains were phylogenetically unrelated. Intriguingly, the strains shared a stress response set of *C. jejuni* variant genes, with proposed roles in oxidative, osmotic and general stress defence, chromosome maintenance and repair, membrane transport, cell wall and capsular biosynthesis and chemotaxis. The results have practical impact on routine typing and on the understanding of the functional adaption to harsh environments, enabling successful spreading and persistence of *Campylobacter*.**

Since 2005, *Campylobacter* is the major zoonotic agent in the European Union, causing 250,161 confirmed campylobacteriosis cases in 2017[1]. Around one third of the cases can be directly attributed to handling, preparation and consumption of broiler meat[2]. Measures for *Campylobacter* reduction focus on virulence mechanisms and persistence factors, enabling the pathogen to successfully circulate within the food chain.

Typing of *Campylobacter* by species differentiation methods and by multi-locus sequence typing (MLST) has become key tools for diagnostics and source attribution. Specific gene targets have proven stable and were, therefore, chosen for this purpose. Two of commonly used species differentiation markers[3–5] are *mapA*, a fitness factor in chicken colonization[6] and *ceuE* playing a role in iron acquisition[7]. For MLST, central enzymatic functions, which are conserved in the genome were defined[8] and are commonly used for phylogenetic analysis.

It was shown that high level of interspecies transfer of genetic material can occur between *C. jejuni* and *C. coli*[9]. Adaptation to hosts can modulate the gene pool and allele variants and was suggested to be of more relevance than geographical location[10].

Here we identified extensive interspecies gene transfer from *C. jejuni* to *C. coli*, impairing species differentiation and MLST analysis. Whole genome sequencing revealed that these hybrid strains shared *C. jejuni* gene variants, involved in stress response. Since the hybrids had predominantly been isolated from egg shells, we suggest that gene variations due to *C. jejuni* sequence introgression might have been a consequence of selection of survivors in a harsh environment.

[1]German Federal Institute for Risk Assessment, Department of Biological Safety, National Reference Laboratory for Campylobacter, Berlin, Germany. [2]German Federal Institute for Risk Assessment, Department of Biological Safety, Study Centre for Genome Sequencing and Analysis, Berlin, Germany. [3]Robert Koch Institute, Microbial Genomics, Berlin, Germany. [4]These authors contributed equally: Julia C. Golz and Lennard Epping. *email: Kerstin.stingl@bfr.bund.de

SCIENTIFIC REPORTS | (2020) 10:3686 | https://doi.org/10.1038/s41598-020-60320-y 1

Isolation source*	# total isolates investigated (*Cj* and *Cc*)	# isolates with ambiguous PCR	% of isolates with ambiguous PCR relative to *Cj* and *Cc*	# total *Cc* isolates	% of isolates with ambiguous PCR relative to *Cc*
eggs	39	5	12.8	11	45.5
duck meat	63	1	1.6	20	5
chicken meat	1245	7	0.6	281	2.5
turkey meat	351	5	1.4	95	5.3
turkey cecum/skin	777	13	1.7	414	3.1

Table 1. Distribution of *Campylobacter* isolation sources of isolates with ambiguous PCR results. *Cj, C. jejuni; Cc, C. coli*; *isolates were obtained in the years 2016–2018; isolation source are displayed, from which isolates with ambiguous PCR results were obtained. Further 1860 isolates from other sources did not result in an ambiguous PCR result and were omitted from the table.

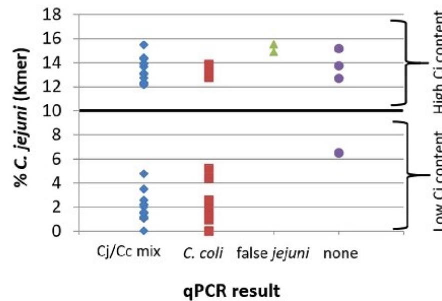


Figure 1. Percentage of *C. jejuni* genome content in *C. coli* isolates detected by k-mer analysis, categorized by qPCR result using *mapA/ceuE* as targets. Cj/Cc mix, both targets for *C. jejuni* and *C. coli* were amplified; *C. coli*, *C. coli* was correctly detected; false *jejuni*, *C. coli* was falsely detected as *C. jejuni*.; none, none of the targets was amplified.

Results

Isolates had been collected from food and animal matrices during routine sampling or zoonosis monitoring by the Federal State Laboratories between January 2016 and December 2018 according to ISO 10272[11]. The isolates were analysed by real-time PCR in the German National Reference Laboratory for *Campylobacter*[4,5]. The target for *C. jejuni* is a fragment of *mapA*, coding for an outer membrane protein. The *C. coli* specific target *ceuE* encodes the enterochelin uptake substrate-binding protein, involved in iron acquisition[3]. Out of 4.335 *C. jejuni* and *C. coli* isolates, 31 delivered ambiguous PCR results (0.72%). Ambiguous PCR results were defined by either amplification of both specific targets for *C. jejuni* and *C. coli* with similar Ct values (Cj/Cc mix, 28/31) or by no amplification at all (none, 3/31). A subsequent gel-based multiplex PCR[12], targeting the *hipO* gene of *C. jejuni* and the *glyA* gene of *C. coli*, indicated that all of these isolates belonged to the species *C. coli*.

The 31 strains with ambiguous real-time PCR signal had been isolated from poultry meat, turkey cecum or skin and eggs. The Federal State Laboratories either did not report any species (9/31), correctly identified *C. coli* (20/31) or in one case falsely reported *C. jejuni* (1/31). Surprisingly, when we compared the number of strains with ambiguous real-time PCR result with the total number of isolates analysed during the same time of collection, proportionally the highest percentage of strains with ambiguous qPCR results was derived from eggs (Table 1), although the total number of analysed eggs was low.

We characterized these isolates by whole genome sequence analysis. In addition, further *C. coli* isolates from previous years (n = 26, 2009–2015) were included. As the prevalence of strains with ambiguous qPCR was highest from eggs, we included those from eggs and additional isolates from laying hens, chicken meat and pig feces.

We performed a k-mer based analysis using the KmerFinder 3.1 (CGE, DTU, Denmark)[13–15]. For a typical *C. coli* it is expected that the k-mers match to different *C. coli* reference genomes. However, as expected from the real-time PCR results, the k-mers of the input *C. coli* sequences with ambiguous PCR results also exhibited *C. jejuni* genomic content. Different percentages of k-mers matching to *C. jejuni* reference genomes were observed, ranging from 0 (undetectable) to 15.5% (Fig. 1). Also correctly PCR-identified *C. coli* exhibited various amounts of *C. jejuni* content (Fig. 1, red squares). From the latter, those with the highest *C. jejuni* content (>10%) were exclusively from eggs, two others with *C. jejuni* content between 4.4–5.3% were from chicken meat and turkey cecum. These isolates apparently harboured an extended amount of *C. jejuni* sequences but maintained *C. coli* sequences at *mapA* and *ceuE*. In total, 29 *C. coli* isolates with a k-mer percentage of more than 10% *C. jejuni* content (Fig. 1, categorized *C. coli* with high *C. jejuni* content, named "hybrids") were identified. "Half hybrid" strains were defined as harbouring <10% *C. jejuni* sequences but displaying an ambiguous qPCR result. According to MALDI-TOF analysis, all hybrid and half hybrid strains belonged to *C. coli* with a score of $\geq$ 2.000, which was

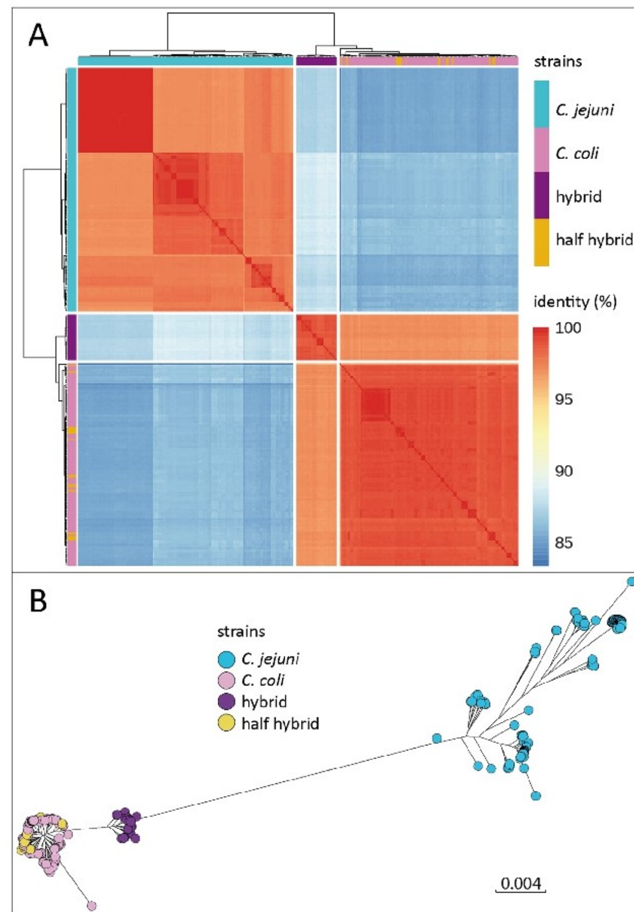


Figure 2. Relatedness of *C. jejuni* (turquoise), *C. coli* (pink), hybrid strains (purple) and half hybrid strains (mustard) according to ANI (**A**) and core genome analysis using Roary (**B**). (**A**) heatmap visualization of ANI values across all isolates. Hybrid strains form a separate cluster but still share ~97% ANI with *C. coli*. Half hybrid isolates are spread across the *C. coli* population. (**B**) phylogeny of the *Campylobacter* core genomes based on Roary analysis. The branch length between *C. coli*, including hybrid and half hybrid strains and *C. jejuni* has been shortened for better visualization.

previously validated as indicative for correct species identification of *Campylobacter* spp.[16–18]. In order to clarify genetic relationship of "hybrid" and "half hybrid" strains within the *Campylobacter* population we performed an average nucleotide identity (ANI) analysis using the tool FastANI[19]. Population and ANI studies of recent years showed that organisms sharing at least 95% ANI among themselves are defined to be of the same species[20,21]. Results of the ANI analysis were visualized in Fig. 2A and reveal that "hybrid" strains form a separate cluster but still share an ANI of 96.95% with *C. coli* isolates, proving them to be part of the *C. coli* population. In comparison, the ANI in the *C. jejuni* population ranges from 97% to 100% and shares 87.92% ANI with the "hybrid" isolates. "Half hybrid" strains are closely related with *C. coli* (98.96% ANI) and cannot be separated by ANI. A very similar observation can be made while looking at the core genome phylogeny in Fig. 2B based on Roary analysis using a sequence identity of at least 80%. The "half hybrid" isolates are found among the original *C. coli* population, whereas the hybrid strains form a separated, but still closely related group nearby *C. coli*. In general, both analyses show that the diversity/identity between *C. coli* including "hybrids" is similar than the diversity within the *C. jejuni* population, confirming that the hybrid strains indeed belong to the species *C. coli*.

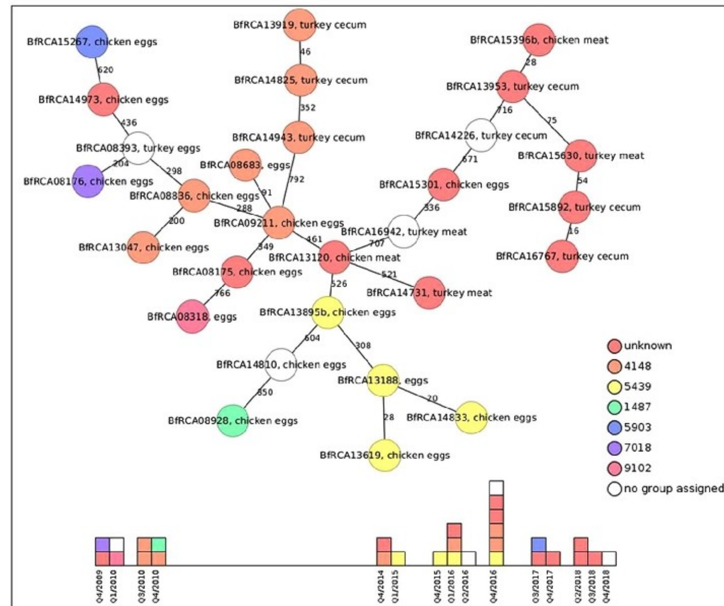


Figure 3. The 29 *C. coli* with at least 10% *C. jejuni* genome content are not related and isolated from different locations and at distinct timepoints. Minimum spanning tree of cgMLST analysis, based on 1343 core genes defined previously[22]. Circles represent *C. coli* isolates; numbers illustrate allele differences between nearest neighbours; isolation matrix is indicated as text; colour code for MLST ST-type as depicted in the legend. Unknown, new ST-type; no group assigned, at least one allele of the 7 housekeeping genes is new. New alleles and ST-types were submitted to PuMLST and numbers are shown in Supplementary Table S1.

We further asked whether the hybrid strains were of clonal origin and disseminated upon the/multiple horizontal *C. jejuni* gene transfer events had occurred. For this purpose, the MLST type was analysed based on the seven housekeeping genes ([8], PubMLST.org). As visualized in Fig. 3 the isolates belonged to different sequence types, from which only two could be attributed to any known clonal complex (both ST-1150 complex), implicating that none of the isolates belonged to the CC-828 complex, which is the most abundant clonal complex of *C. coli*. More specific sequence analysis showed that these housekeeping genes were also affected by *C. jejuni* sequence introgression (see below). Thus, MLST typing apparently has its limitations in those *Campylobacter* with substantial horizontal gene transfer activity.

Analysis via Ridom Seqsphere+ software using allele-based cgMLST[22] of 1343 genes indicated that the 29 *C. coli* hybrids were in majority unrelated. The median number of allele differences between the nearest neighbour was 343 (¼ of all analysed genes, Fig. 3). Thus, most of the strains displayed a phylogenetically diverse origin. This was substantiated by different isolation dates, ranging from 2009 to 2018 and different isolation locations from six federal states of Germany. Taken together, these data indicate that horizontal gene transfer from *C. jejuni* independently occurred in the *C. coli* hybrids.

Where did *C. jejuni* introgression take place? An in-house k-mer analysis was performed. The *C. coli* sequences with >10% of *C. jejuni* introgression were split into 16-mers or 31-mers, which were compared against a set of 95 complete *C. coli* genomes obtained from the NCBI database and 18 further *C. coli* strains without PCR ambiguity, for which whole genome sequencing was performed in the laboratory (Supplementary Table S1). We excluded three genomes from the NCBI database, which were apparently *C. jejuni* strains, falsely annotated as *C. coli* (GCA_001292485.1, GCA_001292205.1 and GCA_001292265.1). K-mers with direct matching were subtracted from the k-mer pool and the residual k-mers were compared against 152 complete *C. jejuni* genomes from the NCBI database and 3 additional sequences from the BfR strain collection (Supplementary Table S1). Those k-mers, which matched sequences present in at least 95% of the *C. jejuni* and maximal 5% of the *C. coli* genomes, were mapped against the reference *C. jejuni* strain NCTC 11168. Example k-mer mappings of a "hybrid" (>10% *C. jejuni* introgression) and a "half hybrid" strain (<10% *C. jejuni* introgression but with ambiguous qPCR result) against the NCTC 11168 reference sequence are depicted in Fig. 4. The recombination events of *C. jejuni* sequences in *C. coli* appear to be distributed all over the chromosome.

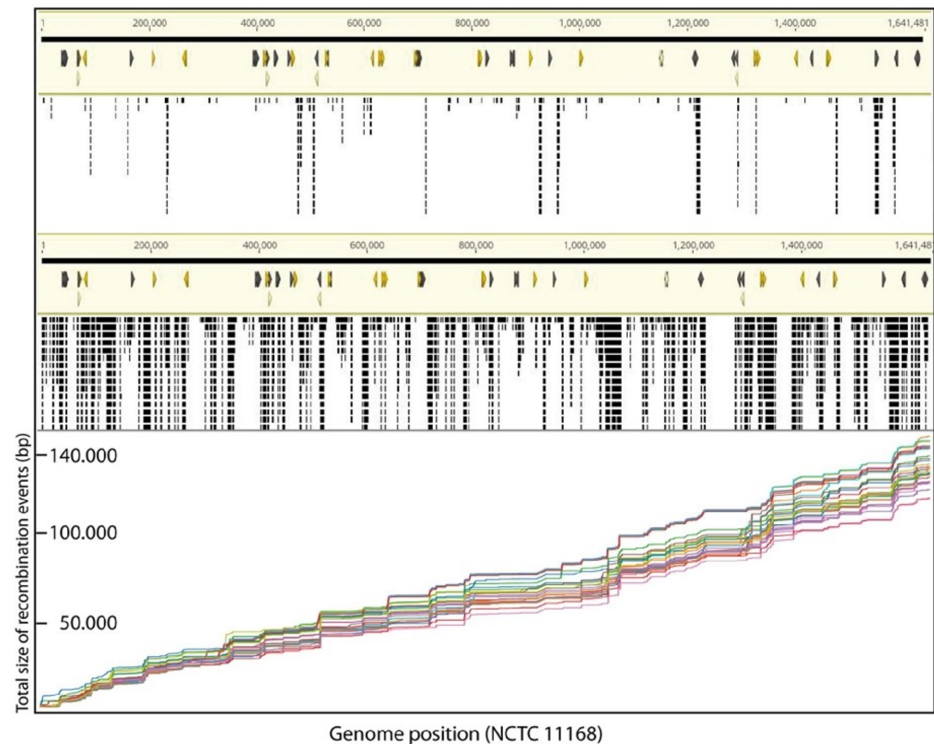


Figure 4. Cc/Cj hybrid strains show recombination events of *C. jejuni* sequences throughout the chromosome. k-mer mapping of *C. jejuni* sequences from two example strains to reference sequence of *C. jejuni* NCTC 11168 (in light yellow, visualized linearly from left (1st base) to right (1,64 Mb)), visualized with Geneious Prime 2019.2.1. Upper panel, "half hybrid" BfR-CA-15281 (<10% *C. jejuni* introgression); middle panel, "hybrid" BfR-CA-14731 (>10% *C. jejuni* introgression); lower panel, cumulative plot of recombination events in 29 "hybrid" strains indicate common locations of *C. jejuni* sequence introgression. Black bars, k-mer matching at the indicated location in the *C. jejuni* genome.

An analysis of the recombination size was performed with a minimal assumed recombination event size of 100 bp and various maximal gaps between events of 100–500 bp between k-mer matchings. As expected, the recombination size was increased with increasing size of maximal gaps. However, the overall median size of recombination events ranged between 297 and 512 bp and the maximal event was between 11.4 and 11.8 kb detected in strain BfR-CA-08318. This might hint at the potential of *C. coli* to incorporate large regions of more than 10 kb within one recombination event but that most of them were below 1 kb. The number of detected recombination events per strain ranged in median between 218 and 230 events. Note that our analysis might underestimate the number and the size due to the fact that only k-mers with exact and unique matches to the reference *C. jejuni* NCTC 11168 and to 95% of all *C. jejuni* strains included in the study were considered. A cumulative plot of recombination events (from gap size analysis of maximal 100 bp) in each strain sorted by the chromosomal location of the reference sequence is depicted in Fig. 4, indicating that common recombination events occurred in multiple *C. coli* hybrid strains and that the overall *C. jejuni* content in these strains was similar as analysed by k-mer analysis via the KmerFinder 3.1 of CGE.

What has happened at *mapA* and *ceuE* loci? In order to find out, why qPCR results led to ambiguous and even false results, k-mer mapping of these isolates to *C. jejuni* NCTC 11168 was visualized with Geneious Prime. The k-mer analysis revealed different patterns of *C. jejuni* introgression in *C. coli mapA* and *ceuE* genes and their gene context (Fig. 5). In *mapA* either several small or large recombination events, covering also the adjacent 3′ genes Cj1028c and *gyrA* and the 5′ upstream located *lepA*, were identified. In the false positive *C. jejuni* isolates, the *ceuE* locus exhibited a mosaic allele structure with the 5′ start of *ceuE* displaying a typical *C. coli* sequence and the 3′ end matching *C. jejuni* sequences. In one strain with no amplification of any of both targets ("none"), the complete *ceuE*, including adjacent *ceuD* and *ceuC* and 3′ downstream located tRNA and Cj1356c

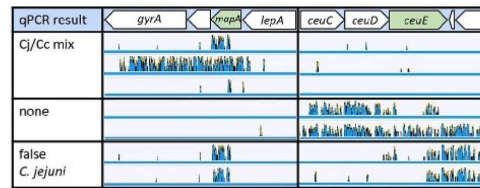


Figure 5. Strains with ambiguous qPCR result display various *C. jejuni* recombination events at the *mapA* and *ceuE* locus. In blue, coverage of k-mers to the reference sequence of *C. jejuni* NCTC 11168 of example strains with ambiguous qPCR results. Cj/Cc mix, integration of *C. jejuni* sequences at the *mapA* locus; none, integration of *C. jejuni* sequences in the *ceuE* locus; false *C. jejuni*, integration of *C. jejuni* sequences in both targets.

were exchanged by *C. jejuni* sequences. Thus, although introgression of *C. jejuni* sequences into *C. coli mapA* locus was more frequently observed, the *ceuE* locus can also partially or fully be introgressed by *C. jejuni* DNA in contrast to previous observations[23].

For a further analysis we looked at the annealing sites of the oligos used for *mapA* and *ceuE* amplification. A multiple alignment of the genes, displaying *C. jejuni* sequence content as identified by k-mer analysis, was done using MegAlign Pro 14 (Supplementary Fig. S1). As expected from the real-time PCR result, those strains from which both targets *mapA* and *ceuE* were amplified, exhibited a sequence at *mapA*, which is typical for *C. jejuni* but maintained a typical *C. coli ceuE* allele. Those four strains with no real-time PCR signal at all ("none" strains), had a *C. coli* allele at *mapA* but either a complete *C. jejuni* sequence of *ceuE* (BfR-CA-15489) or a mosaic gene as indicated in Fig. 5. Besides, this analysis corroborated the idea that independent recombination events led to similar outcome of the PCR result, since the sequence of the annealing sites of oligos and probes at the *mapA* locus was different in the strains with ambiguous PCR results and overall sequence of *mapA* and *ceuE* varied within PCR categories.

Are the strains with ambiguous qPCR result typable by other PCR assays? There are various other PCR assays published for species differentiation of *C. jejuni* and *C. coli* with and without detection of further *Campylobacter* spp[12,24–29]. Thus, the whole-genome sequences of the strains leading to ambiguous species differentiation with *mapA*/*ceuE* targets[3,5] and the correctly identified *C. coli* hybrids with high *C. jejuni* content were further assessed *in silico* (Supplementary Fig. S1). First, a second multiplex PCR targeting *mapA*/*ceuE* was evaluated[25], leading to 56% ambiguous or false results. The sequence data revealed that also *cpn60* detection[24] would lead to false species identification of all *C. coli* hybrid strains but would identify *C. coli* correctly in the "half hybrids", in which *mapA*/*ceuE* were no reliable targets. Besides, strain BfR-CA-17110 harboured a *C. jejuni* sequence in the target *cadF*[28,29], leading to false *C. jejuni* identification of this *C. coli* strain. All other targets *hipO*, *glyA*, *lpxA* and *ccoN* displayed either no or low *C. jejuni* introgression. If *C. jejuni* sequences were detected within the genes, the annealing sites of the PCR oligos were not affected, thus, a correct output of the PCR is expected.

As mentioned above, MLST typing based on the seven housekeeping genes *aspA*, *glyA*, *gltA*, *glnA*, *tkt*, *uncA*, *pgm* was impaired in the *C. coli* hybrid strains with high content of *C. jejuni* sequence introgression (Fig. 3 and Supplementary Table S1). In particular, *aspA* and *tkt* contained *C. jejuni* sequences in all hybrid strains for at least 38 or 26% gene coverage, respectively. *pgm* displayed *C. jejuni* sequences in 24 of the 29 hybrid strains between 13 and 29% of the gene length. Low amount of *C. jejuni* introgression up to 16% were found in *gltA*, *glnA* and *uncA* in 28 strains. *glyA* was the most "stable", since all strains harboured a classical *C. coli* allele or displayed just one *C. jejuni* k-mer match, which was not significant.

Which genes/locations were exchanged by *C. jejuni* sequences in the *C. coli* hybrid strains? Considering the typing results with some genes overrepresented for *C. jejuni* introgression (e. g. *cpn60* or *aspA*) and the observations of distinct recombination regions, the question arises, whether *C. jejuni* sequence exchange had common patterns in the 29 *C. coli* hybrid strains. 346 genes were covered by k-mers in at least 20% of the gene length in minimally one of the high content *C. coli* hybrid genomes. They were depicted as heatmap by the R package pheatmap v.1.0.12. (Supplementary Fig. S2). For better visualization (Fig. 6), the 194 genes were filtered to 50% of gene length exchange by *C. jejuni* sequences in minimally one of the hybrid strains. As shown above, recombination events, i. e. genes with *C. jejuni* content, were distributed throughout the chromosome. Intriguingly, similar genes were exchanged in multiple *C. coli* hybrid strains with similar extent of *C. jejuni* sequence exchange (same coloured lines in Fig. 6). Therefore, we looked at those genes, which were exchanged for at least 20% gene length coverage in at least 25 of the 29 strains and identified 104 genes, identified with both k-mer analysis (16 bp or 31 bp) and additional 8 and 14 genes with 16 bp and 31 bp k-mer analysis, respectively (Supplementary Table S2). Gene categories were annotated by EggNOG 4.5.1[30] and, after integration of additional information on *Campylobacter* homologues, summarized in functional categories (Supplementary Table S2). Assuming that *C. jejuni* sequence introgression into *C. coli* strains was random, we simulated 300 recombination events in 800 core genes of 30 strains by using a Python script (available at https://gitlab.com/microbial_genomics/relative-kmer-project). As expected, and statistically proven significant ($p < 0.05$ by Chi^2 test), the random distribution of genes recombined in multiple strains was distinct from the real distribution of observations in the

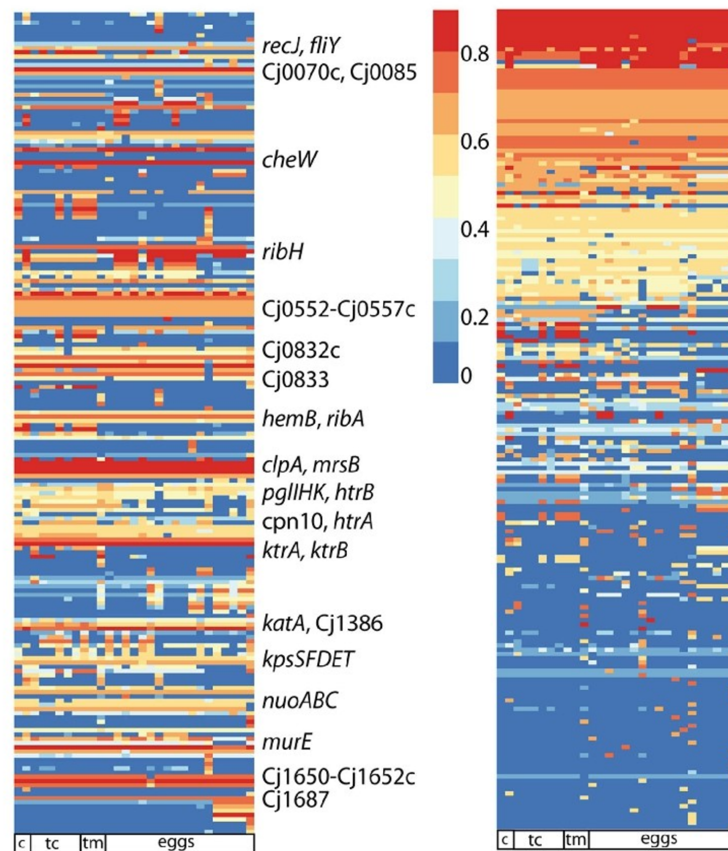


Figure 6. Cc/Cj hybrid strains share common genes with *C. jejuni* sequence content, which are distributed over the chromosome. Visualization of genes with at least 50% k-mer coverage of *C. jejuni* sequences identified in at least one of the 29 Cc/Cj hybrid strains (with >10% *C. jejuni* introgression). X-axis, strains; y-axis, genes. Left, heatmap sorted according to gene location in the reference *C. jejuni* sequence; right, heatmap sorted according to genes with high number of strains and high *C. jejuni* sequence exchange. Colours indicate coverage of gene length by *C. jejuni* sequence specific k-mer (16 bp) matches in % as detailed in the figure. Example genes with high coverage in the majority of strains are indicated. Below heatmaps, isolation source of the strains: c, chicken meat; tc, turkey cecum; tm, turkey meat and eggs.

hybrid strains (Supplementary Fig. S4). This supports our hypothesis of a selective process on the gene set affected by *C. jejuni* introgression in the hybrid strains.

Almost half of all identified genes encode proteins involved in oxidative stress response (*katA*, Cj1386, *mrsB*, *canB*, Cj0833c *hydA*, *hydA2*, *nadD*, *nuoA*, *nuoB*, *nuoC*, Cj0081), stress response in general (*clpA*, *htrB*, *htrA*, *cpn10*, *cpn60*), DNA metabolism and repair (*purF*, *pyrG*, *thyX*, *rarA*, *recJ*, *ung*, *ribA*, *guaB*, *dut*), chemotaxis and flagellar motor switch (*cheA*, *cheV*, *cheW*, *fliY*), signal transduction (Cj1110c, Cj1227c, Cj1258), membrane transporters (*crcB*, cj0832c, *ktrA*, *ktrB*, Cj1257c, Cj1687), cell wall and capsule biosynthesis (*kpsS*, *kpsE*, *kpsF*, *kpsD*, *kpsT*, *murE*) and *metK* encoding a S-adenosylmethionine transferase, involved in providing the substrate for methylation reactions. This suggests that *C. jejuni* sequence recombination in the *C. coli* hybrid genes was not random but might modulate the fitness of the *C. coli* hybrid strains, selected for survival in a harsh environment. Intriguingly, an American isolate, *C. coli* RM4661, from turkey carcass origin (NZ_CP007181.1) was identified as a Cc/Cj-hybrid strain, sharing 106 of the 126 *C. jejuni* introgressed genes revealed in the majority of our hybrid strains (Supplementary Table S2). We propose that this strain underwent a similar selection procedure, which corroborates our hypothesis of independent functional adaptation upon selection in a harsh environment.

Which *C. jejuni* sequence exchange leads to amino acid exchange in the protein and might represent a functional adaptation in *C. coli*? We checked whether the gene variants of the *C. coli* hybrid strains lead to protein variants different from *C. coli* proteins. Since *C. jejuni* and *C. coli* proteins differ in average by nearly 40 amino acids[31], it was expected that most of the observed *C. jejuni* introgression covering at least 20% of the gene length leads to changes in protein sequence. BLAST analysis (https://blast.ncbi.nlm.nih.gov) was performed on a subset of the above-mentioned identified gene translations and in all of the cases amino acid exchanges were detected in the hybrid variants as compared to *C. coli* typical protein sequence. It remains to be investigated in future studies, how these variations impact protein function with respect to *C. coli* survival capacity under stress conditions.

Discussion

The German National Reference Laboratory for *Campylobacter* has access to a large collection of representative isolates from Germany. With this set of isolates in hand we were able to identify multiple strains with ambiguous species differentiation, in particular, isolated from eggs but also from poultry meat and turkey cecum. Further isolates from eggs showed that from this isolation source nearly half of all *C. coli* displayed an extended amount of *C. jejuni* sequences incorporated in the genome.

A study comparing *C. coli* clade 1 (ST-828 and ST-1150) from agriculture with nonagricultural unintrogressed *C. coli* clade 2 and 3 demonstrated the potential of incorporation of substantial *C. jejuni* sequences in clade 1 *C. coli*[31]. The authors identified 26 *C. jejuni* genes present in *C. coli* clade 1 but absent in clade 2 and 3. Our analysis focussed on *C. coli* hybrid strains as a fraction of clade 1 and deciphers ongoing extended *C. jejuni* introgression in these strains. As expected, we only have an overlap of 2 genes (Cj0555 and *htrB*) out of the identified 26 with the study of Sheppard *et al.*[31], since we compared our hybrid strains against 113 *C. coli* sequences (mostly clade 1), including sequences from the NCBI database. This supports the notion that the *C. jejuni* recombination events found in this study represent a further development of *C. coli* strains. Since the *C. coli* hybrids were predominantly isolated from eggs, this supports the notion that the identified *C. jejuni* sequence incorporations might be a consequence of functional adaptation to survival in a harsh environment. *Campylobacter* is transmitted on egg shells via fecal contamination. On the shell, the bacterium encounters oxidative stress but also dryness and, thus, osmotic stress as well as nutrient and cold stress. Usually after 5–6 days, *Campylobacter* are no longer cultivatable from faeces[32,33].

Adaptation to harsh environment might explain shared *C. jejuni* recombinations in *C. coli* hybrids. The hybrid strains carried gene variants of *C. jejuni* or mosaic genes involved in oxidative stress response, such as katalase (*katA*) and Cj1386, which was shown to encode an atypical hemin-binding protein, mediating the trafficking of hemin to katalase[34]. Katalase is one of the key enzymes for protection against oxidative stress by cleaving peroxide to water and oxygen. *mrsB* (cj1112c) encoding a methionine sulphoxide reductase, was shown to protect *C. jejuni* against oxidative and nitrosative stress[35]. Furthermore, *canB* displayed *C. jejuni* sequences in the hybrid strains, encoding carbonic anhydrase, an enzyme important for growth at low CO_2 concentrations[36]. A further oxidoreductase (Cj0833c) and genes encoding for the Ni/Fe hydrogenase small subunit *hydA* (Cj1267c) and *hydA2* (Cj1399c) as well as *nadD* (Cj1404) involved in the synthesis of the redox cofactor NAD + were found to harbour *C. jejuni* sequences. Furthermore, *nuoA*, *nuoB*, *nuoC* implicated in transfer of electrons in the respiration chain and Cj0081, encoding the cyanide-resistant CioAB, which is proposed to lower oxygen levels and maintain microaerobic conditions[37], were identified to bear *C. jejuni* sequences in the hybrid strains. The *htrB* gene encoding a lipid A acyltransferase was proposed to play a role in regulation of cell responses to environmental harsh conditions, such as acid, heat, oxidative and osmotic stress[38]. As mentioned above, also *htrA*, which encodes a protease and chaperone activity with roles in virulence and oxidative stress defence[39–41] was among the genes with *C. jejuni* sequence detected in all hybrid strains. Besides *cgb*, encoding a single-domain haemoglobin, was suggested to protect *Campylobacter* against nitric oxide and nitrosative stress[42].

Interestingly, also genes implicated in general stress response as the *clpA* ATPase and the chaperone genes *cpn10* and *cpn60* were affected by *C. jejuni* introgression. The latter *cpn60* (*groEL*) also serves as target for species differentiation[24], inevitably leading to false species identification of the hybrid strains. Among the genes with *C. jejuni* introgression in the hybrid strains were several with roles in DNA metabolism and repair, such as *purF*, *pyrG*, *thyX*, *rarA*, *recJ*, *ung*, *ribA*, *guaB* and *dut*. Moreover, motility-associated genes, like the chemotaxis genes *cheA*, *cheV*, *cheW* and *fliY*, encoding a flagellar motor switch protein, displayed *C. jejuni* sequences.

In addition, our list of genes with *C. jejuni* content in the hybrid strains, also contained genes implicated in cell wall (*murE*) and capsule biosynthesis (*kpsS*, *kpsE*, *kpsF*, *kpsD*, *kpsT*). Consistently, in *C. jejuni* strains enhanced biofilm formation capacity, which might also be associated with enhanced survival under oxidative stress, was attributed to genes implicated in oxidative stress defence, motility, cell wall and capsular biosynthesis[43].

We suggest that modification of genes by recombination of *C. jejuni* sequences in a common set of genes in most of the hybrid strains, might reflect selection of survivors from harsh environments.

Practical implications for diagnostics. The hybrid strains can elude molecular typing, such as species differentiation using the *mapA*/*ceuE* targets and MLST. It was previously found that *mapA*/*ceuE* targets might lead to ambiguous qPCR results in six identified strains out of a data collection of around 1700 sequences[23]. In our study we identified in total 37 strains (21 "hybrid" strains and 16 "half hybrid" strains), which were not identifiable in the qPCR using *mapA*/*ceuE* targets, including two isolates, which were falsely identified as *C. jejuni*. All "hybrid" strains failed to be typed using the *cpn60* target[24] and one "half-hybrid" would be incorrectly typed as *C. jejuni* using the *cadF* target[28,29].

C. jejuni sequence introgression into *aspA* and adjacent regions (including Cj0081) was previously detected in two *C. coli* strains from turkey[44]. Our data showed that MLST as phylogenetic assay has limitations, since *C. jejuni*

sequences were found in six of the seven housekeeping genes in the majority of "hybrid" strains. Hence, standardized typing methods should consider perturbations due to extended recombination activity in *Campylobacter*. Thus, it is recommendable to include multiple independent species differentiation methods as future molecular annex to ISO 10272-1/2:2017 and to be aware of phylogenetic bias in source attribution analysis.

Conclusions and further aspects. There are various studies dealing with the differential survival of *C. jejuni* and *C. coli* under different environmental and host conditions. It has to be noted, that stress survival of the microaerobic *Campylobacter* is one of the major and still enigmatic topics in order to explain the pathogens widespread dissemination. *C. jejuni* was shown to survive longer in liver juice[45]. Aerotolerant *C. jejuni* strains were identified[46] but also aerotolerant *C. coli* isolates were highly prevalent in other studies[47]. Survival in harsh environments might be a result of various factors and also dependent on the specific genomic background. In aerotolerant *C. coli* point mutations were detected in other genes, not obviously implicated in oxidative stress response[48]. Thus, it remains elusive, how *Campylobacter* species modulate their gene pool in order to adapt to changing environments. However, the identification of hybrid strains, mainly selected from a harsh environment, exhibiting an extended amount of *C. jejuni* sequences in a common gene set, shows the enormous potential of *Campylobacter* for extensive genetic exchange for fitness enhancement.

Materials and Methods

Strains and growth conditions. *C. jejuni* and *C. coli* field strains were isolated from different food matrices and animal samples by the Federal State Laboratories according to ISO 10272[11]. At the National Reference Laboratory, isolates were cultured on Columbia agar (Oxoid, Germany) supplemented with 5% sheep blood (Oxoid, Germany) (ColbA) or passaged through Bolton broth and subcultured on mCCDA in case strains still exhibited non-*Campylobacter* background flora. Incubation was performed for 48 h under microaerobic conditions (5% O_2, 10% CO_2, rest N_2) at 42 °C. Strains were stored at −80 °C using the cryobank system (Mast Diagnostica GmbH, Germany). For DNA extraction strains from −80 °C stocks were grown on ColbA for 24 h under microaerobic conditions at 42 °C and once subcultured for another 24 ± 4 h prior to use.

Species differentiation by PCR. DNA of the strains was extracted by resuspension of the cell pellet in 5% Chelex 100 resin (Bio-Rad Laboratories GmbH, Germany), followed by incubation for 15 min at 95 °C and subsequent centrifugation. The supernatant was used for PCR analysis. For detection and species identification a real-time PCR method, targeting either a *C. jejuni* specific fragment of the *mapA* gene, a *C. coli* specific fragment of the *ceuE* gene or a *C. lari* specific fragment of the *glyA* gene was performed[4,5]. In case of ambiguity of the results, a second gel-based multiplex-PCR was applied, targeting specific fragments of the *hipO* gene for *C. jejuni*, the *glyA* gene for *C. coli* and *C. upsaliensis*, the *cpn60* for *C. lari*, the *sapB2* gene of *C. fetus* and a Campylobacterales specific fragment of the 23 S rRNA gene[12].

Matrix-assisted laser desorption/ionization (MALDI-TOF) analysis. Colony material of a 24 h ColbA plate was spotted onto the target plate (MSP 96 target polished steel (MicroScout Target) plate; Bruker Daltonik, Germany). After air drying the spots were overlaid with 1 µl of saturated α-cyano-4-hydroxy-cinnamic acid matrix solution (200 mg in 2.5% trifluoroacetic acid/50% acetonitrile) and dried completely. MALDI-TOF MS analysis was performed using MALDI-TOF Microflex LT (Bruker Daltonics, Germany) using a range of 2,000–20,000 m/z (mass to charge ratio) following the calibration with Bacterial Test Standard (Bruker Daltonics, Germany). For each spectrum 240 laser shots were summed up in 40 shot steps, but at least 80 shots per raster spot from different positions within the sample were acquired by the AutoXecute method using the software FlexAnalysis 3.4. The spectra were compared with the MBT Compass Library, Revision F (Bruker Daltonics, Germany). Each identification obtains a score value. The identification at the species level with a score ≥ 2.000 was considered correct[16–18].

Whole genome sequence analysis. *Campylobacter* strains grown on ColbA for 24 h under microaerobic atmosphere at 42 °C were harvested and DNA was extracted using the PureLink Genomic DNA Mini Kit (Thermo Fisher Scientific, USA). The quality of the DNA was evaluated by spectral analysis (NanoDrop Spectrophotometer, Thermo Fisher Scientific, USA) and the concentration was fluorimetrically quantified by Qubit 3.0 Fluorometer (dsDNA HS Assay Kit 0.2–100 ng; Thermo Fisher Scientific, USA). DNA libraries were prepared using the Nextera XT DNA Library Prep kit or the Nextera DNA Flex Library Prep Kit according to the manufacturer's instructions (Illumina, San Diego, USA). Quality of the libraries was assessed by gel analysis or on a fragment analyser 3408 (Advanced Analytical Technologies Inc., USA). Paired-end sequencing was performed on the Illumina MiSeq (2 × 301 cycles) or the NextSeq (2 × 151 cycles) platform using the MiSeq v3 (600 cycles) reagent kit or the NextSeq 500/550 Mid Output kit v2.5 (300 cycles), respectively. The sequences were published within the BioProject No. PRJNA595957, BioSample No. SAMN13577876-SAMN13577920, SRA accession No. SRR10698060-SRR10698104 at NCBI sequence read archive (SRA). New MLST alleles and MLST-ST types were uploaded to PubMLST.

Sequence analysis. Sequences were analyzed by Ridom Seqsphere+ v. 6.0.0 (2019–04) (Ridom, Muenster, Germany) using the cgMLST scheme of 1343 gene targets previously proposed[22], with 98% required identity and 98% required percentage of coverage to one of the known alleles (allele library status June 2018). Quality trimming was performed in a window of 20 bp with Phred score 30. The obtained average coverage (processed, unassembled) was >75-fold. Raw reads were *de-novo* assembled via SPAdes 3.11.1[49] with careful option, which performs a mismatch correction. The number of assembled contigs was between 31 and 130, the total size of the assemblies ranged from 1.65 to 1.92 Mb. At least 95% "good targets" were found for cgMLST-based analysis using the previously proposed cgMLST scheme[22]. Average nucleotide identity (ANI) analysis was done using

the tool FastANI[19]. Core genome phylogeny was calculated using Roary v.3.12.0[50] with a sequence identity of at least 80%. This resulted in 800 core genes that were used to build a phylogenetic tree with RAxML v.8.2.10[51] (100 bootstraps). Finally, the phylogenetic tree was adjusted for recombination sites using ClonalFrameML v.1.11[52].

For prescreening of sequences for *C. jejuni* introgression, assembled contigs were analyzed on the web-based KmerFinder 3.1 (Center for Genomic Epidemiology, DTU, Denmark)[13–15], which splits the assembly contigs into overlapping 16-mers and searches for homology matches in sequenced bacterial organisms, filtered on coding sequences (CDS; starting with ATG). The percentage of k-mers matching to distinct reference genomes was received as output data. An in-depth k-mer analysis was performed using an in-house pipeline. For this purpose the assemblies from Ridom Seqsphere+ after SPAdes assembly were used. A k-mer based databases from assembled and closed genomes from *C. jejuni* and *C. coli* (Supplementary Table S1) were built by kmc v.3[53] with a k-mer size of 16 bases or 31 bases. In order to identify *C. jejuni* specific genes, the database of *C. jejuni* includes k-mers present in at least 95% of the genomes whereas the *C. coli* database contains k-mers present in at least 5% of the genomes. K-mers from mixed isolates were subsequently counted and compared against the databases with kmc_tools. K-mers present in *C. jejuni* database and absent in *C. coli* genomes were mapped against a closed reference strain NCTC 11168 (NC_002163.1) of *C. jejuni* by bowtie2[54] and corresponding genes were identified with BEDTools v2.27.1[55]. Since read mappers are optimized for longer sequences, k-mers of size 16 bp were only considered for exact and unique matches for further downstream analysis. Since k-mers of size 31 bp are more specific, mapping results were not filtered at this step.

Genes covered by k-mers in at least 20% or 50% of the gene length and in one of the high content *C. coli* hybrid genome were visualized as heatmap by the R package pheatmap v.1.0.12. K-mers matched in *C. coli* hybrid genomes (as.bam files) were visualized in Geneious v.2019.2.1 using *C. jejuni* strain NCTC 11168 (NC_002163.1) as reference. Source code and scripts used to perform those steps are freely available at https://gitlab.com/microbial_genomics/relative-kmer-project.

Received: 7 October 2019; Accepted: 5 February 2020;
Published online: 28 February 2020

References

1. ECDC. European Centre for Disease Prevention and Control. Campylobacteriosis. In: ECDC. Annual epidemiological report for 2017. Stockholm: ECDC; 201.
2. EFSA. Scientific Opinion on *Campylobacter* in broiler meat production: control options and performance objectives and/or targets at different stages of the food chain. *EFSA Journal 2011* **9**(4):2105. [141 pp.], https://doi.org/10.2903/j.efsa.2011.2105 (2011).
3. Best, E. L., Powell, E. J., Swift, C., Grant, K. A. & Frost, J. A. Applicability of a rapid duplex real-time PCR assay for speciation of *Campylobacter jejuni* and *Campylobacter coli* directly from culture plates. *FEMS Microbiol. Lett.* **229**, 237–241, https://doi.org/10.1016/S0378-1097(03)00845-0 (2003).
4. LFGB. German Food and Feed Code, § 64,L-06.32-1, Detection of *Campylobacter* spp. in minced meat – real-time PCR methods, Federal Office of Consumer Protection and Food Safety (BVL), Beuth GmbH, Germany. (2013).
5. Mayr, A. M. *et al.* Rapid detection and differentiation of *Campylobacter jejuni, Campylobacter coli*, and *Campylobacter lari* in food, using multiplex real-time PCR. *J. Food Prot.* **73**, 241–250 (2010).
6. Johnson, J. G., Livny, J. & Dirita, V. J. High-throughput sequencing of *Campylobacter jejuni* insertion mutant libraries reveals *mapA* as a fitness factor for chicken colonization. *J. Bacteriol.* **196**, 1958–1967, https://doi.org/10.1128/JB.01395-13 (2014).
7. Miller, C. E., Williams, P. H. & Ketley, J. M. Pumping iron: mechanisms for iron uptake by *Campylobacter*. *Microbiology* **155**, 3157–3165, https://doi.org/10.1099/mic.0.032425-0 (2009).
8. Dingle, K. E. *et al.* Multilocus sequence typing system for *Campylobacter jejuni*. *J. Clin. Microbiol.* **39**, 14–23, https://doi.org/10.1128/JCM.39.1.14-23.2001 (2001).
9. Sheppard, S. K., McCarthy, N. D., Falush, D. & Maiden, M. C. Convergence of *Campylobacter* species: implications for bacterial evolution. *Sci.* **320**, 237–239, https://doi.org/10.1126/science.1155532 (2008).
10. Sheppard, S. K. & Maiden, M. C. The evolution of *Campylobacter jejuni* and *Campylobacter coli*. *Cold Spring Harb. Perspect. Biol.* **7**, a018119, https://doi.org/10.1101/cshperspect.a018119 (2015).
11. ISO. 10272-1:2006 revised 2017, Microbiology of the food chain - Horizontal method for detection and enumeration of *Campylobacter* spp. - Part 1: Detection method.
12. Wang, G. *et al.* Colony multiplex PCR assay for identification and differentiation of *Campylobacter jejuni*, *C. coli*, *C. lari*, *C. upsaliensis*, and *C. fetus* subsp. *fetus*. *J. Clin. Microbiol.* **40**, 4744–4747, https://doi.org/10.1128/jcm.40.12.4744-4747.2002 (2002).
13. Clausen, P., Aarestrup, F. M. & Lund, O. Rapid and precise alignment of raw reads against redundant databases with KMA. *Bmc Bioinforma.* **19**, 307, https://doi.org/10.1186/s12859-018-2336-6 (2018).
14. Hasman, H. *et al.* Rapid whole-genome sequencing for detection and characterization of microorganisms directly from clinical samples. *J. Clin. Microbiol.* **52**, 139–146, https://doi.org/10.1128/JCM.02452-13 (2014).
15. Larsen, M. V. *et al.* Benchmarking of methods for genomic taxonomy. *J. Clin. Microbiol.* **52**, 1529–1539, https://doi.org/10.1128/JCM.02981-13 (2014).
16. Bessede, E., Solecki, O., Sifre, E., Labadi, L. & Megraud, F. Identification of *Campylobacter* species and related organisms by matrix assisted laser desorption ionization-time of flight (MALDI-TOF) mass spectrometry. *Clin. Microbiol. Infect.* **17**, 1735–1739, https://doi.org/10.1111/j.1469-0691.2011.03468.x (2011).
17. Emele, M. F. *et al.* Proteotyping as alternate typing method to differentiate *Campylobacter coli* clades. *Sci. Rep.* **9**, 4244, https://doi.org/10.1038/s41598-019-40842-w (2019).
18. Hsieh, Y. H. *et al.* Application of MALDI-TOF MS systems in the rapid identification of *Campylobacter* spp. of public health importance. *J. AOAC Int.* **101**, 761–768, https://doi.org/10.5740/jaoacint.17-0266 (2018).
19. Jain, C., Rodriguez, R. L., Phillippy, A. M., Konstantinidis, K. T. & Aluru, S. High throughput ANI analysis of 90K prokaryotic genomes reveals clear species boundaries. *Nat. Commun.* **9**, 5114, https://doi.org/10.1038/s41467-018-07641-9 (2018).
20. Figueras, M. J., Beaz-Hidalgo, R., Hossain, M. J. & Liles, M. R. Taxonomic affiliation of new genomes should be verified using average nucleotide identity and multilocus phylogenetic analysis. *Genome Announc* **2**, https://doi.org/10.1128/genomeA.00927-14 (2014).
21. Goris, J. *et al.* DNA-DNA hybridization values and their relationship to whole-genome sequence similarities. *Int. J. Syst. Evol. Microbiol.* **57**, 81–91, https://doi.org/10.1099/ijs.0.64483-0 (2007).
22. Cody, A. J., Bray, J. E., Jolley, K. A., McCarthy, N. D. & Maiden, M. C. J. Core genome multilocus sequence typing scheme for stable, comparative analyses of *Campylobacter jejuni* and *C. coli* human disease isolates. *J. Clin. Microbiol.* **55**, 2086–2097, https://doi.org/10.1128/JCM.00080-17 (2017).

23. Jansen van Rensburg, M. J., Swift, C., Cody, A. J., Jenkins, C. & Maiden, M. C. Exploiting bacterial whole-genome sequencing data for evaluation of diagnostic assays: *Campylobacter* species identification as a case study. *J. Clin. Microbiol.* **54**, 2882–2890, https://doi.org/10.1128/JCM.01522-16 (2016).
24. Chaban, B., Musil, K. M., Himsworth, C. G. & Hill, J. E. Development of cpn60-based real-time quantitative PCR assays for the detection of 14 *Campylobacter* species and application to screening of canine fecal samples. *Appl. Env. Microbiol.* **75**, 3055–3061, https://doi.org/10.1128/AEM.00101-09 (2009).
25. Denis, M. *et al*. Development of a m-PCR assay for simultaneous identification of *Campylobacter jejuni* and *C. coli*. *Lett. Appl. Microbiol.* **29**, 406–410, https://doi.org/10.1046/j.1472-765x.1999.00658.x (1999).
26. Klena, J. D. *et al*. Differentiation of *Campylobacter coli*, *Campylobacter jejuni*, *Campylobacter lari*, and *Campylobacter upsaliensis* by a multiplex PCR developed from the nucleotide sequence of the lipid A gene *lpxA*. *J. Clin. Microbiol.* **42**, 5549–5557, https://doi.org/10.1128/JCM.42.12.5549-5557.2004 (2004).
27. LaGier, M. J., Joseph, L. A., Passaretti, T. V., Musser, K. A. & Cirino, N. M. A real-time multiplexed PCR assay for rapid detection and differentiation of *Campylobacter jejuni* and *Campylobacter coli*. *Mol. Cell Probes* **18**, 275–282, https://doi.org/10.1016/j.mcp.2004.04.002 (2004).
28. Shams, S., Bakhshi, B. & Tohidi Moghadam, T. In silico analysis of the *cadF* gene and development of a duplex polymerase chain reaction for species-specific identification of *Campylobacter jejuni* and *Campylobacter coli*. *Jundishapur J. Microbiol.* **9**, e29645, https://doi.org/10.5812/jjm.29645 (2016).
29. Toplak, N., Kovac, M., Piskernik, S., Mozina, S. S. & Jersek, B. Detection and quantification of *Campylobacter jejuni* and *Campylobacter coli* using real-time multiplex PCR. *J. Appl. Microbiol.* **112**, 752–764, https://doi.org/10.1111/j.1365-2672.2012.05235.x (2012).
30. Huerta-Cepas, J. *et al*. eggNOG 4.5: a hierarchical orthology framework with improved functional annotations for eukaryotic, prokaryotic and viral sequences. *Nucleic Acids Res.* **44**, D286–293, https://doi.org/10.1093/nar/gkv1248 (2016).
31. Sheppard, S. K. *et al*. Progressive genome-wide introgression in agricultural *Campylobacter coli*. *Mol. Ecol.* **22**, 1051–1064, https://doi.org/10.1111/mec.12162 (2013).
32. Ahmed, M. F., Schulz, J. & Hartung, J. Survival of *Campylobacter jejuni* in naturally and artificially contaminated laying hen feces. *Poult. Sci.* **92**, 364–369, https://doi.org/10.3382/ps.2012-02496 (2013).
33. Bui, X. T., Wolff, A., Madsen, M. & Bang, D. D. Reverse transcriptase real-time PCR for detection and quantification of viable *Campylobacter jejuni* directly from poultry faecal samples. *Res. Microbiol.* **163**, 64–72, https://doi.org/10.1016/j.resmic.2011.10.007 (2012).
34. Flint, A. & Stintzi, A. Cj1386, an atypical hemin-binding protein, mediates hemin trafficking to KatA in *Campylobacter jejuni*. *J. Bacteriol.* **197**, 1002–1011, https://doi.org/10.1128/JB.02346-14 (2015).
35. Atack, J. M. & Kelly, D. J. Contribution of the stereospecific methionine sulphoxide reductases MsrA and MsrB to oxidative and nitrosative stress resistance in the food-borne pathogen *Campylobacter jejuni*. *Microbiology* **154**, 2219–2230, https://doi.org/10.1099/mic.0.2008/019711-0 (2008).
36. Al-Haideri, H., White, M. A. & Kelly, D. J. Major contribution of the type II beta carbonic anhydrase CanB (Cj0237) to the capnophilic growth phenotype of *Campylobacter jejuni*. *Env. Microbiol.* **18**, 721–735, https://doi.org/10.1111/1462-2920.13092 (2016).
37. Jackson, R. J. *et al*. Oxygen reactivity of both respiratory oxidases in *Campylobacter jejuni*: the *cydAB* genes encode a cyanide-resistant, low-affinity oxidase that is not of the cytochrome bd type. *J. Bacteriol.* **189**, 1604–1615, https://doi.org/10.1128/JB.00897-06 (2007).
38. Phongsisay, V., Perera, V. N. & Fry, B. N. Expression of the *htrB* gene is essential for responsiveness of *Salmonella typhimurium* and *Campylobacter jejuni* to harsh environments. *Microbiology* **153**, 254–262, https://doi.org/10.1099/mic.0.29230-0 (2007).
39. Baek, K. T., Vegge, C. S., Skorko-Glonek, J. & Brondsted, L. Different contributions of HtrA protease and chaperone activities to *Campylobacter jejuni* stress tolerance and physiology. *Appl. Env. Microbiol.* **77**, 57–66, https://doi.org/10.1128/AEM.01603-10 (2011).
40. Boehm, M. *et al*. Rapid paracellular transmigration of *Campylobacter jejuni* across polarized epithelial cells without affecting TER: role of proteolytic-active HtrA cleaving E-cadherin but not fibronectin. *Gut Pathog.* **4**, 3, https://doi.org/10.1186/1757-4749-4-3 (2012).
41. Boehm, M., Lind, J., Backert, S. & Tegtmeyer, N. *Campylobacter jejuni* serine protease HtrA plays an important role in heat tolerance, oxygen resistance, host cell adhesion, invasion, and transmigration. *Eur. J. Microbiol. Immunol.* **5**, 68–80, https://doi.org/10.1556/EUJMI-D-15-00003 (2015).
42. Elvers, K. T., Wu, G., Gilberthorpe, N. J., Poole, R. K. & Park, S. F. Role of an inducible single-domain hemoglobin in mediating resistance to nitric oxide and nitrosative stress in *Campylobacter jejuni* and *Campylobacter coli*. *J. Bacteriol.* **186**, 5332–5341, https://doi.org/10.1128/JB.186.16.5332-5341.2004 (2004).
43. Pascoe, B. *et al*. Enhanced biofilm formation and multi-host transmission evolve from divergent genetic backgrounds in *Campylobacter jejuni*. *Env. Microbiol.* **17**, 4779–4789, https://doi.org/10.1111/1462-2920.13051 (2015).
44. Chan, K., Elhanafi, D. & Kathariou, S. Genomic evidence for interspecies acquisition of chromosomal DNA from *Campylobacter jejuni* by *Campylobacter coli* strains of a turkey-associated clonal group (cluster II). *Foodborne Pathog. Dis.* **5**, 387–398, https://doi.org/10.1089/fpd.2008.0113 (2008).
45. Karki, A. B., Wells, H. & Fakhr, M. K. Retail liver juices enhance the survivability of *Campylobacter jejuni* and *Campylobacter coli* at low temperatures. *Sci. Rep.* **9**, 2733, https://doi.org/10.1038/s41598-018-35820-7 (2019).
46. Oh, E., McMullen, L. & Jeon, B. High prevalence of hyper-aerotolerant *Campylobacter jejuni* in retail poultry with potential implication in human infection. *Front. Microbiol.* **6**, 1263, https://doi.org/10.3389/fmicb.2015.01263 (2015).
47. Karki, A. B., Marasini, D., Oakey, C. K., Mar, K. & Fakhr, M. K. *Campylobacter coli* from retail liver and meat products is more aerotolerant than *Campylobacter jejuni*. *Front. Microbiol.* **9**, 2951, https://doi.org/10.3389/fmicb.2018.02951 (2018).
48. O'Kane, P. M. & Connerton, I. F. Characterisation of aerotolerant forms of a robust chicken colonizing *Campylobacter coli*. *Front. Microbiol.* **8**, 513, https://doi.org/10.3389/fmicb.2017.00513 (2017).
49. Bankevich, A. *et al*. SPAdes: a new genome assembly algorithm and its applications to single-cell sequencing. *J. Comput. Biol.* **19**, 455–477, https://doi.org/10.1089/cmb.2012.0021 (2012).
50. Page, A. J. *et al*. Roary: rapid large-scale prokaryote pan genome analysis. *Bioinforma.* **31**, 3691–3693, https://doi.org/10.1093/bioinformatics/btv421 (2015).
51. Stamatakis, A. RAxML version 8: a tool for phylogenetic analysis and post-analysis of large phylogenies. *Bioinforma.* **30**, 1312–1313, https://doi.org/10.1093/bioinformatics/btu033 (2014).
52. Didelot, X. & Wilson, D. J. ClonalFrameML: efficient inference of recombination in whole bacterial genomes. *PLoS Comput. Biol.* **11**, e1004041, https://doi.org/10.1371/journal.pcbi.1004041 (2015).
53. Kokot, M., Dlugosz, M. & Deorowicz, S. KMC 3: counting and manipulating k-mer statistics. *Bioinforma.* **33**, 2759–2761, https://doi.org/10.1093/bioinformatics/btx304 (2017).
54. Langmead, B. & Salzberg, S. L. Fast gapped-read alignment with Bowtie 2. *Nat. Methods* **9**, 357–359, https://doi.org/10.1038/nmeth.1923 (2012).
55. Quinlan, A. R. & Hall, I. M. BEDTools: a flexible suite of utilities for comparing genomic features. *Bioinforma.* **26**, 841–842, https://doi.org/10.1093/bioinformatics/btq033 (2010).

Acknowledgements
J.G. was financed by the Federal Ministry of Education and Research (BMBF) within the PAC-CAMPY project IP3/01KI1725B, whereas L.E. was financed by IP10/01KI1725F. The study was supported by the 1322–646 SFB project of BfR. The funders had no influence on the design, data collection, analysis and conclusions of the study. We thank Nicole vom Ort from the BfR section Diagnostics, Pathogen Characterization, Parasites in Food and all members of the National Reference Laboratory for excellent technical assistance. We are grateful for the continuous collaboration with the Federal State Laboratories. We thank Dag Harmsen and Joerg Rothgaenger for implementation of the cgMLST scheme[22] into the Ridom Seqsphere+ software. The authors declare that they have no conflict of interest.

Author contributions
J.G. performed experiments, analysed data and co-wrote the manuscript; L.E. designed the k-mer analysis, analysed data, co-wrote the manuscript; M.K., performed experiments; F.H., analysed data; M.B., C.D., B.M. and T.S. provided critical advice in experimental design and edited the paper; K.S. designed the study, analysed data and wrote the manuscript. All authors reviewed the manuscript.

Competing interests
The authors declare no competing interests.

Additional information
Supplementary information is available for this paper at https://doi.org/10.1038/s41598-020-60320-y.

Correspondence and requests for materials should be addressed to K.S.

Reprints and permissions information is available at www.nature.com/reprints.

Publisher's note Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

Open Access This article is licensed under a Creative Commons Attribution 4.0 International License, which permits use, sharing, adaptation, distribution and reproduction in any medium or format, as long as you give appropriate credit to the original author(s) and the source, provide a link to the Creative Commons license, and indicate if changes were made. The images or other third party material in this article are included in the article's Creative Commons license, unless indicated otherwise in a credit line to the material. If material is not included in the article's Creative Commons license and your intended use is not permitted by statutory regulation or exceeds the permitted use, you will need to obtain permission directly from the copyright holder. To view a copy of this license, visit http://creativecommons.org/licenses/by/4.0/.

© The Author(s) 2020

5.5 Publication 4: Natural competence and horizontal gene transfer in *Campylobacter*.

Natural Competence and Horizontal Gene Transfer in *Campylobacter*

Julia Carolin Golz and Kerstin Stingl

Contents

1 Introduction 266
2 Mechanisms of Horizontal Gene Transfer 267
2.1 Natural Transformation and Uptake of Free DNA 269
2.2 Conjugative Gene Transfer 274
2.3 Phage Transduction and Genomic Rearrangements 275
3 Barriers to Horizontal Gene Transfer 277
3.1 CRISPR-Cas and Nucleases 278
3.2 Methylation-Dependent DNA Recognition 279
4 Impact of Gene Transfer on *Campylobacter* Fitness 280
4.1 Spread of Resistomes and Persistence Factors 280
4.2 Interspecies Gene Transfer 282
5 Concluding Remarks 283
References 284

Abstract Thermophilic *Campylobacter*, in particular *Campylobacter jejuni*, *C. coli* and *C. lari* are the main relevant *Campylobacter* species for human infections. Due to their high capacity of genetic exchange by horizontal gene transfer (HGT), rapid adaptation to changing environmental and host conditions contribute to successful spreading and persistence of these foodborne pathogens. However, extensive HGT can exert dangerous side effects for the bacterium, such as the incorporation of gene fragments leading to disturbed gene functions. Here we discuss mechanisms of HGT, notably natural transformation, conjugation and bacteriophage transduction and limiting regulatory strategies of gene transfer. In particular, we summarize the current knowledge on how the DNA macromolecule is exchanged between single cells. Mechanisms to stimulate and to limit HGT obviously coevolved and maintained an optimal balance. Chromosomal rearrangements and incorporation of harmful mutations are risk factors for survival and can result in drastic loss of fitness. In *Campylobacter*, the restricted recognition and preferential uptake of free DNA

J. C. Golz · K. Stingl (✉)
Department of Biological Safety, National Reference Laboratory for Campylobacter, German Federal Institute for Risk Assessment (BfR), Diedersdorfer Weg 1, 12277 Berlin, Germany
e-mail: kerstin.stingl@bfr.bund.de

J. C. Golz
e-mail: julia.golz@bfr.bund.de

© The Author(s) 2021 265
S. Backert (ed.), *Fighting Campylobacter Infections*, Current Topics in Microbiology and Immunology 431, https://doi.org/10.1007/978-3-030-65481-8_10

from relatives are mediated by a short methylated DNA pattern and not by a classical DNA uptake sequence as found in other bacteria. A class two CRISPR-Cas system is present but also other DNases and restriction–modification systems appear to be important for *Campylobacter* genome integrity. Several lytic and integrated bacteriophages have been identified, which contribute to genome diversity. Furthermore, we focus on the impact of gene transfer on the spread of antibiotic resistance genes (resistome) and persistence factors. We discuss remaining open questions in the HGT field, supposed to be answered in the future by current technologies like whole-genome sequencing and single-cell approaches.

1 Introduction

Horizontal gene transfer (HGT) is the exchange of genetic material and plays a major role in genetic diversity of pathogens (Lawrence 2005; Daubin and Szollosi 2016). Therefore, HGT in *Campylobacter jejuni* is thought to lead to host adaptation and fitness enhancement. There are three types of HGT, natural transformation, conjugation and phage transduction (Fig. 1). During natural transformation, free environmental DNA is taken up and incorporated into the genome upon homologous recombination or in case of plasmids by plasmid reconstitution and replication. Free DNA might occur in the environment by active secretion from bacterial cells or by cell lysis. Conjugation, however, is limited to DNA exchange between donor and recipient cells being in physical contact with each other. Transduction describes the genetic exchange mediated by bacteriophages. HGT in *Campylobacter* is the main driving force for the outstanding genetic diversity of this pathogen (Wilson et al. 2009; Sheppard et al. 2008). In Sect. 2, we discuss various HGT mechanisms in thermophilic *Campylobacter*, including *C. jejuni*, *C. coli* and *C. lari*, which are the *Campylobacter* species most frequently implicated in human gut disease.

However, genetic changes harbor the risk of harmful mutations or unfavorable chromosomal rearrangements for the bacteria. Therefore, mechanisms for the regulation of DNA entry and recombination into the bacterial chromosome co-evolved. CRISPR-Cas can be considered as the bacterial immune system protecting cells form invading bacteriophages or plasmids (Hille et al. 2018). However, other nucleases including restriction–modification systems play an important role for limiting harmful transfer of genetic material into the foodborne pathogen and are discussed in Sect. 3. Nevertheless, HGT bears the advantage of rapid host adaptation due to fitness enhancement and, e.g., spread of antibiotic resistances. Hence, in Sect. 4, we focus on the current knowledge of interspecies gene transfer and acquisition of novel beneficial genetic traits in thermophilic *Campylobacter* spp.

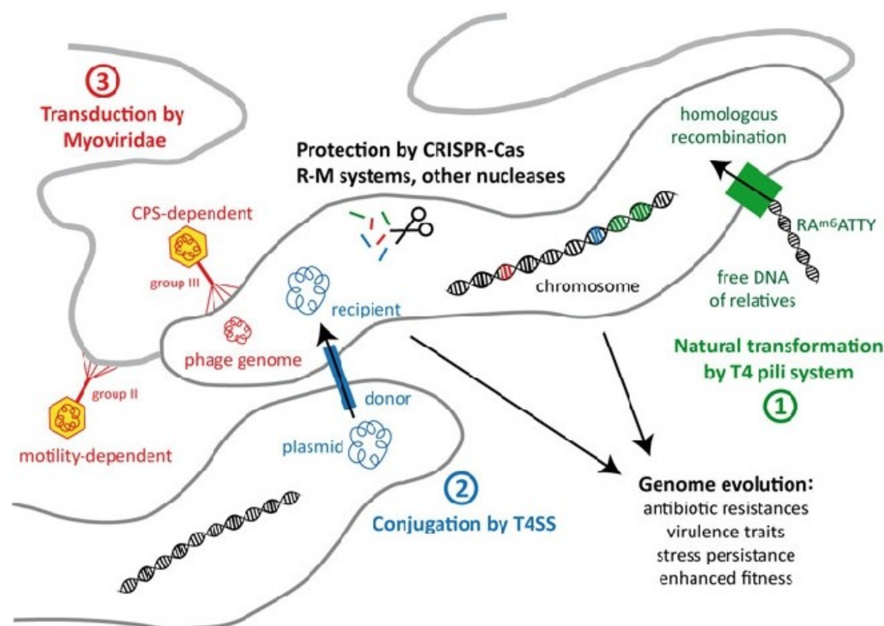


Fig. 1 Overview of horizontal gene transfer (HGT) mechanisms, genetic barriers and impact on pathogen adaptation. The three mechanisms of HGT are depicted for thermophilic *Campylobacter* spp. Natural transformation of free external DNA (in green) occurs via a type II secretion/T4 pili system, which displays homology to the competence machinery of *Neisseria* (detailed in Fig. 2). Transfer of plasmids via conjugation (in blue) is mediated by type IV secretion systems (T4SS) in direct cell–cell contact. Two main classes of bacteriophages of the family Myoviridae mediate genetic diversity by transduction (in red). The Fletcherviruses, group III, CP8-like phages depend on capsular polysaccharide (CPS) modifications as receptor for host entry. The Firehammer, group II, CP220-like phages need motile bacteria for infection. *Campylobacter* limits natural transformation by selection of DNA from relatives harboring a methylated RA^{m6}ATTY profile, provided by activity of the CtsM methylase. Periplasmic nucleases and cytoplasmic restriction–modification systems as well as the CRISPR-Cas type II-C system provide additional barriers for incoming DNA. HGT leads to frequent homologous and rare non-homologous recombination of genetic material, acquisition of plasmids and/or rearrangements of chromosomal loci, which in turn shapes genome evolution. Hence, *Campylobacter* populations genetically diversify providing preadaptation to changing environments, such as presence of antimicrobials, switch of hosts and environmental stress, thereby enhancing the bacterium's overall fitness for survival and transmission

2 Mechanisms of Horizontal Gene Transfer

Most studies are based on classical approaches, in which HGT is followed using a selective marker and phenotypic characterization of resulting bacterial colonies after incorporation of the transferred marker gene (Table 1, first column). The enormous capacity of transfer of a chloramphenicol selection marker by natural transformation in *C. jejuni* was impressively shown by establishing a plate DNA transformation assays for screening mutants (Wiesner et al. 2003). The principle of the assay was the

Table 1 Overview on experimental approaches to decipher HGT mechanisms in bacteria and evolutionary impact

	Classical approaches	Single-cell assays	Whole-genome sequencing
Principle	Transfer of (antibiotic) markers for phenotypic selection	Uptake of (fluorescently) labeled DNA and epifluorescent or electron microscopy (bacteriophage, conjugation)	Bioinformatic analysis of whole genome before/after gene transfer
Methods	Mixture of donor/recipient cells, addition of free DNA or bacteriophages	Addition of fluorescently labeled DNA, donor/recipient cells or bacteriophages	DNA extraction from field strains or after *in vitro* gene transfer events; Fragment library preparation
Analysis	Selection with antimicrobial agent; phenotypic characterization	Analysis with fluorescence microscope or by TEM with/without antibodies	MiSeq, NextSeq, PacBio; Analysis: K-mer, SNP or cgMLST analysis for phylogeny; annotation tools
Readout	Number of CFU with marker vs. total CFU; frequency of gene transfer	Microscopic photo for localization of gene transfer; detection and quantification of gene transfer in single cells	Genome diversity; recombination frequency; chromosomal rearrangements; virulence/antibiotic resistance determinants
Power/advantage of approach	Detection and quantification of the final result of gene transfer processes	Localization of components for gene transfer visible at single-cell level; direct monitoring of different steps of gene transfer possible; parameters for induction of gene transfer can be identified	High throughput method; identification of multiple gene transfer events and impact of gene transfer on whole bacterium/bacterial population
Drawback/disadvantage of approach	Readout does not distinguish between different steps of gene transfer; CFU is biased due to fastidious nature of *Campylobacter*	Accessibility/visibility of DNA during transfer limited; detection and quantification need differential approaches for visualization	Mechanisms of gene transfer are only indirectly visible

TEM, transmission electron microscopy; MiSeq and NextSeq, middle-scale and large-scale short-read, massive parallel whole-genome sequencing methods of Illumina; PacBio, single-molecule real-time sequencing technique of Pacific Biosciences; SNP, single nucleotide polymorphism; cgMLST, core genome multi-locus sequence typing; CFU, colony-forming units

spreading of a countable number of *C. jejuni* cells on an agar plate, which had been overlaid by 2.5 μg of transforming DNA, harboring a given selection marker. After two days of growth, bacterial colonies were patched on agar plates with and without antibiotic. Intriguingly, the authors observed that nearly all colonies comprised transformed cells. Assuming growth from single cells to visible colonies of around 10^6-10^7 cells, natural transformation occurred within approximately 20–25 generations. Two days of incubation were sufficient to generate a bacterial population with adequate capacity of adaptive survival based on a former single cell. The final result of HGT

is monitored in classical approaches but the readout cannot distinguish between different steps of gene transfer. Furthermore, in some settings in which the cells are exposed to stress conditions, the parameter colony-forming units (CFU) can be biased due to the fastidious nature of *Campylobacter* spp. and might not reflect full capacity of gene transfer.

Single-cell approaches have the advantage of dissecting different steps of HGT and to localize DNA uptake/transfer complexes (Table 1, second column). The detection and quantification of gene transfer are feasible at the level of single cells, displaying phenotypic heterogeneity. Parameters for induction of gene transfer can more directly be identified, since the assay does not depend on the complete process including the incorporation and expression of a marker gene. However, accessibility and, thus, visibility of DNA during the transfer event are limited. For example, covalently labeled DNA can only be followed into the periplasm and transfer of DNA into the cytoplasm is only indirectly monitored by disappearance of fluorescence of non-covalently labeled DNA (Stingl et al. 2010). For conjugation and transduction, DNA is steadily protected within biological compartments, and the detection by antibodies using transmission electron microscopy (TEM) is a stochastic event. Thus, differential approaches combined with the construction and characterization of mutants are necessary for complete monitoring and quantification of DNA transfer.

A recent approach focusses on whole-genome analysis in order to monitor the overall effects of HGT on population dynamics (Table 1, third column). Different platforms for whole-genome sequencing are used and quality as well as interpretation parameters are currently harmonized in order to optimally compare datasets of different laboratories. Ideally, all three approaches are combined to reveal the complete process and impact of HGT in the foodborne pathogen.

2.1 Natural Transformation and Uptake of Free DNA

Natural transformation was first discovered almost one century ago in *Streptococcus pneumoniae*, when phenotypic changes upon addition of heat-inactivated virulent bacteria to a recipient non-virulent culture were observed (Griffith 1928). Avery and colleagues (1944) pinpointed the transforming agent as DNA. The term “competence” depicts the state, in which cells are able to take up free DNA and naturally transform, i.e., integrate genetic material into their genome or replicate epichromosomal elements autonomously. Potential benefits of natural transformation include the repair of mutations by incoming homologous DNA and the acquisition of new genes and, therefore, new functions, e.g., antibiotic resistance genes or virulence factors. In addition, DNA might serve as nutrient supply by offering a reservoir for recycling of nucleotides. Besides extracellular DNA might serve as a matrix for the formation of biofilms and can enhance persistence of the pathogen outside the host (Feng et al. 2018; Svensson et al. 2014). For comprehensive reviews on natural transformation in other bacteria refer e. g. to Dubnau and Blokesch (2019) and Bakkali and colleagues (2013). Since uptake of foreign DNA might represent a

danger of acquiring harmful mutations, competence development is usually a highly regulated process (Johnston et al. 2014). Only few information is available on parameters controlling competence development in *Campylobacter* spp. *C. jejuni* seems to show the highest transformation levels under optimal growth conditions, but transformation also occurred, when growth was restricted at higher pH (Vegge et al. 2012). However, it is unclear, if already expressed DNA uptake complexes still functioned under growth limiting conditions or if competence development still occurred. Prolonged incubation times in the presence of DNA were performed in this study, which do not allow distinguishing activity of DNA uptake complexes from transcriptional regulation of competence genes. Wilson and colleagues (2003) suggested that lower CO_2 levels led to decreased competence in *C. jejuni* strains, although also here pH effects cannot be ruled out.

Since *Campylobacter* are Gram-negative bacteria, free DNA for natural transformation has to be transported i) over the outer membrane into the periplasm and ii) across the inner membrane into the cytoplasm. *Campylobacter* harbors gene homologues of a type II secretion/type IV pilus system that were shown to be essential for DNA uptake in other organisms (Table 2, Fig. 2) (Parkhill et al. 2000; Gundogdu et al. 2007). An at least 1000-fold reduction in transformation frequency was observed by Wiesner and colleagues (2003) using a transposon-based mutagenesis approach in eleven genes, nine of them were named *Campylobacter* transformation system (*cts*) genes (Table 2). Six of the genes are located in an operon, *ctsF-ctsE-ctsX-ctsP-ctsD-ctsR*. The remaining three *cts* genes, *ctsG, ctsT* and *ctsW* are separately located on the chromosome. CtsP and CtsE harbor nucleotide-binding sites (Walker A and B boxes) and are proper candidates for empowering uptake of the DNA macromolecule and/or assembly of a (pseudo-)pilus, like ComGA or PilF/T in *B. subtilis* or *Neisseria*, respectively (Beauchamp et al. 2015). CtsP physically interacts with the unique CtsX protein, both located in the membrane, while CtsE seems to be located in the soluble fraction (Beauchamp et al. 2015). *Campylobacter* recognizes DNA from relatives by using the methylated RAATTY site (see Sect. 3). In *N. gonorrhoeae*, PilQ constitutes the outer membrane pore (Drake and Koomey 1995), mediating entry of external DNA into the periplasm. *C. jejuni* harbors the *pilQ* homolog *ctsD* (Wiesner et al. 2003), which might have similar function as outer membrane DNA pore in *C. jejuni*. The genes *ctsF*, *ctsG* and *ctsT* have homology to *comGB*, *comGC* (*pilE* in Gram-negative bacteria) and *comGD* of *B. subtilis*, playing putative roles in function and assembly of the type IV (pseudo-)pilus system. In particular, it was suggested that ComGB displays an integral membrane protein forming the base for pilus assembly, with ComGC as major and ComGD as minor pilins (Chen et al. 2006). Retraction of DNA bound to type IV competence pili in *Vibrio* was recently demonstrated (Ellison et al. 2018). It remains to be shown, if a similar mechanism for “grabbing” external DNA is present in *Campylobacter* or if a pseudopilus is sufficient for DNA uptake as shown for *Neisseria* (Obergfell and Seifert 2016).

In addition, transposon insertion in *ceuB,* which is located in an operon structure with *ceuC, ceuD, ceuE,* encoding the enterochelin uptake system important for iron acquisition, resulted in impaired natural transformation. Furthermore, also *ctsW*, *proC* and the downstream region of *ansA* led to reduced transformation rates (Wiesner

Table 2 Genes implicated in natural transformation by *C. jejuni*

Gene name[1]	Putative function in natural transformation	Cc	Cl	Reference
comEC (Cj1211)	Competence family protein; predicted integral membrane channel for transport of DNA into cytoplasm	✓	✓	Jeon et al. 2008
comE (Cj0011c)	Periplasmic DNA-binding protein; generates force for pulling DNA macromolecule over the outer membrane in other bacteria; role unclear in *Campylobacter* spp.	×	×	Jeon and Zhang 2007; Meric et al. 2014
ctsD/pilQ (Cj1474c)	Type II secretion/T4 pilus system; potential outer membrane pore/secretin for transport of DNA into periplasm	✓	✓	Wiesner et al. 2003
ctsP (Cj1473c)	Type II secretion/T4 pilus system; ATP/GTP-binding protein with Walker A and B boxes; peripheral membrane protein	✓	×	Wiesner et al. 2003; Beauchamp et al. 2015
ctsX (Cj1472c)	Unique membrane protein; transformation system protein with unknown function; interacts with CtsP	✓	✓	Wiesner et al. 2003; Beauchamp et al. 2015
ctsE/pilF/comGA (Cj1471c)	Type II secretion/T4 pilus system; ATP/GTP-binding protein with Walker A and B boxes; present in soluble fraction	✓	✓	Wiesner et al. 2003; Beauchamp et al. 2015
ctsF/pilG/comGB (Cj1470c)	Type II secretion/T4 pilus system, membrane protein, putatively constitutes platform for pilus/pseudopilus assembly	✓	✓	Wiesner et al. 2003
ctsG/pilE/comGC (Cj1343c)	Type II secretion/T4 pilus system, periplasmic protein; major (?) pre-pilin	✓	✓	Wiesner et al. 2003
ctsT/comGD (Cj1077)	Type II secretion/T4 pilus system; Periplasmic protein; pre-pilin	✓	×	Wiesner et al. 2003

(continued)

Table 2 (continued)

Gene name[1]	Putative function in natural transformation	Cc	Cl	Reference
Cj1078*	Type II secretion/T4 pilus system; Periplasmic protein; prepilin; not yet been demonstrated to function in natural transformation in *C. jejuni*	✓	✕	Wiesner et al. 2003
Cj0825**/pilD/comC*	Putative prepilin peptidase with transmembrane helices	✓	✓	Wiesner et al. 2003
ctsW (Cj1028c)	Not involved in DNA uptake (maybe role in cytoplasmic transport or recombination); purine/pyrimidine phosphoribosyltransferase	✓	✓	Wiesner et al. 2003
ctsM (Cj0208)	DNA modification methylase of RAATTY motif; important for recognition of free DNA	✓	✓	Beauchamp et al. 2017

[1] Gene names as annotated for *C. jejuni* strain NCTC11168 with homologs in *Neisseria* or *Bacillus subtilis* are depicted with putative function in natural transformation
*, evidence only by BLAST homology; BLAST searches based on the translated nucleotide database using a protein query; accession 13.04.2020; Cc, *C. coli*, Cl, *C. lari*
✓, presence and ✕, absence of homologous genes is depicted

et al. 2003). The role of these genes in natural transformation is still unknown. Fry and colleagues reported that a *galE* mutant, with defects in lipopolysaccharide (LPS) synthesis, showed a mild 20-fold reduction in DNA uptake and chromosomal integration, which might be indicative of LPS influencing the function of the DNA uptake machinery (Fry et al. 2000).

The periplasmic DNA-binding competence protein (Com) ComE was shown to facilitate DNA entry into the periplasm in *Neisseria* and *Vibrio* by generation of a pulling force on double-stranded (ds) DNA upon binding (Hepp and Maier 2016; Seitz et al. 2014). The role of the *C. jejuni comE* homolog (Cj0011c) has not been unraveled completely. Transformation rates in Cj0011c knockout mutants were only decreased by 10- to 50-fold (Jeon and Zhang 2007). Interestingly, *C. coli*, which displays similar gene homologs of a type II secretion/type IV pilus system as *C. jejuni* (Table 2), lacks a *comE* homolog (Meric et al. 2014). *comE* is also missing in C. *lari* (Table 2). Hence, in *C. coli* and *C. lari* a role of ComE in natural transformation can be ruled out.

Once the DNA reaches the periplasm, dsDNA has to be unzipped for import of single-stranded (ss) DNA into the cytoplasm mediated by the inner membrane channel ComEC in all so far known competent bacteria (Dubnau and Blokesch 2019). Absence of transformation activity in *C. jejuni comEC* (Cj1211) insertional mutants was demonstrated, whereas binding and uptake of radiolabeled DNA were not impaired (Jeon et al. 2008). Depending on homology, incoming DNA will be

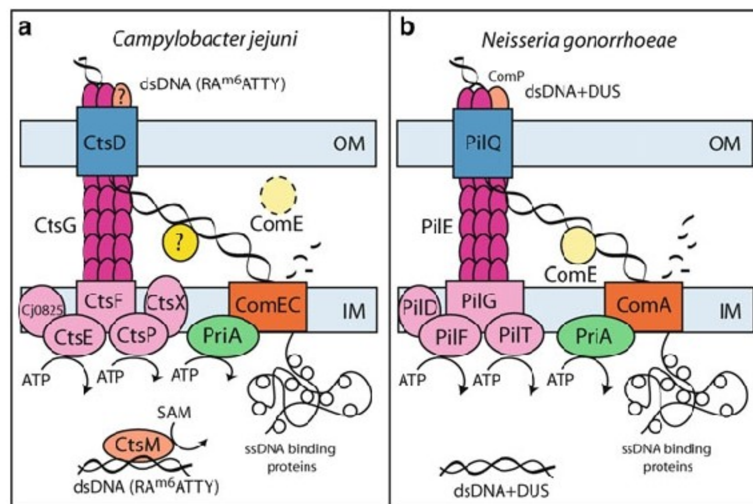


Fig. 2 Working model of the DNA uptake complex for natural transformation in *Campylobacter jejuni* A compared to the system in *Neisseria gonorrhoeae* B Uptake of free external DNA probably occurs in two steps. Transport into the periplasm is mediated by a type II secretion/T4 pili system. Homology analysis suggests CtsD as the outer membrane porin. CtsG might be the major pilin but further pilin proteins CtsT and Cj1078 were identified in *C. jejuni* (see also Table 2). CtsF might form the basis for pilus/pseudopilus. CtsE and CtsP were proposed as ATPases, eventually empowering the DNA uptake process and/or pili assembly. The unique membrane protein CtsX was shown to interact with CtsP. The role of ComE as DNA-binding protein in the periplasm is enigmatic, since a homolog is lacking in *C. coli* and *C. lari*. ComEC appears to be the inner membrane channel as proposed for all competent bacteria, leading to import of single-stranded DNA into the cytoplasm, eventually empowered by PriA. In *Neisseria,* the minor pilin ComP recognizes a specific DNA uptake sequence (DUS) for selective uptake of DNA from relatives. In *C. jejuni,* the methylated $RA^{m6}ATTY$ motif is recognized by a yet unknown receptor. Methylation is mediated by the CtsM methylase. OM, outer membrane; IM, inner membrane; SAM, S-adenosylmethionine; ATP, adenosine triphosphate; dsDNA, double-stranded DNA; ssDNA, single-stranded DNA

incorporated into the genome by homologous recombination. Site-directed homologous recombination of transformed plasmid DNA was observed with homologous regions of at least 286 bp, whereas 125-270 bp homology only led to a random and rare non-homologous insertion into the chromosome (Richardson and Park 1997). However, although non-homologous integration of DNA is infrequent, this mechanism guarantees the incorporation of completely novel genes.

C. jejuni strain 81–176 carries the plasmid pVir, encoding homologs to *Helicobacter pylori cag* pathogenicity island as well as homologs to type IV secretion systems (Bacon et al. 2002; Fischer et al. 2020). The role of pVir in natural transformation is not completely understood. Nevertheless, Bacon and colleagues (2000) showed an 80% reduction in transformation frequency in a *comB3* mutant, whereas *virB11* inactivation did not show a reduced transformation activity. Mutation of one of the glycosylation sites in the glycoprotein VirB10 or deletion of *virB10* showed a mild ~ tenfold decrease in transformation efficiency (Larsen et al. 2004). Knockout of the

N-linked protein glycosylation system (*pgl*), e.g., by deletion of *pglB* or *pglE* led to a drastic 10,000-fold decreased transformation rate (Larsen et al. 2004), suggesting that glycosylation of proteins is essential for natural transformation. Mutations in *virD4* and *comB1* led to wild-type transformation activity (Wiesner et al. 2003), thus, unlike the situation in the close relative *H. pylori*, the VirB/ComB system does not seem to play a major role for DNA uptake in *C. jejuni*.

2.2 Conjugative Gene Transfer

In conjugation processes, DNA is transferred from a donor to a recipient cell through cell-to-cell contact (Lederberg and Tatum 1946). To date 177 plasmid sequences from *Campylobacter* spp. are released at NCBI (https://www.ncbi.nlm.nih.gov/genome/browse#!/plasmids/campylobacter; accession on 22.09.2020). The size of currently identified *C. jejuni* and *C. coli* plasmids ranges from 1.3 to 190 kb. The presence of megaplasmids was shown in various strains from retail (Marasini and Fakhr 2016, 2014; Ghatak et al. 2017; Gunther et al. 2016). The transfer of plasmid-encoded antibiotic resistances in *Campylobacter* has frequently been reported (Taylor et al. 1981; Velazquez et al. 1995; Gibreel et al. 2004; Batchelor et al. 2004; Pratt and Korolik 2005; Zeng et al. 2015; Tang et al. 2017). Type-1 plasmids (pTet) harboring *tetO* are most prevalent in *C. jejuni* and *C. coli* (Schmidt-Ott et al. 2005; Marasini et al. 2018). Although only the *tetO* gene is representative for all pTet plasmids, most of them carry a VirB-type IV secretions system for conjugation. Type-2 plasmids were primarily found in *C. coli* strains, which are characterized to have a size between 24 and 32 kb and bear several *trb* genes for conjugative transfer as well as *virD4*, *traI* and *traQ* (Marasini et al. 2018).

The pVir plasmid of *C. jejuni* strain 81-176 mentioned above is categorized as the "prototype" of the type-3 plasmids. Small plasmids < 6 kb were categorized as type-4 plasmids despite absence of homologous genes shared between them. They contain genes with hypothetical function and replication initiator genes and await further investigations.

Pratt and Korolik (2005) showed that conjugation frequencies of a plasmids encoding *tetO* from donor strains to a recipient strain varied between ~10^{-8} and 10^{-6} within 6 h of mating. Interestingly, also co-transfer of a smaller plasmid was observed together with a larger plasmid conferring resistance to tetracycline (Pratt and Korolik 2005). Absence of conjugation in some strains was observed, indicative of barriers, e.g., restriction–modification systems and/or inability of plasmids to replicate in specific strains. Strain dependency of conjugation rates was identified in a different study, showing variations from 10^{-8} to 10^{-3} (Zeng et al. 2015). Hence, it might be concluded that natural transformation with transformation rates of ~ 10^{-3}–10^{-2} is a more efficient way of HGT in *Campylobacter* spp. However, further studies are needed to collect more data on different field strains and to correlate in vitro with in vivo HGT frequencies.

Conjugation efficiency was induced 100–1000-fold in strains with low-frequency conjugation (LFC) upon 30 min heat shock at around 50 °C (Zeng et al. 2015). Recently, Zeng and colleagues (2018) identified the restriction–modification enzyme CjeI (Cj1051c) as crucial factor for reduced conjugation rate in the LFC strain NCTC11168. In high conjugation frequency (HCF) strains, 1000-fold reduced conjugation frequency was observed upon chromosomal complementation with *cjeI*. The *cjeI* mutants showed enhanced conjugation efficiency, which was nearly independent of heat shock, suggesting that CjeI was the heat-inactivated limiting factor of successful conjugational transfer of plasmids in LFC strains. It was previously observed that CjeI also restricted incoming DNA during natural transformation (Holt et al. 2012). Restriction barriers are discussed in more detail in Sect. 3.

Interestingly, unidirectional DNaseI-resistant conjugation-like transfer of a chromosomal resistance gene was observed from *H. pylori* to *C. jejuni* (Oyarzabal et al. 2007), demonstrating the potential of bacteria of the class Campylobacterales for genetic exchange (Fernandez-Gonzalez et al. 2014).

2.3 Phage Transduction and Genomic Rearrangements

Campylobacter bacteriophages have been isolated from diverse matrices, including food, animals and environments (for a recent review on isolation methods, see Jäckel et al. 2019), indicating that the pathogen is constantly exposed to phages in its natural habitat. *Campylobacter* bacteriophages were first reported in 1968 in *C. fetus* (formerly *Vibrio fetus*) upon induction of lytic phase by the bactericidal agent mitomycin C (Firehammer and Border 1968). For details on the application of bacteriophages for *Campylobacter* infection control, the reader should refer to Chap. 6 of this book.

Most sequenced *Campylobacter* phages (CP) belong to the family Myoviridae, displaying long contractile tails (Javed et al. 2014; NCBI Taxonomy Browser, accession 22.09.2020). They are categorized into two main groups, the Firehammervirus, group II, CP220-like and the Fletcherviruses, group III, CP8-like phages. Group I phages with large genomes of ~320 kb are, however, rare. DNA from *Campylobacter* phages was observed to be refractory to digestion by several restriction enzymes (Sails et al. 1998), which was recently attributed to complete exchange of deoxyguanosine (dG) by modified bases in phage DNA (Crippen et al. 2019).

In general, bacteriophage predation was shown to lead to chromosomal rearrangements in bacteria and, therefore, phages could also contribute to *Campylobacter* genomic evolution. For example, up to 590 kb in *C. jejuni* were inverted due to inversions caused by Mu-like phages (Scott et al. 2007). Interestingly, *C. jejuni* carrying the bacteriophage in its chromosome were resistant to infections by other bacteriophages but revealed an inefficient colonization of the chicken. Besides, the integration of phage-like elements into the genome can lead to genomic changes, visible by altered pulsed-field gel electrophoresis (PFGE) patterns of cleaved chromosomal DNA (Barton et al. 2007).

The process of phage transduction can be divided into several steps. Initially, the bacteriophage has to interact with a receptor on the bacterial cell. Principally, *Campylobacter* phage infection was shown to be either dependent on modifications of the capsular polysaccharides (Sorensen et al. 2011) or on motile flagella (Baldvinsson et al. 2014). Receptor-type dependency could be correlated with phage genus (Sorensen et al. 2015). While CP81-like Fletcherviruses were dependent on capsular polysaccharide (CPS), thereby unable to infect acapsular ($\Delta kpsM$) mutants, the CP220-like Firehammerviruses were deficient of infecting non-motile ($\Delta motA$) *C. jejuni* strains. The receptor in *C. jejuni* NCTC 11168 for the Myoviridae phage F336 was shown to be an *O*-methyl phosphoramidate attached to 2-acetamido-2-deoxy-D-galactofuranose (GalfNAc) on the capsular polysaccharide (Sorensen et al. 2011). A frameshift in the phase variable homopolymeric G tract of gene Cj1421 resulting in a non-functional O-methyl phosphoramidate (MeOPN) transferase conferred resistance against phage F336. This is because the receptor is unavailable due to lack of receptor attachment to CPS. In addition, Cj1422, another phase variable gene, was shown to attach MeOPN to a heptose in CPS in *C. jejuni*, which confers resistance to F336 (Holst Sorensen et al. 2012; Aidley et al. 2017). The existence of further CPS receptors independent of MeOPN in CPS-dependent phages was suggested recently (Gencay et al. 2018).

In addition, it was shown that a conserved glycan-specific phage protein, Gp047 renamed FlaGrab, recognizes 7-acetamidino-modified pseudaminic acid residues on *Campylobacter* flagella, inhibiting bacterial growth (Javed et al. 2015). In particular, FlaGrab exposure led *C. jejuni* cells to downregulate expression of energy metabolism genes, which was dependent on a functional flagellar motor and was host strain-dependent, irrespective of the level of motility (Sacher et al. 2020). However, FlaGrab is also present in CPS-dependent phages, but is not part of the phage capsule. Thus, it was speculated that FlaGrab is not involved in phage entry, but presents an important protein in the phage lifecycle. It may either function as extracellular effector molecule upon phage-induced cell lysis, improving new infection by reduction of host motility or intracellularly during phage infection (Javed et al. 2015).

The transcriptional bacterial response upon infection of a CP8-like type-III phage NCTC 12673 revealed regulation of an unknown operon with some homology to T4 phage superinfection exclusion and antitoxin genes, as well as multidrug efflux pumps and oxidative stress defense genes (Sacher et al. 2018). Mutants of the *cmeABC* efflux pump were more susceptible for phage infections, while loss of catalase and superoxide dismutase genes led to enhanced phage resistance (Sacher et al. 2018). Thus, it seems that phage infection modulates the capacity of the host to resist antimicrobial treatment and oxidative stress, probably as part of phage–host dynamics. Interestingly, RidA, previously shown to play a role in flagella–flagella interactions due to regulation of flagellar glycan modification and motility (Reuter et al. 2015), was observed to also function in bacteriophage infectivity (Irons et al. 2019). However, the exact molecular mechanism is not yet clear. Taken together, more studies are clearly needed to fully understand *Campylobacter* phage lifecycle and the complex interaction with their host.

The ganglioside-like structures GM1 and GD1 generated by the *cts-II*-encoded sialyltransferase play a role in resistance against bacteriophages (Louwen et al. 2013). This was first suggested by the observation that isolates involved in Guillain-Barré syndrome induction showed lower susceptibility to a panel of 29 bacteriophages. Furthermore, a *ΔctsII* mutant showed increased susceptibility to bacteriophage than the wild-type bacteria. Bioinformatic screening revealed a correlation between the presence of *ctsII* and a degenerated CRISPR-Cas system (see also Sect. 3) in *C. jejuni* strains, indicating that virulence-associated ganglioside-like structures might serve as bacteriophage defense system.

While above we have discussed the current knowledge on lytic phages, also chromosomally integrated prophages have been described in various *Campylobacter* strains. For example, *C. jejuni* strain RM1221 carries four so-called *Campylobacter jejuni*-integrated elements (CJIEs), three of which (CJIE1, 2 and 4) seem to originate from phages (Barton et al. 2007) and the fourth (CJIE3) putatively from an integrated plasmid (Fouts et al. 2005; Parker et al. 2006). The Mu-like phage CJIE1 is integrated upstream of the *argC* gene, encoding an N-acetyl-γ-glutamyl-phosphate reductase. CJIE2 and CJIE4 are integrated at the 3'end of arginyl- and methionyl-tRNA genes. CJIE3 is integrated into the 3' end of an arginyl-tRNA. CJIE1 encodes typical Mu and Mu-like phage proteins. CJIE2 and CJIE4 potentially encode methylases, endonucleases and repressors. CJIE1 was present among ~ 1/7 of *Campylobacter* isolates obtained from surveillance programs in Canada (Clark 2011). Most of these isolates were *C. jejuni* but CJIE1 was also present in *C. coli* and *C. upsaliensis*. The sequence and structure of the integrated CJIE1 varied, leading to protein alterations (Clark and Ng 2008). Furthermore, integration loci varied in different *C. jejuni* strains (Parker et al. 2006). Similarly to CJIE1, also CJIE2 and CJIE4 were inserted at different loci in the *Campylobacter* chromosome in different strains (Clark and Ng 2008). However, until now, induction of these CJIE prophages to lytic phase was unsuccessful (Clark and Ng 2008).

3 Barriers to Horizontal Gene Transfer

While HGT is crucial for the acquisition of novel genetic material and beneficial adaptation to changing environments, introgression of foreign DNA in bacterial genomes can also lead to tremendous fitness loss. The fact that *Campylobacter* is well protected by HGT barriers becomes obvious, since genetic manipulation of *Campylobacter* is hampered using constructs amplified in cloning strains of *Escherichia coli* (Gardner and Olson 2012). In the following, we address the aspect of barriers to HGT and focus on the CRISPR-Cas system and on other nucleases, including restriction–modification systems protecting *Campylobacter* against incoming foreign DNA. Furthermore, we address the question how *C. jejuni* can select for DNA of relatives, without using a classical DNA uptake sequence as demonstrated for other bacteria.

3.1 CRISPR-Cas and Nucleases

"Bacterial immunity" based on clustered regularly interspaces short palindromic repeats (CRISPR) and CRISPR-associated (Cas) proteins might be a powerful mechanism for restriction of horizontal gene transfer in *Campylobacter*. These systems are present in ~ 40% of complete bacterial and ~ 85% of archaeal genomes (Makarova et al. 2020). The principle is that incoming foreign DNA is memorized by incorporation of small fragments in CRISPR regions. Upon repeated entry, complementary CRISPR rRNAs (crRNA) in complex with Cas proteins target invading DNA for degradation. CRISPR-Cas systems are classified into two classes, six types and at least 33 subtypes (Makarova et al. 2020). While class 1 systems use multiple Cas proteins building up the effector complex, the class 2 system uses a single-protein effector, e.g., Cas9 in case of *Campylobacter* spp. Class 2 systems currently include three types and 17 subtypes. *C. jejuni* harbors a class 2, type II-C CRISPR-Cas system. It consists of the genes *cas1*, *cas2* and *cas9* as well as a trans-activating CRISPR RNA (TracrRNA). Cas1 and Cas2 are suggested to acquire and integrate new protospacers (Yosef et al. 2012). Cas9 participates in spacer acquisition (Heler et al. 2015; Wei et al. 2015). CRISPR loci are transcribed as a single pre-crRNA precursor, which is processed to crRNAs by the bacterial non-Cas RNase III in type II systems. In turn, crRNAs in complex with Cas9 silence invading plasmid or phage DNA, which bear sequence homology to the integrated spacer sequences. DNA strand breaks at stalled replication forks induce RecBCD-dependent spacer acquisition. In order to avoid autoimmunity, chromosomal loci were protected against spacer acquisition by relatively abundant Chi sites in *E. coli*, at which dsDNA break repair is stimulated in bacteria (Levy et al. 2015). However, self-DNA might be integrated into the CRISPR loci at very low frequency (Stern et al. 2010). Cas4-like proteins in *Campylobacter* bacteriophages were suggested to modify spacer element acquisition in favor of phage evasion due to preferential integration of host sequences in CRISPR loci (Hooton and Connerton 2014). Thus, coevolution of phages with the host leads to continuous modulation of genome dynamics.

The optimal size of the bacterial memory is dependent on the diversity of threats, i.e., phages. Since the effectiveness of response is dependent on the number/concentration of crRNA-Cas complexes with matching specificity, the depth of memory was proposed to be limited to 10–100 spacers in bacteria (Bradde et al. 2020). Based on the current database called CRISPRCasFinder, hosted at the University of Paris-Saclay, the numbers of predicted CRISPR loci in *C. jejuni* and *C. coli* range from 0 to 11 (median = 1; nCj = 207, nCc = 37, accession 22.09.2020 (at https://crisprcas.i2bc.paris-saclay.fr/MainDb/StrainList), harboring each one to multiple spacers (Grissa et al. 2007; Couvin et al. 2018). However, low transcription of crRNAs and TracrRNA was observed in *C. jejuni* RM1221 due to a stop-mutation in *cas9* (Dugar et al. 2013). Thus, the authors suggested that absence of CRISPR loci or truncation of *cas9* enabled acquisition of prophages or plasmids and that

active CRISPR and mobile elements are mutually exclusive. Although Cas9 nucleases usually target dsDNA, a recent study demonstrated that in *C. jejuni* also endogenous ssRNA was targeted by CjCas9 (Dugar et al. 2018). Hence, it was proposed by the authors that CjCas9 may also serve to target RNA viruses or even regulate endogenous gene expression, which should be investigated in the future.

Apart from the CRISPR-Cas system, periplasmic nucleases were reported to degrade incoming genomic DNA, thereby inhibiting natural transformation. The periplasmic DNase, encoded by the *dns* gene (CJE0256) from Mu-like prophage CJIEI, inhibits natural transformation in RM1221 (Gaasbeek et al. 2009). Transformability of field strains correlated with presence or absence of *dns*. Homologs of DNA/RNA non-specific endonucleases were subsequently also detected on the prophages CJIE2 and CJIE4 and inhibition of natural transformation levels by around 30–40-fold were demonstrated (Gaasbeek et al. 2010).

3.2 Methylation-Dependent DNA Recognition

It has been reported long time ago that *C. jejuni* preferentially takes up DNA from siblings, although the mechanisms were completely unknown (Wang and Taylor 1990). However, *C. jejuni* does not have a typical DNA uptake sequence (DUS), like it was demonstrated for *N. gonorrhoeae* (Goodman and Scocca 1988), with the minor pilin protein ComP identified as specific receptor (Cehovin et al. 2013). Nevertheless, *C. jejuni* selects DNA of relatives and discriminates against foreign DNA. By single-molecule real-time sequencing (SMRT), a high degree of methylation of chromosomal DNA became apparent, with the RAATTY site being the only methylation site shared between *C. coli* BfR-CA-09557 and other *C. jejuni* strains (Zautner et al. 2015). By deletion of the respective methylase gene *ctsM* (named as *Campylobacter* transformation system methyltransferase), it was shown that *C. jejuni* recognizes the adenine N6 (exocyclic NH_2-group at the sixth position of the purine ring) methylated RAATTY site of free external DNA as first step of natural transformation (Beauchamp et al. 2017). *ctsM* mutants were not impaired in DNA uptake, indicating that CtsM itself is not involved in recognition and/or transport of methylated DNA. The authors also demonstrated that *E. coli* plasmids could successfully be transformed into *C. jejuni* after methylation with the *E. coli* *Eco*RI methylase. In this case, one of the four RAATTY-sites, namely GAATTC is methylated, which was sufficient for DNA uptake in *Campylobacter*. This study presented a major advantage for future genetic manipulation of *Campylobacter* spp., since researchers can substantially improve genetic manipulation by methylation of plasmid constructs via commercially available *Eco*RI methylase prior to transformation in the respective *Campylobacter* host. The native *Eco*RI system is restricted to a special strain of *E. coli* and not ubiquitously found in this species, explaining that DNA from *E. coli* does not present a substrate for natural transformation of *Campylobacter* spp. It remains to be investigated, which components of the DNA

uptake complex recognize the methylated RAATTY motif, in order to decipher the mechanism of selective DNA uptake in the foodborne pathogen.

Apart from CtsM as methylase, other restriction–modification systems are thought to constitute a genetic barrier for incoming DNA. The restriction–modification type IIG enzyme Cj1051c was shown to lower transformation efficiency using a *C. jejuni* derived plasmid by 1000-fold (Holt et al. 2012). Cj1051c was shown to also drastically reduce conjugation efficiency among *C. jejuni* strains (Zeng et al. 2018). Since *Campylobacter* genomes harbor diverse methylation profiles and various restriction-methylation genes, that are also strain-dependent (O'Loughlin et al. 2015; Zautner et al. 2015), it is expected that several other restriction–modification systems play crucial roles in establishing genetic barriers, even against relatives, favoring clonal spreading.

4 Impact of Gene Transfer on *Campylobacter* Fitness

As discussed above, during evolution *Campylobacter* spp. have developed powerful means for HGT and coevolved with incoming genetic material in order to balance the acquisition of novel material and putative detrimental effects. In the following, we address the beneficial impact of gene transfer and report on fitness advantages due to enormous genetic plasticity of the foodborne pathogen.

4.1 Spread of Resistomes and Persistence Factors

Human infections by *Campylobacters* are commonly caused by consumption and handling of raw poultry meat (for more details see Chap. 1 of this book). While most human campylobacteriosis cases are self-limiting, antibiotic treatment, in particular the use of macrolides or fluoroquinolones, was reported in around one-third of the patients (Rosner et al. 2017). The spread of antibiotic resistances by HGT enables preadaptation to changing environments and leads to diversification of the bacterial population. The observation of different resistances shared between *C. jejuni* and *C. coli* strains isolated from livestock, sewage and human disease indicated frequent spread of plasmids and multidrug-resistant genomic islands (MDRGIs) by HGT (Mourkas et al. 2019). Spread of antibiotic resistance between *C. jejuni* strains by natural transformation was reinforced in biofilms versus planktonic environments (Bae et al. 2014). For details on biofilm formation and quorum sensing, the reader should refer to Chap. 11 of this book. Biofilms contain extracellular DNA and are thought to convey enhanced persistence of host-associated pathogens in the environment. Their role in the dissemination of antibiotic resistances remains to be studied in more detail.

One of the well-known and the most prevailing resistance mechanisms of *Campylobacter* against macrolides in European strains is the point mutation A2075G in

the 23S rRNA gene. This mutation is associated with a substantial decrease in bacterial fitness (Wang et al. 2014; Luangtongkum et al. 2012), probably leading to the currently observed low rates of macrolide resistance in *Campylobacter* spp. from livestock (EFSA 2020). Recently, *C. jejuni* and *C. coli* strains were isolated carrying the gene *ermB*, encoding an rRNA methylase, conferring resistance against macrolides in Asia (Qin et al. 2014; Du et al. 2018; Cheng et al. 2020; Liu et al. 2017), Europe (Florez-Cuadrado et al. 2016) and USA (Chen et al. 2018). Up to now, nine types of *ermB*-carrying MDRGI have been identified in *Campylobacter* spp. Besides *ermB*, these islands include resistances against aminoglycosides, such as gentamicin, kanamycin, streptomycin, spectinomycin or streptothricin, as well as ampicillins and tetracyclines (Wang et al. 2014; Florez-Cuadrado et al. 2016; Chen et al. 2018). *C. coli* strains were also identified, harboring *ermB* on different plasmids (Wang et al. 2014). The published NCBI sequences of *Campylobacter ermB* present four different allele variants. Comparative genome analysis revealed identical *ermB* sequences in *Campylobacter*, *Streptococcus suis*, *Enterococcus faecium* and *Clostridium difficile* isolates from different matrices (Florez-Cuadrado et al. 2017), suggesting multiple HGT events among different species. Especially the spread of macrolide resistance is of great danger, since macrolides are often drugs of choice to treat campylobacteriosis in humans (Rosner et al. 2017).

A variant of the multidrug efflux pump RE-CmeABC (for resistance-enhancing *Campylobacter* multidrug efflux system ABC), displaying sequence variation and enhanced expression due to a mutation in the promotor region, was shown to be spread via natural transformation and homologous recombination (Yao et al. 2016). This "super" pump conveys increased minimal inhibitory concentrations (MICs) against antimicrobials, such as ciprofloxacin, erythromycin, phenicols and tetracycline.

As long as the acquired antibiotic resistance determinant does not lead to fitness decrease, it can stably remain in a strain and is readily spread to other strains. For example, the transfer of *tetO* in *C. jejuni* was demonstrated to occur in vivo in chicken even without selection pressure (Avrain et al. 2004). This is especially important because it demonstrates that in case a long-term antimicrobial is discontinued, the resistance might persist and even spread to other strains. It is further stated that tetracyline resistance determinant, *tetO*, originated from Gram-positive cocci (Sougakoff et al. 1987) and kanamycin resistance seems to originate from Gram-positive cocci or from *Enterobacteriaceae* (Ouellette et al. 1987; Gibreel and Skold 1998). Apart from tetracycline resistance, resistance against (fluoro-)quinolones was observed to be another example of resistance determinant, not necessarily vanishing upon cease of antibiotic use. The resistance is conferred by the C257T point mutation in the gene encoding gyrase subunit A (*gyrA*). This point mutation was shown to even exert a fitness advantage on certain *Campylobacter* strains in the in vivo chicken gut environment (Luo et al. 2005). The spread of fluoroquinolone resistance in distinct clonal lineages might at least partially be explained by this fitness enhancement, although an additional selective pressure by antibiotic usage cannot be ruled out (Kovac et al. 2015; Leekitcharoenphon et al. 2018).

Not only the dissemination of antibiotic resistances bears risks for human health, but also the spread of bacterial persistence factors can increase the adaptive potential

favoring the pathogens' survival and transmission. However, since the function of gene variations is mostly unknown and it is expected that multiple gene exchanges synergistically lead to a beneficial adaptation, reports are scarce on the acquisition of novel traits other than antibiotic resistances. Mosaic sequence exchange in the highly similar flagellin genes *flaA* and *flaB* was observed on the intra- and intergenomic level (Wassenaar et al. 1995; Harrington et al. 1997). *Campylobacter* virulence is dependent on motility and, thus, a functional flagellar system. Hence, variations of the involved structural genes lead to variants, putatively evading host immune response.

Phongsisay and colleagues (2006) showed that human ganglioside-like structures, such as GM1, were readily transformable to strains not associated with Guillain-Barré syndrome induction in humans. The resulting transformants had acquired large DNA fragments and presented a high degree of genetic and phenotypic variation, corroborating the enormous potential of *C. jejuni* for genome plasticity upon natural transformation. Another interesting study highlighted successful HGT of genes with metabolic functions (Vorwerk et al. 2015). In particular, most *Campylobacter* strains are not capable of catabolizing glucose. Nevertheless, some *C. coli* strains harbor a genomic island, which allows using glucose as an energy source through the metabolic Entner–Doudoroff pathway. This locus was transferred between *C. coli* strains as well as between *C. coli* and *C. jejuni*, conferring glycolytic activity (Vorwerk et al. 2015), suggesting that this metabolic trait was acquired in order to optimize energy supply in distinct niches.

4.2 Interspecies Gene Transfer

As reported above, *C. jejuni* differentiates DNA of relatives by recognition of the methylated RAATTY profile, mediated by the N-adenine specific methylase CstM (Beauchamp et al. 2017). *ctsM* homologs are present in thermophilic *Campylobacter* spp., suggesting that gene transfer is enabled between different species. The manifestation of incoming DNA is further dependent on the degree of homology and of strain-specific restriction–modification systems as well as nucleases, which function as genetic barriers (see Sect. 3).

Genetic exchanges can also be analyzed using genome analysis of bacterial populations. The population structure of *C. jejuni* is different from *C. coli* even though their core genomes show a nucleotide sequence identity of ~ 85%, and they colonize similar habitats (Dingle et al. 2005). From nearly 3,000 MLST types, 11% of *C. coli* sequence types showed *C. jejuni* origin, vice versa this was only estimated for 0.6% of the *C. jejuni* types (Sheppard et al. 2008). This indicated a considerable but asymmetric gene flow between the two major thermophilic *Campylobacter* species. *C. jejuni* has a very diverse structure, with over 40 clonal complexes. In *C. coli,* only three different clades were identified (Sheppard et al. 2012). Clade 1 is predominantly found in clinical and animal farm samples and comprises the majority of all isolated and sequenced *C. coli* strains, whereas clade 2 and 3 were found in waterfowl and riparian environment. A genetic exchange between *C. jejuni* and *C. coli* of clade 1

was observed previously (Sheppard et al. 2011), while clade 2 and 3 were unaffected by *C. jejuni* introgression, probably due to separated niches and lack of contact with *C. jejuni*. A separation of individual clones with rare or no contact to others and a host tropism can explain why some strains isolated from the same host in different geographic location are more related than strains from different hosts (Sheppard and Maiden 2015). The study by Epping and colleagues (2020) analyzing whole-genome sequences of more than 490 *C. jejuni* strains obtained from Germany and Canada showed a strong host association and enables to further study host adaptation on the level of subsets of variant genes. For more details, the reader should refer to Chap. 3 of this book. Frequent HGT events might also give rise to a population of *Campylobacter* strains that are called "generalists," able to colonize multiple hosts.

Introgression can occur as mosaic recombination of gene alleles. Consistent with asymmetric gene flow between the two species, the exchange from *C. jejuni* into *C. coli* was 17 times more frequently observed than from *C. coli* to *C. jejuni* (Sheppard et al. 2011). However, based on frequent genetic exchange, a convergence between the species *C. coli* and *C. jejuni* was postulated (Sheppard et al. 2008). For *C. jejuni* and *C. coli,* there are 44 clonal complexes and 11,111 sequence types defined (https://pubmlst.org, accession on 22.09.2020). Interestingly, nearly 40% of sequence types are not assigned to a clonal complex, demonstrating the diverse genome structure of these two major species. However, *C. jejuni* diversity is much greater than *C. coli* as defined by core genome phylogeny (Golz et al. 2020), with yet unknown reason.

We have identified *C. coli* strains as a fraction of clade 1, which have undergone recent ongoing extended introgression by *C. jejuni* sequences (Golz et al. 2020). These strains were particularly isolated from chicken eggs, i.e., from fecal contamination on egg shells. K-mer analysis on whole-genome sequences revealed that these "hybrid" strains had incorporated up to 15% of genomic sequences from *C. jejuni* along the whole genome. However, a more in-depth analysis showed that recombination events were not random but followed a common pattern. In particular, *C. jejuni* introgression occurred in a common set of genes, implicated in stress defense. Hence, this genome alteration might represent a functional adaptation to survival in a harsh environment and confirms the enormous potential of natural transformation in shaping *Campylobacter* genomes.

5 Concluding Remarks

Due to high levels of genetic exchange by natural transformation, conjugation or transduction, *Campylobacter* shows an enormous genome diversity. This widens the pathogens adaptive potential and enables colonization of multiple hosts and successful survival in the environment, although the microaerobic bacterium is generally stress-sensitive and fastidious. Also, spread of antibiotic resistances endangers therapy options for treatment of campylobacteriosis (Oyarzabal and Backert 2012). The mechanisms of HGT in *Campylobacter* are yet poorly understood, and there is an urgent need to understand more in detail how the pathogen adapts by gene acquisition

and/or gene variation. For example, open questions remain of how HGT is regulated in the pathogen, i.e., under which conditions gene transfer is most active and efficient. Once parameters are revealed that inhibit competence development and/or the function of HGT mechanisms, those critical elements could serve as target for the development of HGT inhibition. Especially in the context of control strategies such as chemical decontamination, bacteriophage treatment or vaccine development, it will be crucial to have a second-line strategy for prevention of pathogen adaptation. Therefore, the inhibition of HGT in *Campylobacter* is a promising approach in combating *Campylobacter*.

Acknowledgements This work is supported by the German Federal Ministry of Education and Research (BMBF) in frame of the zoonoses research consortium PAC-CAMPY to K.S. (project IP3/01KI1725B).

References

Aidley J, Sorensen MCH, Bayliss CD, Brondsted L (2017) Phage exposure causes dynamic shifts in the expression states of specific phase-variable genes of *Campylobacter jejuni*. Microbiology 163(6):911–919. https://doi.org/10.1099/mic.0.000470

Avery OT, Macleod CM, McCarty M (1944) Studies on the chemical nature of the substance inducing transformation of pneumococcal types: induction of transformation by a desoxyribonucleic acid fraction isolated from pneumococcus type III. J Exp Med 79(2):137–158. https://doi.org/10.1084/jem.79.2.137

Avrain L, Vernozy-Rozand C, Kempf I (2004) Evidence for natural horizontal transfer of *tetO* gene between *Campylobacter jejuni* strains in chickens. J Appl Microbiol 97(1):134–140. https://doi.org/10.1111/j.1365-2672.2004.02306.x

Bacon DJ, Alm RA, Burr DH, Hu L, Kopecko DJ, Ewing CP, Trust TJ, Guerry P (2000) Involvement of a plasmid in virulence of *Campylobacter jejuni* 81-176. Infect Immun 68(8):4384–4390. https://doi.org/10.1128/iai.68.8.4384-4390.2000

Bacon DJ, Alm RA, Hu L, Hickey TE, Ewing CP, Batchelor RA, Trust TJ, Guerry P (2002) DNA sequence and mutational analyses of the pVir plasmid of *Campylobacter jejuni* 81-176. Infect Immun 70(11):6242–6250. https://doi.org/10.1128/iai.70.11.6242-6250.2002

Bae J, Oh E, Jeon B (2014) Enhanced transmission of antibiotic resistance in *Campylobacter jejuni* biofilms by natural transformation. Antimicrob Agents Chemother 58(12):7573–7575. https://doi.org/10.1128/AAC.04066-14

Bakkali M (2013) Could DNA uptake be a side effect of bacterial adhesion and twitching motility? Arch Microbiol 195(4):279–289. https://doi.org/10.1007/s00203-013-0870-1

Baldvinsson SB, Sorensen MC, Vegge CS, Clokie MR, Brondsted L (2014) *Campylobacter jejuni* motility is required for infection of the flagellotropic bacteriophage F341. Appl Environ Microbiol 80(22):7096–7106. https://doi.org/10.1128/AEM.02057-14

Barton C, Ng L-K, Tyler SD, Clark CG (2007) Temperate bacteriophages affect pulsed-field gel electrophoresis patterns of *Campylobacter jejuni*. J Clin Microbiol 45(2):386–391. https://doi.org/10.1128/JCM.01513-06

Batchelor RA, Pearson BM, Friis LM, Guerry P, Wells JM (2004) Nucleotide sequences and comparison of two large conjugative plasmids from different *Campylobacter* species. Microbiology 150(Pt 10):3507–3517. https://doi.org/10.1099/mic.0.27112-0

Beauchamp JM, Erfurt RS, DiRita VJ (2015) Characterization and localization of the *Campylobacter jejuni* transformation system proteins CtsE, CtsP, and CtsX. J Bacteriol 197(3):636–645. https://doi.org/10.1128/JB.02434-14

Beauchamp JM, Leveque RM, Dawid S, DiRita VJ (2017) Methylation-dependent DNA discrimination in natural transformation of *Campylobacter jejuni*. Proc Natl Acad Sci U S A 114(38):E8053–E8061. https://doi.org/10.1073/pnas.1703331114

Bradde S, Nourmohammad A, Goyal S, Balasubramanian V (2020) The size of the immune repertoire of bacteria. Proc Natl Acad Sci U S A 117(10):5144–5151. https://doi.org/10.1073/pnas.1903666117

Cehovin A, Simpson PJ, McDowell MA, Brown DR, Noschese R, Pallett M, Brady J, Baldwin GS, Lea SM, Matthews SJ, Pelicic V (2013) Specific DNA recognition mediated by a type IV pilin. Proc Natl Acad Sci U S A 110(8):3065–3070. https://doi.org/10.1073/pnas.1218832110

Chen I, Provvedi R, Dubnau D (2006) A macromolecular complex formed by a pilin-like protein in competent *Bacillus subtilis*. J Biol Chem 281(31):21720–21727. https://doi.org/10.1074/jbc.M604071200

Chen JC, Tagg KA, Joung YJ, Bennett C, Francois Watkins L, Eikmeier D, Folster JP (2018) Report of *erm*(B)(+) *Campylobacter jejuni* in the United States. Antimicrobial agents and chemotherapy 62(6). https://doi.org/10.1128/AAC.02615-17

Cheng Y, Zhang W, Lu Q, Wen G, Zhao Z, Luo Q, Shao H, Zhang T (2020) Point deletion or insertion in CmeR-Box, A2075G substitution in 23S rRNA, and presence of *erm*(B): are key factors of erythromycin resistance in *Campylobacter jejuni* and *Campylobacter coli* isolated from central China. Front Microbiol 11:203. https://doi.org/10.3389/fmicb.2020.00203

Clark CG (2011) Sequencing of CJIE1 prophages from *Campylobacter jejuni* isolates reveals the presence of inserted and (or) deleted genes. Can J Microbiol 57(10):795–808. https://doi.org/10.1139/w11-069

Clark CG, Ng L-K (2008) Sequence variability of *Campylobacter* temperate bacteriophages. BMC Microbiol 8(1):49. https://doi.org/10.1186/1471-2180-8-49

Couvin D, Bernheim A, Toffano-Nioche C, Touchon M, Michalik J, Neron B, Rocha EPC, Vergnaud G, Gautheret D, Pourcel C (2018) CRISPRCasFinder, an update of CRISRFinder, includes a portable version, enhanced performance and integrates search for Cas proteins. Nucleic Acids Res 46(W1):W246–W251. https://doi.org/10.1093/nar/gky425

Crippen CS, Lee YJ, Hutinet G, Shajahan A, Sacher JC, Azadi P, de Crecy-Lagard V, Weigele PR, Szymanski CM (2019) Deoxyinosine and 7-Deaza-2-Deoxyguanosine as carriers of genetic information in the DNA of *Campylobacter* Viruses. J Virol 93(23). https://doi.org/10.1128/JVI.01111-19

Daubin V, Szollosi GJ (2016) Horizontal Gene transfer and the history of life. Cold Spring Harb Perspect Biol 8(4):a018036. https://doi.org/10.1101/cshperspect.a018036

Dingle KE, Colles FM, Falush D, Maiden MC (2005) Sequence typing and comparison of population biology of *Campylobacter coli* and *Campylobacter jejuni*. J Clin Microbiol 43(1):340–347. https://doi.org/10.1128/JCM.43.1.340-347.2005

Drake SL, Koomey M (1995) The product of the *pilQ* gene is essential for the biogenesis of type IV pili in *Neisseria gonorrhoeae*. Mol Microbiol 18(5):975–986. https://doi.org/10.1111/j.1365-2958.1995.18050975.x

Du Y, Wang C, Ye Y, Liu Y, Wang A, Li Y, Zhou X, Pan H, Zhang J, Xu X (2018) Molecular identification of multidrug-resistant *Campylobacter* species from diarrheal patients and poultry meat in Shanghai China. Front Microbiol 9:1642. https://doi.org/10.3389/fmicb.2018.01642

Dubnau D, Blokesch M (2019) Mechanisms of DNA uptake by naturally competent bacteria. Annu Rev Genet 53:217–237. https://doi.org/10.1146/annurev-genet-112618-043641

Dugar G, Herbig A, Forstner KU, Heidrich N, Reinhardt R, Nieselt K, Sharma CM (2013) High-resolution transcriptome maps reveal strain-specific regulatory features of multiple *Campylobacter jejuni* isolates. PLoS Genet 9(5):e1003495. https://doi.org/10.1371/journal.pgen.1003495

Dugar G, Leenay RT, Eisenbart SK, Bischler T, Aul BU, Beisel CL, Sharma CM (2018) CRISPR RNA-dependent binding and cleavage of endogenous RNAs by the *Campylobacter jejuni* Cas9. Mol Cell 69(5):893–905 e897. https://doi.org/10.1016/j.molcel.2018.01.032

EFSA (2020) The European union summary report on antimicrobial resistance in zoonotic and indicator bacteria from humans, animals and food in 2017/2018. EFSA Journal 18(3):e06007. https://doi.org/10.2903/j.efsa.2020.6007

Ellison CK, Dalia TN, Vidal Ceballos A, Wang JC, Biais N, Brun YV, Dalia AB (2018) Retraction of DNA-bound type IV competence pili initiates DNA uptake during natural transformation in *Vibrio cholerae*. Nat Microbiol 3(7):773–780. https://doi.org/10.1038/s41564-018-0174-y

Epping L, Piro RM, Knüver MT, Borowiak M, Huber C, Thürmer A, Malorny B, Stingl K, Fruth A, Wieler LH, Semmler T (2020) Genome-wide identification of Host-Specificity of *Campylobacter jejuni* in Germany based on Whole Genome Data. In: 6th Joint conference of DGHM and VAAM, Leipzig, Germany

Feng J, Ma L, Nie J, Konkel ME, Lu X (2018) Environmental stress-induced bacterial lysis and extracellular DNA release contribute to *Campylobacter jejuni* biofilm formation. Appl Environ Microbiol 84(5). https://doi.org/10.1128/AEM.02068-17

Fernandez-Gonzalez E, Backert S (2014) DNA transfer in the gastric pathogen *Helicobacter pylori*. J Gastroenterol. 49(4):594–604. https://doi.org/10.1007/s00535-014-0938-y

Firehammer B, Border M (1968) Isolation of temperate bacteriophages from *Vibrio fetus*. Am J Vet Res 29(11):2229–2235

Fischer W, Tegtmeyer N, Stingl K, Backert S (2020) Four chromosomal type IV secretion systems in *Helicobacter pylori*: composition, structure and function. Front Microbiol 11:1592. https://doi.org/10.3389/fmicb.2020.01592

Florez-Cuadrado D, Ugarte-Ruiz M, Meric G, Quesada A, Porrero MC, Pascoe B, Saez-Llorente JL, Orozco GL, Dominguez L, Sheppard SK (2017) Genome comparison of erythromycin resistant *Campylobacter* from turkeys identifies hosts and pathways for horizontal spread of *erm*(B) Genes. Front Microbiol 8:2240. https://doi.org/10.3389/fmicb.2017.02240

Florez-Cuadrado D, Ugarte-Ruiz M, Quesada A, Palomo G, Dominguez L, Porrero MC (2016) Description of an *erm*(B)-carrying *Campylobacter coli* isolate in Europe. J Antimicrob Chemother 71(3):841–843. https://doi.org/10.1093/jac/dkv383

Fouts DE, Mongodin EF, Mandrell RE, Miller WG, Rasko DA, Ravel J, Brinkac LM, DeBoy RT, Parker CT, Daugherty SC, Dodson RJ, Durkin AS, Madupu R, Sullivan SA, Shetty JU, Ayodeji MA, Shvartsbeyn A, Schatz MC, Badger JH, Fraser CM, Nelson KE (2005) Major structural differences and novel potential virulence mechanisms from the genomes of multiple *Campylobacter* species. PLoS Biol 3(1):e15. https://doi.org/10.1371/journal.pbio.0030015

Fry BN, Feng S, Chen YY, Newell DG, Coloe PJ, Korolik V (2000) The *galE* gene of *Campylobacter jejuni* is involved in lipopolysaccharide synthesis and virulence. Infect Immun 68(5):2594–2601. https://doi.org/10.1128/iai.68.5.2594-2601.2000

Gaasbeek EJ, Wagenaar JA, Guilhabert MR, van Putten JP, Parker CT, van der Wal FJ (2010) Nucleases encoded by the integrated elements CJIE2 and CJIE4 inhibit natural transformation of *Campylobacter jejuni*. J Bacteriol 192(4):936–941. https://doi.org/10.1128/jb.00867-09

Gaasbeek EJ, Wagenaar JA, Guilhabert MR, Wosten MM, van Putten JP, van der Graaf-van Bloois L, Parker CT, van der Wal FJ (2009) A DNase encoded by integrated element CJIE1 inhibits natural transformation of *Campylobacter jejuni*. J Bacteriol 191(7):2296–2306. https://doi.org/10.1128/jb.01430-08

Gardner SP, Olson JW (2012) Barriers to horizontal gene transfer in *Campylobacter jejuni*. Adv Appl Microbiol 79:19–42. https://doi.org/10.1016/B978-0-12-394318-7.00002-4

Gencay YE, Sorensen MCH, Wenzel CQ, Szymanski CM, Brondsted L (2018) Phase variable expression of a single phage receptor in *Campylobacter jejuni* NCTC12662 influences sensitivity toward several diverse CPS-dependent phages. Front Microbiol 9:82. https://doi.org/10.3389/fmicb.2018.00082

Ghatak S, He Y, Reed S, Strobaugh T Jr, Irwin P (2017) Whole genome sequencing and analysis of *Campylobacter coli* YH502 from retail chicken reveals a plasmid-borne type VI secretion system. Genom Data 11:128–131. https://doi.org/10.1016/j.gdata.2017.02.005

Gibreel A, Skold O (1998) High-level resistance to trimethoprim in clinical isolates of *Campylobacter jejuni* by acquisition of foreign genes (*dfr1* and *dfr9*) expressing drug-insensitive dihydrofolate reductases. Antimicrob Agents Chemother 42(12):3059–3064. https://doi.org/10.1128/AAC.42.12.3059

Gibreel A, Skold O, Taylor DE (2004) Characterization of plasmid-mediated *aphA-3* kanamycin resistance in *Campylobacter jejuni*. Microb Drug Resist 10(2):98–105. https://doi.org/10.1089/1076629041310127

Golz JC, Epping L, Knuver MT, Borowiak M, Hartkopf F, Deneke C, Malorny B, Semmler T, Stingl K (2020) Whole genome sequencing reveals extended natural transformation in *Campylobacter* impacting diagnostics and the pathogens adaptive potential. Sci Rep 10(1):3686. https://doi.org/10.1038/s41598-020-60320-y

Goodman SD, Scocca JJ (1988) Identification and arrangement of the DNA sequence recognized in specific transformation of *Neisseria gonorrhoeae*. Proc Natl Acad Sci U S A 85(18):6982–6986. https://doi.org/10.1073/pnas.85.18.6982

Griffith F (1928) The significance of pneumococcal types. J Hyg (Lond) 27(2):113–159. https://doi.org/10.1017/s0022172400031879

Grissa I, Vergnaud G, Pourcel C (2007) The CRISPRdb database and tools to display CRISPRs and to generate dictionaries of spacers and repeats. BMC Bioinformatics 8:172. https://doi.org/10.1186/1471-2105-8-172

Gundogdu O, Bentley SD, Holden MT, Parkhill J, Dorrell N, Wren BW (2007) Re-annotation and re-analysis of the *Campylobacter jejuni* NCTC11168 genome sequence. BMC Genom 8:162. https://doi.org/10.1186/1471-2164-8-162

Gunther NWt, Reichenberger ER, Bono JL (2016) Complete genome sequence of UV-resistant *Campylobacter jejuni* RM3194, Including an 81.08-Kilobase Plasmid. Genome Announc 4(2). https://doi.org/10.1128/genomeA.00305-16

Harrington CS, Thomson-Carter FM, Carter PE (1997) Evidence for recombination in the flagellin locus of *Campylobacter jejuni*: implications for the flagellin gene typing scheme. J Clin Microbiol 35(9):2386–2392. https://doi.org/10.1128/JCM.35.9.2386-2392.1997

Heler R, Samai P, Modell JW, Weiner C, Goldberg GW, Bikard D, Marraffini LA (2015) Cas9 specifies functional viral targets during CRISPR-Cas adaptation. Nature 519(7542):199–202. https://doi.org/10.1038/nature14245

Hepp C, Maier B (2016) Kinetics of DNA uptake during transformation provide evidence for a translocation ratchet mechanism. Proc Natl Acad Sci U S A 113(44):12467–12472. https://doi.org/10.1073/pnas.1608110113

Hille F, Richter H, Wong SP, Bratovic M, Ressel S, Charpentier E (2018) The biology of CRISPR-Cas: backward and forward. Cell 172(6):1239–59. https://doi.org/10.1016/j.cell.2017.11.032

Holst Sorensen MC, van Alphen LB, Fodor C, Crowley SM, Christensen BB, Szymanski CM, Brondsted L (2012) Phase variable expression of capsular polysaccharide modifications allows *Campylobacter jejuni* to avoid bacteriophage infection in chickens. Front Cell Infect Microbiol 2:11. https://doi.org/10.3389/fcimb.2012.00011

Holt JP, Grant AJ, Coward C, Maskell DJ, Quinlan JJ (2012) Identification of Cj1051c as a major determinant for the restriction barrier of *Campylobacter jejuni* strain NCTC11168. Appl Environ Microbiol 78(22):7841–7848. https://doi.org/10.1128/AEM.01799-12

Hooton SP, Connerton IF (2014) *Campylobacter jejuni* acquire new host-derived CRISPR spacers when in association with bacteriophages harboring a CRISPR-like Cas4 protein. Front Microbiol 5:744. https://doi.org/10.3389/fmicb.2014.00744

Irons J, Sacher JC, Szymanski CM, Downs DM (2019) Cj1388 is a RidA homolog and is required for flagella biosynthesis and/or function in *Campylobacter jejuni*. Front Microbiol 10:2058. https://doi.org/10.3389/fmicb.2019.02058

Jäckel C, Hammerl JA, Hertwig S (2019) *Campylobacter* phage isolation and characterization: what we have learned so far. Methods Protoc 2(1). https://doi.org/10.3390/mps2010018

Javed MA, Ackermann HW, Azeredo J, Carvalho CM, Connerton I, Evoy S, Hammerl JA, Hertwig S, Lavigne R, Singh A, Szymanski CM, Timms A, Kropinski AM (2014) A suggested classification for two groups of *Campylobacter* myoviruses. Arch Virol 159(1):181–190. https://doi.org/10.1007/s00705-013-1788-2

Javed MA, Sacher JC, van Alphen LB, Patry RT, Szymanski CM (2015) A flagellar glycan-specific protein encoded by *Campylobacter* phages inhibits host cell growth. Viruses 7(12):6661–6674. https://doi.org/10.3390/v7122964

Jeon B, Muraoka W, Sahin O, Zhang Q (2008) Role of Cj1211 in natural transformation and transfer of antibiotic resistance determinants in *Campylobacter jejuni*. Antimicrob Agents Chemother 52(8):2699–2708. https://doi.org/10.1128/aac.01607-07

Jeon B, Zhang Q (2007) Cj0011c, a periplasmic single- and double-stranded DNA-binding protein, contributes to natural transformation in *Campylobacter jejuni*. J Bacteriol 189(20):7399–7407. https://doi.org/10.1128/jb.01012-07

Johnston C, Martin B, Fichant G, Polard P, Claverys JP (2014) Bacterial transformation: distribution, shared mechanisms and divergent control. Nat Rev Microbiol 12(3):181–196. https://doi.org/10.1038/nrmicro3199

Kovac J, Cadez N, Stessl B, Stingl K, Gruntar I, Ocepek M, Trkov M, Wagner M, Smole Mozina S (2015) High genetic similarity of ciprofloxacin-resistant *Campylobacter jejuni* in central Europe. Front Microbiol 6:1169. https://doi.org/10.3389/fmicb.2015.01169

Larsen JC, Szymanski C, Guerry P (2004) N-linked protein glycosylation is required for full competence in *Campylobacter jejuni* 81-176. J Bacteriol 186(19):6508–6514. https://doi.org/10.1128/jb.186.19.6508-6514.2004

Lawrence JG (2005) Horizontal and vertical gene transfer: the life history of pathogens. Contrib Microbiol 12:255–271. https://doi.org/10.1159/000081699

Lederberg J, Tatum EL (1946) Gene recombination in *Escherichia coli*. Nature 158(4016):558. https://doi.org/10.1038/158558a0

Leekitcharoenphon P, Garcia-Graells C, Botteldoorn N, Dierick K, Kempf I, Olkkola S, Rossi M, Nykäsenoja S, Malorny B, Stingl K (2018) Comparative genomics of quinolone-resistant and susceptible Campylobacter jejuni of poultry origin from major poultry producing European countries (GENCAMP). EFSA Support Publ 15(5):1398E. https://doi.org/10.2903/sp.efsa.2018.EN-1398

Levy A, Goren MG, Yosef I, Auster O, Manor M, Amitai G, Edgar R, Qimron U, Sorek R (2015) CRISPR adaptation biases explain preference for acquisition of foreign DNA. Nature 520(7548):505–510. https://doi.org/10.1038/nature14302

Liu D, Deng F, Gao Y, Yao H, Shen Z, Wu C, Wang Y, Shen J (2017) Dissemination of *erm*(B) and its associated multidrug-resistance genomic islands in *Campylobacter* from 2013 to 2015. Vet Microbiol 204:20–24. https://doi.org/10.1016/j.vetmic.2017.02.022

Louwen R, Horst-Kreft D, de Boer AG, van der Graaf L, de Knegt G, Hamersma M, Heikema AP, Timms AR, Jacobs BC, Wagenaar JA, Endtz HP, van der Oost J, Wells JM, Nieuwenhuis EE, van Vliet AH, Willemsen PT, van Baarlen P, van Belkum A (2013) A novel link between *Campylobacter jejuni* bacteriophage defence, virulence and Guillain-Barre syndrome. Eur J Clin Microbiol Infect Dis 32(2):207–226. https://doi.org/10.1007/s10096-012-1733-4

Luangtongkum T, Shen Z, Seng VW, Sahin O, Jeon B, Liu P, Zhang Q (2012) Impaired fitness and transmission of macrolide-resistant *Campylobacter jejuni* in its natural host. Antimicrob Agents Chemother 56(3):1300–1308. https://doi.org/10.1128/AAC.05516-11

Luo N, Pereira S, Sahin O, Lin J, Huang S, Michel L, Zhang Q (2005) Enhanced *in vivo* fitness of fluoroquinolone-resistant *Campylobacter jejuni* in the absence of antibiotic selection pressure. Proc Natl Acad Sci U S A 102(3):541–546. https://doi.org/10.1073/pnas.0408966102

Makarova KS, Wolf YI, Iranzo J, Shmakov SA, Alkhnbashi OS, Brouns SJJ, Charpentier E, Cheng D, Haft DH, Horvath P, Moineau S, Mojica FJM, Scott D, Shah SA, Siksnys V, Terns MP, Venclovas C, White MF, Yakunin AF, Yan W, Zhang F, Garrett RA, Backofen R, van der Oost

J, Barrangou R, Koonin EV (2020) Evolutionary classification of CRISPR-Cas systems: a burst of class 2 and derived variants. Nat Rev Microbiol 18(2):67–83. https://doi.org/10.1038/s41579-019-0299-x

Marasini D, Fakhr MK (2014) Exploring PFGE for detecting large plasmids in *Campylobacter jejuni* and *Campylobacter coli* isolated from various retail meats. Pathogens 3(4):833–844. https://doi.org/10.3390/pathogens3040833

Marasini D, Fakhr MK (2016) Whole-genome sequencing of a *Campylobacter jejuni* strain isolated from retail chicken meat reveals the presence of a megaplasmid with Mu-like prophage and multidrug resistance genes. Genome Announc 4(3). https://doi.org/10.1128/genomeA.00460-16

Marasini D, Karki AB, Buchheim MA, Fakhr MK (2018) Phylogenetic relatedness among plasmids harbored by *Campylobacter jejuni* and *Campylobacter coli* isolated from retail meats. Front Microbiol 9:2167. https://doi.org/10.3389/fmicb.2018.02167

Meric G, Yahara K, Mageiros L, Pascoe B, Maiden MC, Jolley KA, Sheppard SK (2014) A reference pan-genome approach to comparative bacterial genomics: identification of novel epidemiological markers in pathogenic *Campylobacter*. PLoS ONE 9(3):e92798. https://doi.org/10.1371/journal.pone.0092798

Mourkas E, Florez-Cuadrado D, Pascoe B, Calland JK, Bayliss SC, Mageiros L, Meric G, Hitchings MD, Quesada A, Porrero C, Ugarte-Ruiz M, Gutierrez-Fernandez J, Dominguez L, Sheppard SK (2019) Gene pool transmission of multidrug resistance among *Campylobacter* from livestock, sewage and human disease. Environ Microbiol 21(12):4597–4613. https://doi.org/10.1111/1462-2920.14760

O'Loughlin JL, Eucker TP, Chavez JD, Samuelson DR, Neal-McKinney J, Gourley CR, Bruce JE, Konkel ME (2015) Analysis of the *Campylobacter jejuni* genome by SMRT DNA sequencing identifies restriction-modification motifs. PLoS ONE 10(2):e0118533. https://doi.org/10.1371/journal.pone.0118533

Obergfell KP, Seifert HS (2016) The pilin N-terminal domain maintains *Neisseria gonorrhoeae* transformation competence during pilus phase variation. PLoS Genet 12(5):e1006069. https://doi.org/10.1371/journal.pgen.1006069

Ouellette M, Gerbaud G, Lambert T, Courvalin P (1987) Acquisition by a *Campylobacter*-like strain of aphA-1, a kanamycin resistance determinant from members of the family *Enterobacteriaceae*. Antimicrob Agents Chemother 31(7):1021–1026. https://doi.org/10.1128/aac.31.7.1021

Oyarzabal OA, Backert S (2012) Microbial Food Safety: An Introduction (Food Science Text Series). Springer, Heidelberg

Oyarzabal OA, Rad R, Backert S (2007) Conjugative transfer of chromosomally encoded antibiotic resistance from *Helicobacter pylori* to *Campylobacter jejuni*. J Clin Microbiol 45(2):402–408. https://doi.org/10.1128/JCM.01456-06

Parker CT, Quiñones B, Miller WG, Horn ST, Mandrell RE (2006) Comparative genomic analysis of *Campylobacter jejuni* strains reveals diversity due to genomic elements similar to those present in *C. jejuni* strain RM1221. J Clinical Microbiol 44(11):4125–4135. https://doi.org/10.1128/JCM.01231-06

Parkhill J, Wren BW, Mungall K, Ketley JM, Churcher C, Basham D, Chillingworth T, Davies RM, Feltwell T, Holroyd S, Jagels K, Karlyshev AV, Moule S, Pallen MJ, Penn CW, Quail MA, Rajandream MA, Rutherford KM, van Vliet AH, Whitehead S, Barrell BG (2000) The genome sequence of the food-borne pathogen *Campylobacter jejuni* reveals hypervariable sequences. Nature 403(6770):665–668. https://doi.org/10.1038/35001088

Phongsisay V, Perera VN, Fry BN (2006) Exchange of lipooligosaccharide synthesis genes creates potential Guillain-Barre syndrome-inducible strains of *Campylobacter jejuni*. Infect Immun 74(2):1368–1372. https://doi.org/10.1128/IAI.74.2.1368-1372.2006

Pratt A, Korolik V (2005) Tetracycline resistance of Australian *Campylobacter jejuni* and *Campylobacter coli* isolates. J Antimicrob Chemother 55(4):452–460. https://doi.org/10.1093/jac/dki040

Qin S, Wang Y, Zhang Q, Zhang M, Deng F, Shen Z, Wu C, Wang S, Zhang J, Shen J (2014) Report of ribosomal RNA methylase gene *erm*(B) in multidrug-resistant *Campylobacter coli*. J Antimicrob Chemother 69(4):964–968. https://doi.org/10.1093/jac/dkt492

Reuter M, Periago PM, Mulholland F, Brown HL, van Vliet AH (2015) A PAS domain-containing regulator controls flagella-flagella interactions in *Campylobacter jejuni*. Front Microbiol 6:770. https://doi.org/10.3389/fmicb.2015.00770

Richardson PT, Park SF (1997) Integration of heterologous plasmid DNA into multiple sites on the genome of *Campylobacter coli* following natural transformation. J Bacteriol 179(5):1809–1812. https://doi.org/10.1128/jb.179.5.1809-1812.1997

Rosner BM, Schielke A, Didelot X, Kops F, Breidenbach J, Willrich N, Golz G, Alter T, Stingl K, Josenhans C, Suerbaum S, Stark K (2017) A combined case-control and molecular source attribution study of human *Campylobacter* infections in Germany, 2011-2014. Sci Rep 7(1):5139. https://doi.org/10.1038/s41598-017-05227-x

Sacher JC, Flint A, Butcher J, Blasdel B, Reynolds HM, Lavigne R, Stintzi A, Szymanski CM (2018) Transcriptomic analysis of the *Campylobacter jejuni* response to T4-Like phage NCTC 12673 infection. Viruses 10(6). https://doi.org/10.3390/v10060332

Sacher JC, Shajahan A, Butcher J, Patry RT, Flint A, Hendrixson DR, Stintzi A, Azadi P, Szymanski CM (2020) Binding of phage-encoded FlaGrab to motile *Campylobacter jejuni* flagella inhibits growth, downregulates energy metabolism, and requires specific flagellar glycans. Front Microbiol 11:397. https://doi.org/10.3389/fmicb.2020.00397

Sails A, Wareing D, Bolton F, Fox A, Curry A (1998) Characterisation of 16 *Campylobacter jejuni* and *C. coli* typing bacteriophages. J Med Microbiol 47(2):123–128. https://doi.org/10.1099/00222615-47-2-123

Schmidt-Ott R, Pohl S, Burghard S, Weig M, Gross U (2005) Identification and characterization of a major subgroup of conjugative *Campylobacter jejuni* plasmids. J Infect 50(1):12–21. https://doi.org/10.1016/j.jinf.2004.02.013

Scott AE, Timms AR, Connerton PL, Carrillo CL, Radzum KA, Connerton IF (2007) Genome dynamics of *Campylobacter jejuni* in response to bacteriophage predation. PLoS Pathog 3(8):e119. https://doi.org/10.1371/journal.ppat.0030119

Seitz P, Pezeshgi Modarres H, Borgeaud S, Bulushev RD, Steinbock LJ, Radenovic A, Dal Peraro M, Blokesch M (2014) ComEA is essential for the transfer of external DNA into the periplasm in naturally transformable *Vibrio cholerae* cells. PLoS Genet 10(1):e1004066. https://doi.org/10.1371/journal.pgen.1004066

Sheppard SK, Jolley KA, Maiden MC (2012) A Gene-By-Gene Approach to Bacterial Population Genomics: Whole Genome MLST of *Campylobacter*. Genes (Basel) 3(2):261–277. https://doi.org/10.3390/genes3020261

Sheppard SK, Maiden MC (2015) The evolution of *Campylobacter jejuni* and *Campylobacter coli*. Cold Spring Harb Perspect Biol 7(8):a018119. https://doi.org/10.1101/cshperspect.a018119

Sheppard SK, McCarthy ND, Falush D, Maiden MC (2008) Convergence of *Campylobacter* species: implications for bacterial evolution. Science 320(5873):237–239. https://doi.org/10.1126/science.1155532

Sheppard SK, McCarthy ND, Jolley KA, Maiden MC (2011) Introgression in the genus *Campylobacter*: generation and spread of mosaic alleles. Microbiology 157(Pt 4):1066–1074. https://doi.org/10.1099/mic.0.045153-0

Sorensen MC, Gencay YE, Birk T, Baldvinsson SB, Jackel C, Hammerl JA, Vegge CS, Neve H, Brondsted L (2015) Primary isolation strain determines both phage type and receptors recognised by *Campylobacter jejuni* bacteriophages. PLoS ONE 10(1):e0116287. https://doi.org/10.1371/journal.pone.0116287

Sorensen MC, van Alphen LB, Harboe A, Li J, Christensen BB, Szymanski CM, Brondsted L (2011) Bacteriophage F336 recognizes the capsular phosphoramidate modification of *Campylobacter jejuni* NCTC11168. J Bacteriol 193(23):6742–6749. https://doi.org/10.1128/JB.05276-11

Sougakoff W, Papadopoulou B, Nordmann P, Courvalin P (1987) Nucleotide sequence and distribution of gene *tetO* encoding tetracycline resistance in *Campylobacter coli*. FEMS Microbiol Lett 44(1):153–159. https://doi.org/10.1111/j.1574-6968.1987.tb02260.x

Stern A, Keren L, Wurtzel O, Amitai G, Sorek R (2010) Self-targeting by CRISPR: gene regulation or autoimmunity? Trends Genet 26(8):335–340. https://doi.org/10.1016/j.tig.2010.05.008

Stingl K, Muller S, Scheidgen-Kleyboldt G, Clausen M, Maier B (2010) Composite system mediates two-step DNA uptake into *Helicobacter pylori*. Proc Natl Acad Sci U S A 107(3):1184–1189. https://doi.org/10.1073/pnas.0909955107

Svensson SL, Pryjma M, Gaynor EC (2014) Flagella-mediated adhesion and extracellular DNA release contribute to biofilm formation and stress tolerance of *Campylobacter jejuni*. PLoS ONE 9(8):e106063. https://doi.org/10.1371/journal.pone.0106063

Tang Y, Dai L, Sahin O, Wu Z, Liu M, Zhang Q (2017) Emergence of a plasmid-borne multidrug resistance gene *cfr(C)* in foodborne pathogen *Campylobacter*. J Antimicrob Chemother 72(6):1581–1588. https://doi.org/10.1093/jac/dkx023

Taylor DE, De Grandis SA, Karmali MA, Fleming PC (1981) Transmissible plasmids from *Campylobacter jejuni*. Antimicrob Agents Chemother 19(5):831–835. https://doi.org/10.1128/aac.19.5.831

Vegge CS, Brondsted L, Ligowska-Marzeta M, Ingmer H (2012) Natural transformation of *Campylobacter jejuni* occurs beyond limits of growth. PLoS ONE 7(9):e45467. https://doi.org/10.1371/journal.pone.0045467

Velazquez JB, Jimenez A, Chomon B, Villa TG (1995) Incidence and transmission of antibiotic resistance in *Campylobacter jejuni* and *Campylobacter coli*. J Antimicrob Chemother 35(1):173–178. https://doi.org/10.1093/jac/35.1.173

Vorwerk H, Huber C, Mohr J, Bunk B, Bhuju S, Wensel O, Sproer C, Fruth A, Flieger A, Schmidt-Hohagen K, Schomburg D, Eisenreich W, Hofreuter D (2015) A transferable plasticity region in *Campylobacter coli* allows isolates of an otherwise non-glycolytic food-borne pathogen to catabolize glucose. Mol Microbiol 98(5):809–830. https://doi.org/10.1111/mmi.13159

Wang Y, Taylor DE (1990) Natural transformation in *Campylobacter* species. J Bacteriol 172(2):949–955. https://doi.org/10.1128/jb.172.2.949-955.1990

Wang Y, Zhang M, Deng F, Shen Z, Wu C, Zhang J, Zhang Q, Shen J (2014) Emergence of multidrug-resistant *Campylobacter* species isolates with a horizontally acquired rRNA methylase. Antimicrob Agents Chemother 58(9):5405–5412. https://doi.org/10.1128/AAC.03039-14

Wassenaar TM, Fry BN, van der Zeijst BA (1995) Variation of the flagellin gene locus of *Campylobacter jejuni* by recombination and horizontal gene transfer. Microbiology 141(Pt 1):95–101. https://doi.org/10.1099/00221287-141-1-95

Wei Y, Terns RM, Terns MP (2015) Cas9 function and host genome sampling in Type II-A CRISPR-Cas adaptation. Genes Dev 29(4):356–361. https://doi.org/10.1101/gad.257550.114

Wiesner RS, Hendrixson DR, DiRita VJ (2003) Natural Transformation of *Campylobacter jejuni* Requires Components of a Type II Secretion System. J Bacteriol 185(18):5408–5418. https://doi.org/10.1128/jb.185.18.5408-5418.2003

Wilson DJ, Gabriel E, Leatherbarrow AJ, Cheesbrough J, Gee S, Bolton E, Fox A, Hart CA, Diggle PJ, Fearnhead P (2009) Rapid evolution and the importance of recombination to the gastroenteric pathogen *Campylobacter jejuni*. Mol Biol Evol 26(2):385–397. https://doi.org/10.1093/molbev/msn264

Wilson DL, Bell JA, Young VB, Wilder SR, Mansfield LS, Linz JE (2003) Variation of the natural transformation frequency of *Campylobacter jejuni* in liquid shake culture. Microbiology 149(Pt 12):3603–3615. https://doi.org/10.1099/mic.0.26531-0

Yao H, Shen Z, Wang Y, Deng F, Liu D, Naren G, Dai L, Su CC, Wang B, Wang S, Wu C, Yu EW, Zhang Q, Shen J (2016) Emergence of a potent multidrug efflux pump variant that enhances *Campylobacter* resistance to multiple antibiotics. mBio 7(5). https://doi.org/10.1128/mBio.01543-16

Yosef I, Goren MG, Qimron U (2012) Proteins and DNA elements essential for the CRISPR adaptation process in *Escherichia coli*. Nucleic Acids Res 40(12):5569–5576. https://doi.org/10.1093/nar/gks216

Zautner AE, Goldschmidt AM, Thurmer A, Schuldes J, Bader O, Lugert R, Gross U, Stingl K, Salinas G, Lingner T (2015) SMRT sequencing of the *Campylobacter coli* BfR-CA-9557 genome sequence reveals unique methylation motifs. BMC Genom 16:1088. https://doi.org/10.1186/s12864-015-2317-3

Zeng X, Ardeshna D, Lin J (2015) Heat shock-enhanced conjugation efficiency in standard *Campylobacter jejuni* strains. Appl Environ Microbiol 81(13):4546–4552. https://doi.org/10.1128/aem.00346-15

Zeng X, Wu Z, Zhang Q, Lin J (2018) A cotransformation method to identify a restriction-modification enzyme that reduces conjugation efficiency in *Campylobacter jejuni*. Appl Environ Microbiol 84(23). https://doi.org/10.1128/AEM.02004-18

Open Access This chapter is licensed under the terms of the Creative Commons Attribution 4.0 International License (http://creativecommons.org/licenses/by/4.0/), which permits use, sharing, adaptation, distribution and reproduction in any medium or format, as long as you give appropriate credit to the original author(s) and the source, provide a link to the Creative Commons license and indicate if changes were made.

The images or other third party material in this chapter are included in the chapter's Creative Commons license, unless indicated otherwise in a credit line to the material. If material is not included in the chapter's Creative Commons license and your intended use is not permitted by statutory regulation or exceeds the permitted use, you will need to obtain permission directly from the copyright holder.

5.6 Publication 5: "Take it or leave it"-Factors regulating competence development and DNA uptake in *Campylobacter jejuni*.

International Journal of
Molecular Sciences

Article

"Take It or Leave It"—Factors Regulating Competence Development and DNA Uptake in *Campylobacter jejuni*

Julia C. Golz and Kerstin Stingl *

German Federal Institute for Risk Assessment, Department of Biological Safety, National Reference Laboratory for Campylobacter, 12277 Berlin, Germany; Julia.Golz@bfr.bund.de
* Correspondence: Kerstin.Stingl@bfr.bund.de; Tel.: +49-30-18412-24206

Abstract: *Campylobacter jejuni* has a large adaptive potential due to enormous genetic exchange. Factors regulating natural transformation in this food-borne pathogen are largely unknown but of interest for the application of sustained reduction strategies in the food-processing industry. Using a single cell DNA uptake assay, we visualized that recognition of methylated *C. jejuni* DNA was essential for the first step of DNA uptake into a DNase resistant state. Transformation rates using a resistance marker correlated with the fraction of competent bacteria, harboring one to maximally four locations of active DNA uptake, not necessarily being located at the cell pole. Competence developed with rising pH between 6.5 and 7.5 under microaerobic conditions and was nearly insensitive towards growth temperatures between 32 °C and 42 °C, CO_2 concentrations ranging from 0 to 50% and growth rates. However, competence development was abolished at pH 5 or under aerobic stress conditions, in which the bacteria ceased growth but fully survived. The DNA uptake machinery in competent bacteria shut down at slightly acidic pH and was reversibly switched on upon neutralization. It was dependent on the proton motive force and, in contrast to competence development, slightly enhanced under aerobic conditions. The results suggest that natural transformation in *C. jejuni* occurs in the neutral and microaerobic intestinal environment for enhanced genetic diversity and pre-adaption before host switch. In addition, highly competent bacteria might be shed into the environment, still able to acquire genetic material for increased survival.

Keywords: natural transformation; genetic diversity; adaptation; pH regulation

Citation: Golz, J.C.; Stingl, K. "Take It or Leave It"—Factors Regulating Competence Development and DNA Uptake in *Campylobacter jejuni*. *Int. J. Mol. Sci.* **2021**, 22, 10169. https://doi.org/10.3390/ijms221810169

Academic Editor: Kunio Takeyasu

Received: 17 August 2021
Accepted: 15 September 2021
Published: 21 September 2021

Publisher's Note: MDPI stays neutral with regard to jurisdictional claims in published maps and institutional affiliations.

Copyright: © 2021 by the authors. Licensee MDPI, Basel, Switzerland. This article is an open access article distributed under the terms and conditions of the Creative Commons Attribution (CC BY) license (https://creativecommons.org/licenses/by/4.0/).

1. Introduction

Campylobacter jejuni is a human foodborne pathogen causing the majority of bacterially induced gastroenteritis in the European Union, with 220,682 reported cases in 2019 [1]. The pathogen resides in birds, but also pigs, cattle and other animals are frequently colonized by the bacterium [2–4]. *C. jejuni* infection of humans primarily occurs via contaminated food, in particular due to cross-contamination from raw poultry meat to ready-to-eat fresh food and by consumption of undercooked poultry, other raw meat and milk [5,6].

Campylobacter is highly sensitive to environmental stress. Microaerobic conditions and temperatures between 37 °C and 42 °C are optimal growth conditions for *C. jejuni*, while the bacterium cannot grow below 30 °C or under atmospheric oxygen levels [7,8]. However, *Campylobacter* exhibits enormous survival strategies and is highly adapted to surviving harsh conditions by undergoing the viable but non-culturable state [9], but also by adaptation mechanisms [10–12] and by genetic exchange [13]. Recently, we identified by whole genome sequencing with *k*-mer analysis, that some *C. coli* strains harbored 15% introgression of *C. jejuni* sequences [14]. The gene set with *C. jejuni* introgression displayed potential roles in stress defense, suggesting enormous adaptation by natural transformation.

Although natural transformation is widespread among bacteria and offers the chance to acquire new genes or gene variants, it may be harmful for genome integrity [15–17].

Therefore, the process is usually regulated and limited to a subset of the bacterial population. Bistability of bacteria, tightly regulated using a main competence regulator or silencing of DNA uptake genes by non-coding small RNA have been identified to integrate various internal and external stimuli for the control of competence development and DNA uptake in some example bacteria, like *Bacillus subtilis*, *Streptococcus*, *Vibrio* and *Legionella* [18–20]. However, for most other bacteria, even the parameters for stimulating or inhibiting competence development are largely unknown.

The expression of the competence genes encoding a type IV secretion system in a close relative of *C. jejuni*, *Helicobacter pylori*, was differently expressed in a strain-dependent manner [21]. Natural transformation in *H. pylori* was shown to be tightly regulated by pH and oxidative stress [22]. A pH above 6.5 opened up a window for transformation, in which increase in pH and oxygen levels drastically stimulated DNA uptake, conditions prevailing at the multiplication site of the pathogen. In *Campylobacter*, natural transformation was observed to be most effective in exponential growth phase and parameters were, thereafter, varied within a 4 h assay with contact to chromosomal DNA and selection of resistant transformants [23]. In that study alkaline pH, 42 °C and aerobic as well as microaerobic conditions led to increase in the number of transformants. In another study, a positive correlation between lower CO_2 concentrations and higher transformation levels was described [24]. A growth condition, in which *C. jejuni* did not take up any DNA was not identified.

In contrast to *H. pylori*, *C. jejuni* also restricts DNA uptake by differentiation between own and foreign external DNA [25] by recognition of a CtsM methylated RATTY motif frequently present in *C. jejuni* chromosomal DNA [26]. The mechanism of DNA uptake in *C. jejuni* is similar to other bacteria, using a type II-secretion/type IV pilus system in combination with the ComEC master inner membrane channel, which is common to all transformable bacteria [27–30].

Here we used a single cell-based assay to directly monitor DNA uptake in the food-borne pathogen *C. jejuni*. For the first time we were able to independently test parameters for stimulating or inhibiting competence development or DNA uptake. As observed for *H. pylori*, competence development was dependent on neutral to slightly alkaline pH. In contrast, aerobic conditions prevented competence development but not DNA uptake. Interestingly, *C. jejuni* grew in the absence of CO_2 despite of increased generation times, with competence development being unaffected. The DNA uptake itself was energy-dependent, insensitive against oxygen but shut down at slightly acidic pH. More close understanding of the mechanisms and the parameters influencing natural transformation in *C. jejuni* are useful for the development of strategies inhibiting the pathogen's adaptive potential. This may support the design of sustainably effective reduction strategies against the pathogen in food-supplying animals.

2. Results

2.1. DNA Uptake in C. jejuni Occurs at Distinct Locations, Is DNA Substrate Specific and Energy-Dependent

In contrast to *H. pylori*, *C. jejuni* DNA uptake is limited to DNA substrates, which harbor RATTY sites methylated by *Campylobacter* spp. specific CtsM methylase [26]. Thus, in order to establish the single cell assay for *C. jejuni*, genomic DNA extracted from *C. jejuni* served as DNA substrate, rather than DNA of the *E. coli* bacteriophage λ, previously used for *H. pylori*. As proof-of-principle, both DNA substrates were labelled with fluorescein and *C. jejuni* cells in exponential growth phase were challenged by these two sorts of covalently labeled DNA. After incubation at 37 °C for 30 min and subsequent degradation of external non-incorporated DNA by 5 min of DNaseI digestion at 37 °C, bacteria were immobilized using an agarose gel surface and microscopically analyzed. The bacteria harboring at least one DNase-resistant fluorescent focus were considered competent for natural transformation (Figure 1). Using λ-DNA lacking methylation at the recognition motif RATTY, no DNA focus was detected in *C. jejuni* BfR-CA-14430 grown for 18 ± 4 h in Bolton broth (final pH of 7.5) (Figure 1A,B). When *C. jejuni* genomic DNA was used

as substrate, bacteria showed competence levels of around 45.1 ± 5.8% (Figure 1B,C). Analyzing this competent fraction in detail, 70.8 ± 6.5% of the competent cells harbored one DNase-resistant fluorescent focus, 24.8 ± 4.3% showed two foci and 4.1 ± 3.0% even displayed three visually separated locations of DNA uptake (Figure 1D). Only very few bacteria were observed with four DNA foci (0.3 ± 0.3%).

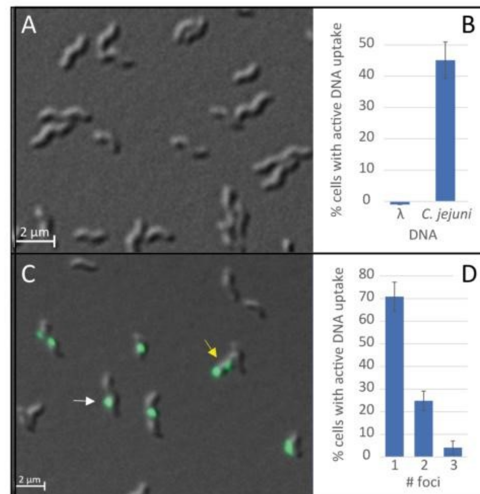


Figure 1. DNA uptake in *C. jejuni* BfR-CA-14430 occurs at distinct locations and is dependent on the DNA substrate. *C. jejuni* were incubated with fluorescently labelled DNA of the bacteriophage λ (**A**) or *C. jejuni* DNA (**C**) and the fraction of cells with active DNA uptake (**B**) and the distribution of number of DNA foci (**D**) after contact to *C. jejuni* DNA were quantified. Arrows in (**C**) indicate example bacteria with one (white) and two (yellow) DNA uptake locations. Overlay image of differential phase contrast (DIC) and Fluorescein channel. Scale bar, 2 µm. Experiments were performed at least three times. Error bars indicate standard deviation. #, number.

This result suggests that recognition of methylated RATTY is necessary at the very first step of DNA uptake, namely transport of DNA from the environment into a DNase-resistant state (periplasm). It also corroborates the idea that the assay was suitable for detection of DNA uptake into *C. jejuni*.

We further checked if the process of DNA uptake was dependent on the proton motive force (pmf), as shown for other bacteria [31,32]. For this purpose, we modulated the conditions during the time period, in which competent cells were in contact with the fluorescent genomic *C. jejuni* DNA. Under "standard DNA uptake conditions" using brain heart infusion (BHI), nearly half of the bacteria (49.1 ± 2.2%) showed active DNA uptake within 30 min of uptake period (Figure 2). When *C. jejuni* were pre-incubated for 10 min with 250 µM of the protonophore, carbonyl cyanide m-chlorophenyl hydrazine (CCCP), which abolishes the proton gradient across the inner membrane [33], DNA uptake capacity was completely lost (Figure 2).

Furthermore, we limited the nutrient supply by incubation of competent cells for 2 h in phosphate-buffered saline (PBS) under microaerobic conditions. Under these conditions, the bacteria were non-motile, as also noticed in the presence of CCCP, indicating that pmf is limiting, which drives flagellar movement [34]. Those nutrient-depleted *C. jejuni* were subsequently incubated with fluorescent DNA in the presence of PBS or in re-energizing BHI. While *C. jejuni* were not able to take up DNA in PBS, 29.3 ± 5.7% of the bacteria in BHI took up DNA into a DNase resistant state (Figure 2). These re-energized bacteria also regained motility under this condition. We conclude that *C. jejuni* requires the proton

motive force for DNA uptake. It also indicated that uptake is favored under nutrient-rich conditions. Consistently, DNA uptake performed under aerobic conditions led to a 1.28 ± 0.26-fold increased fraction of bacteria with a visible DNA focus than under microaerobic conditions (n = 20; Figure S1).

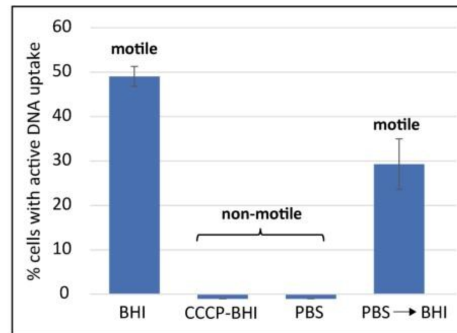


Figure 2. DNA uptake requires proton motive force. Competent *C. jejuni* pre-incubated for 10 min in the presence of the protonophore CCCP or nutrient-depleted for 2 h in PBS showed abolished DNA uptake capacity and motility. Re-energized cells (PBS→BHI; 2 h in PBS shifted to BHI during DNA uptake) regained motility and the ability to take up DNA. Experiments were performed at least three times. Error bars indicate standard deviation. PBS, phosphate-buffered saline; BHI, brain heart infusion; CCCP, carbonyl cyanide m-chlorophenyl hydrazine.

2.2. Competence Development in C. jejuni Is Dependent on External pH and Shut Down under Aerobic Conditions

In order to decipher parameters regulating competence development in *C. jejuni* we used a distinct experimental setup, in which we monitored "long-term" and "short-term" effects on competence development, such as growth phase, pH, temperature, oxidative stress and CO_2 concentration (Figure 3). For fastidious bacteria, it is important to adapt optimal growth conditions in order to obtain physiological relevant data. For this purpose, *C. jejuni* were pre-cultured from a fresh 18 ± 4 h culture on Columbia blood agar (ColBA) at an initial OD_{600nm} of around 0.3 in liquid Bolton broth basis (without selective supplements) for 5 to 9 h to OD_{600nm} of ~1.5 before sub-culturing the bacteria in the same medium. The sub-culture was modified with respect to inoculum so that *C. jejuni* reached either exponential (OD_{600nm} of 0.1 to 0.5) or stationary growth phase (OD_{600nm} of 0.8 to 1.5) after 18 ± 4 h of incubation ("long-term" setup). Therefore, very low initial OD_{600nm} between 3×10^{-7} and 2.5×10^{-5} for exponential or $1–5 \times 10^{-3}$ for stationary phase cultures were inoculated. In Bolton broth at pH 7.5 growth rate of *C. jejuni* was highly reproducible with a mean generation time of 1.2 ± 0.1 h (exponential phase, Table S1). To determine the effect of pH on competence development, pH of the medium was adjusted with sodium hydroxide (NaOH) or hydrochloric acid (HCl).

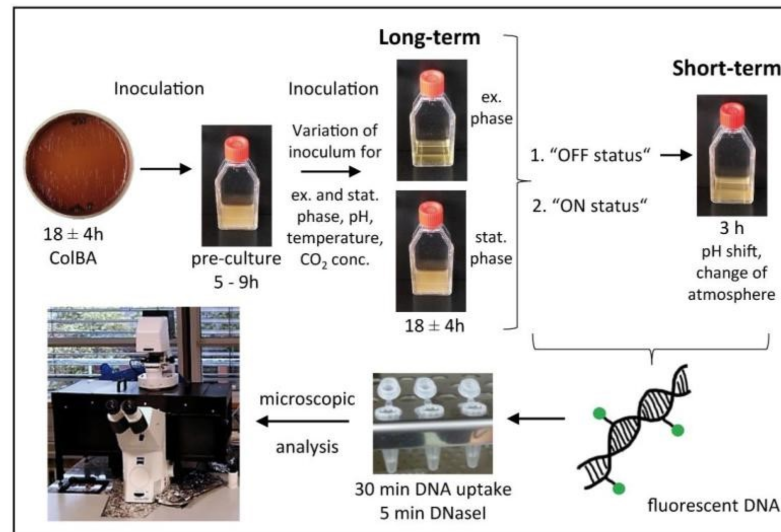


Figure 3. Schematic overview of the experimental setup of the single cell assay. All cells grown on ColBA plates were pre-cultured in Bolton broth for 5–9 h to stationary phase. A subculture was prepared with variation of inoculum (to reach exponential or stationary phase), pH, temperature, CO_2 concentration and incubated microaerobically for 18 ± 4 h. These cells were analyzed in the framework of the "long-term" setup. Cultures (from the "long-term" setup) in "OFF status" were challenged for 3 h by "short-term" pH elevation and/or atmospheric shift. After adding fluorescently labelled DNA and subsequent DNaseI digestion, bacteria were analyzed by fluorescence microscopy. Ex. phase, exponential phase culture with OD_{600nm} of 0.1–0.5; stat. phase, stationary phase culture with OD_{600nm} of 0.8–1.5; conc., concentration.

Indicated pH values in the text and in Figure 4 are final pH values after growth ± 0.2. The titrated pH at standard microaerobic conditions did not change during growth from inoculation until exponential growth phase (Figure S2, yellow squares) but was slightly increased in stationary growth phase (Figure 4, pH values in brackets) due to *C. jejuni* metabolic activity at higher cell densities. The fraction of competent bacteria in exponential phase at pH 7.5 was 45.1 ± 5.8% (Figure 4). In stationary growth phase at a final pH of 7.7 this fraction of bacteria was lower comprising 27.7 ± 5.8%. When the pH was lowered to 6.3, a drastic decrease of the fraction of competent bacteria was observed in exponential growth phase, since only 2.3 ± 0.9% of bacteria were observed with at least one fluorescent DNA focus (Figure 4A). A parallel culture with initial pH of 6.3 but grown to stationary growth phase was likewise only marginally competent with 3.5 ± 2.1% of bacteria (Figure 4A). For all conditions depicted in Figure 4, the incubation of bacteria with fluorescent DNA was done at pH 7.5. An exception to this rule was the culture at pH 5.7, which was challenged with DNA at the same pH of 5.7 in order to see, if DNA uptake in *C. jejuni* could be completely abolished under these conditions. Indeed, not a single bacterium was observed to take up fluorescent DNA under these conditions, suggesting that slight acidic pH is sufficient for complete shut-down of natural transformation in *C. jejuni*. However, we chose the exponential pH 6.3 and the corresponding stationary culture at 7.2 as "OFF-status" cultures for the "short-term" setup (as indicated in Figure 4A), since the generation time of *C. jejuni* at pH 5.7 was considerably extended (Table S1).

Table 1. Transformation rates of *C. jejuni* correlate with the fraction of competent cells, harboring at least one fluorescent DNA focus.

Condition	Transformation Rate ± SD	Fraction Competent Cells ± SD (%)	Change in Transformation Rate Compared to pH 6.3	Change in Fraction of Competent Cells Relative to pH 6.3	Ratio Change Transformation Rate vs. Change in Fraction Competent Cells
pH 5.7	$\leq 6.9 \times 10^{-9} \pm 6.2 \times 10^{-10}$	0	≤0.0053-fold	∞	n.a.
pH 6.3	$1.3 \times 10^{-6} \pm 2.5 \times 10^{-6}$	2.3 ± 0.9	1.0-fold	1.0-fold	1.0
pH 6.3 +3 h at pH 7.6	$1.4 \times 10^{-5} \pm 7.8 \times 10^{-6}$	12.2 ± 3.5	10.8-fold	5.3-fold	2.0
pH 6.3 +3 h with fresh medium	$2.9 \times 10^{-5} \pm 2.1 \times 10^{-5}$	33.9 ± 5.0	22.3-fold	14.7-fold	1.5
pH 6.3 +3 h aerobic at pH 7.9	$1.3 \times 10^{-6} \pm 6.4 \times 10^{-7}$	0.8 ± 0.4	1.0-fold	0.3-fold	2.9
pH 6.3 +3 h aerobic with fresh medium	$3.7 \times 10^{-6} \pm 1.2 \times 10^{-5}$	3.4 ± 1.1	2.8-fold	1.5-fold	1.9
pH 7.5	$1.3 \times 10^{-4} \pm 2.3 \times 10^{-4}$	45.1 ± 5.8	100-fold	19.6-fold	5.1
pH 7.5 without DNA	$<6.4 \times 10^{-9} \pm 2.5 \times 10^{-9}$	0	<0.0049-fold	∞	n. a.
pH 7.3 (stat phase)	$6.6 \times 10^{-6} \pm 4.3 \times 10^{-6}$	3.5 ± 2.1	5.1-fold	1.5-fold	3.3
pH 7.3 (stat phase) without DNA	$<6.5 \times 10^{-9} \pm 8.0 \times 10^{-10}$	0	<0.005-fold	∞	n.a.

After growth in Bolton broth at different pH values for 18 ± 4 h, bacteria were transformed with either genomic *C. jejuni* DNA carrying the streptomycin resistance marker A128G in the ribosomal *rpsL* gene or with fluorescent DNA in parallel. Number of transformants relative to CFU and fraction of competent cells identified by the presence of fluorescent DNaseI resistant foci were compared. Conditions are detailed in Figure 4. For estimation of spontaneous mutation rate, controls without DNA were included. Experiments were performed at least three times; ∞, infinite; n.a., not applicable; <, no transformant detected within indicated detection limit.

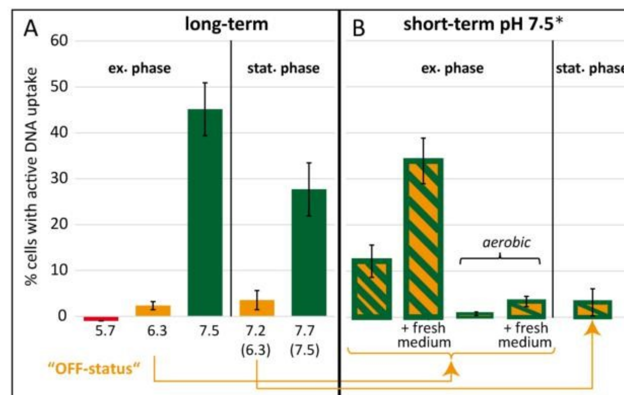


Figure 4. pH and atmosphere determine competence development in *C. jejuni*. Experimental setups for "long-term pH" (**A**) and "short-term pH shift" (**B**) of *C. jejuni* BfR-CA-14430 were as described in Figure 3. Final pH values ± 0.2 of cultures tested in the DNA uptake assay were as indicated, with initial pH-values ± 0.2 of original (exponential) cultures in brackets. The exponential culture with a final pH 6.3 and its corresponding stationary culture without pH adjustment (pH 7.2) were used as "OFF-status" cultures for short-term pH shifts. Ex. phase, exponential phase culture with OD_{600nm} of 0.1–0.5; stat. phase, stationary phase culture with OD_{600nm} of 0.8–1.5. Fresh medium, cultures were diluted 3 to 5-fold with Bolton broth; aerobic, atmosphere was changed to aerobic conditions for short-term challenge; *, final pH 7.9 instead of 7.5 due to additional release of CO_2 from the medium. DNA uptake occurred under aerobic conditions at pH 7.5 ± 0.1 except for culture pH 5.7, for which uptake was kept at the same pH value of 5.7 (see also Table 1). Experiments were performed at least three times. Error bars indicate standard deviation.

"OFF-status" bacteria were challenged by a pH shift to 7.5 for 3 h in the "short-term" setup (Figure 4B). When the pH upshift was established by direct titration of the culture with NaOH, 12.2 ± 3.5% of the cells took up fluorescently labelled DNA. In case we upshifted the exponential phase culture by 3- to 5-fold dilution in fresh Bolton broth at pH 7.5 to OD_{600nm} 0.05, even 33.9 ± 5.0% of the bacteria displayed active DNA uptake capacity (Figure 4B), indicating that fresh medium stimulated competence development. The stationary "OFF-status" culture did not respond during the 3 h, indicating that those cells lost capacity to switch into the competent phase (Figure 4B).

Interestingly, when the exponential "OFF-status" culture was pH-upshifted under aerobic conditions, competence levels even decreased to 0.8 ± 0.4% or were stable in the presence of fresh medium at low levels (Figure 4B). Since under aerobic conditions, *C. jejuni* is stressed by high oxygen levels and does not grow, survival of the bacterium under these conditions was confirmed. We observed that colony-forming units (CFU) remained constant, with $7.7 \times 10^8 \pm 2.9 \times 10^8$ CFU/mL before and $9.3 \times 10^8 \pm 3.2 \times 10^8$ CFU/mL after 3 h of aerobic incubation. Hence, the results suggested that atmospheric oxygen levels inhibited competence development even at permissive neutral to slightly alkaline pH values, while the bacterium arrested growth but was fully viable.

The observed increase of competence levels after exchange with fresh medium motivated us to test if several sub-cultivation steps within the exponential growth phase with fresh medium could enhance the proportion of competent cells. Therefore, we sub-cultured *C. jejuni* BfR-CA-14430 in Bolton broth (final pH7.5) three times with an overall growth period of more than 60 h. We kept cells in exponential phase with maximal OD_{600nm} of 0.4, never entering stationary phase. At each step of sub-cultivation, we determined the competence level, which was maintained at 43.4 ± 3.4%.

2.3. Uptake of DNA into a DNase-Resistant State Is a Main Factor for Regulation of Natural Transformation

The single cell uptake assay using covalently labelled DNA, visualizes the first step of natural transformation, i.e., DNA uptake from the environment into the cell (periplasm). In order to show its relevance for the whole process of natural transformation, we determined uptake rates and transformation rates in parallel (Table 1). Fluorescently labeled DNA and in parallel genomic *C. jejuni* DNA carrying the mutation A128G in *rps*L conferring streptomycin resistance, were added to a *C. jejuni* cell suspension. As depicted in Figure 3, these cells had been grown for 18 ± 4 h in liquid culture under several different conditions including growth to exponential vs. stationary phase, varying pH values and microaerobic vs. aerobic conditions (Figure 4). Transformation rates were calculated from the number of transformants on streptomycin-containing ColBA relative to the number of CFU under non-selective conditions. Under optimal conditions at pH 7.5 a transformation rate of $1.3 \times 10^{-4} \pm 2.3 \times 10^{-4}$ was observed (Table 1).

Consistently, bacteria incubated at pH 5.7, which did not display any DNA uptake activity, occasionally showed one resistant colony, leading to a theoretical transformation rate of $\leq 6.9 \times 10^{-9} \pm 6.2 \times 10^{-10}$ (Table 1). The latter rate, however, was indistinguishable from a spontaneous mutation rate, since our detection limit was one resistant colony within ~7×10^9 CFU (Table 1, values without DNA). At a slightly less acidic pH of 6.3 *C. jejuni* competent fraction of bacteria was 2.3 ± 0.9%, corresponding to an approximately 200-fold increased transformation rate of $1.3 \times 10^{-6} \pm 2.5 \times 10^{-6}$ in comparison to pH 5.7.

Furthermore, we correlated the changes in transformation rate relative to the fraction of bacteria with DNA foci, normalized to the "OFF-status" culture at pH 6.3. The higher the fraction of competent bacteria, displaying DNA uptake, the higher was the transformation rate. The correlation was not strictly linear, but had a maximal ratio of change in transformation rate versus change in fraction of competent cells in different experimental settings of 5.1-fold (Table 1). This suggested that the DNA uptake assay is a reliable tool to monitor natural transformation.

2.4. The Concentration of CO_2 or Carbonate and Temperature Do Not Play a Pivotal Role in Competence Regulation

During exponential growth phase, an increase in pH between pH 5.7 and pH 7.5 resulted in enhanced levels of bacteria that displayed competence (Figure 4). Under microaerobic conditions, it is expected that a more alkaline pH results in higher levels of dissolved CO_2 and, thus, enhanced concentration of carbonate. We asked whether the amount of dissolved CO_2 and the concentration of carbonate also triggered competence development in *C. jejuni*. Therefore, we used different CO_2 concentrations in the gas atmosphere during growth at different pH values, keeping levels of H_2 and O_2 constant (Figure S3). CO_2 concentrations varied between 0% and 50%, with intermediate concentrations of 1%, 7%, 15% and 35%. Doubling times during *C. jejuni* growth were optimal with around 1.1 h in the presence of 1% to 15% CO_2 (Table S1), suggesting that the latter concentration range perfectly supports growth of *C. jejuni* in liquid broth culture. At higher concentrations of 35% and 50% CO_2, mean generation times were marginally increased to 1.5 and 1.6 h, respectively (Table S1). Interestingly, even without gaseous CO_2 *C. jejuni* was able to grow, although with 3-fold increased mean generation times (3.4 h, Table S1). For better comparison, the fraction of competent bacteria grown under standard condition at 7% CO_2 and pH 7.5 was set to 100% for the experimental day. Although, the day-to-day variation at low concentrations of CO_2 (0% and 1%) seemed to be large, the CO_2 concentration itself did not correlate with the kinetics of pH-dependent switch of bacteria into the competent state (Figure S3). We also cross-checked the influence of carbonate levels on competence development in a control experiment by addition of 12 or 48 mM of sodium hydrogen carbonate to the medium and could confirm that pH-dependent switch into competent state was unaffected by the concentration of carbonate (Figure S4).

Furthermore, we investigated whether temperature played a role in competence development of *C. jejuni*, as indicated by others [23]. We checked the impact of growth temperature at pH 7.5 on competence development in *C. jejuni*, choosing 32 °C as minimal growth temperature and 37 °C and 42 °C as typical host temperatures (Figure 5). The generation time of *C. jejuni* at the different temperatures was 1.2 ± 0.1 h (37 °C), 0.9 ± 0.2 h (42 °C) and 3.1 ± 0.3 h (32 °C). We observed that competence development was optimal at 37 °C with 45.1 ± 5.8% (Figure 5A).

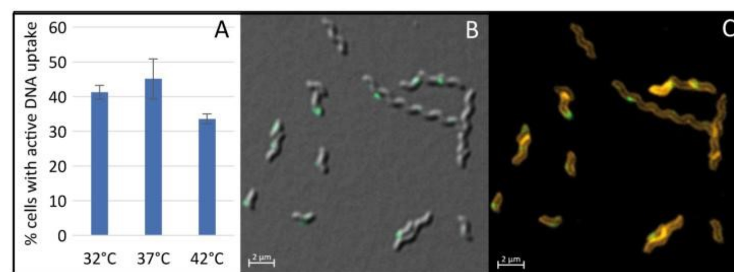


Figure 5. Growth temperature only marginally influences competence development in *C. jejuni*. Fraction of competent *C. jejuni* grown to exponential phase at pH 7.5 at 32 °C, 37 °C and 42 °C (**A**). Bacterial morphology and DNA uptake locations of bacteria grown at 32 °C were displayed by an overlay differential phase contrast (DIC) image with the Fluorescein channel (labelled DNA, in green) (**B**) or by an overlay image of the Fluorescein channel (labelled DNA) and the Cyanine-3 (Cy3) channel (membranes stained with the commercial dye, SynaptoRed C2) (**C**). Scale bar, 2 µm. DNA uptake was performed at 37 °C under aerobic conditions. Experiments were performed at least three times. Error bars indicate standard deviation.

An increase in growth temperature to 42 °C resulted in a slight reduction of competence levels to 33.6 ± 1.4%. As expected for non-optimal growth temperature of

32 °C, the culture harbored normal length bacteria but also several elongated phenotypes (Figure 5B,C). However, competence levels were only marginally altered (41.3 ± 2.0%), if elongated cells were counted as one bacterium. Since in other bacteria, DNA uptake was localized at the cell poles or at the newly synthetized septum, we wondered, if this was also true for *C. jejuni*. We used the SynaptoRed C2 for in vivo staining of *C. jejuni* membranes in order to detect putative septa in elongated cells (Figure 5C). However, it appeared that the elongated cells did not yet harbor a septum and that localization of DNA uptake did not necessarily occur directly at the cell pole or at the next division site.

2.5. DNA Uptake Machinery of Competent Cells Was Shut Down at Slightly Acidic pH

In order to evaluate the direct effect of pH on the DNA uptake process in already competent bacteria, multiple aliquots of a competent *C. jejuni* culture grown in BHI at pH 7.2 and displaying a competent cell fraction of 23.7 ± 6.2% and a transformation rate of $1.3 \times 10^{-5} \pm 8.0 \times 10^{-7}$ in BHI at pH 7.2 were centrifuged. Subsequently, the bacteria were resuspended in pH-adjusted medium and incubated with fluorescently labelled DNA. DNA uptake was performed for 30 min at microaerobic conditions and the fraction of competent cells observed in non-titrated BHI at pH 7.2 set to 100% per experimental day. Not only competence development (see above) but also the DNA uptake process was highly pH-sensitive, with optimal transport at pH values between 7.2 and 8 (Figure 6).

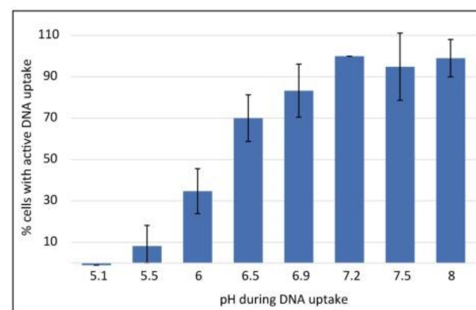


Figure 6. DNA uptake is completely shut down at slightly acidic pH. Competent *C. jejuni* BfR-CA-14430 bacteria were incubated with fluorescently labelled DNA in BHI titrated to the indicated pH values (±0.1) under microaerobic conditions. The fraction of bacteria showing active DNA uptake are depicted, normalized to untitrated BHI, pH 7.2 condition (set to 100%). Experiments were performed at least three times with independent cultures. Error bars indicate standard deviation. BHI, brain-heart infusion.

Between pH 7.2 and pH 5.5 the fraction of cells with active DNA uptake decreased to less than 10% and at pH 5.1 DNA uptake was abolished. Likewise, transformation activity at pH 5.1 was also drastically reduced to levels reaching the detection limit ($2.2 \times 10^{-9} \pm 3.8 \times 10^{-9}$). By pre-incubation of the bacteria with fluorescent DNA at pH 5.1 for 30 min and subsequent titration to pH 7.5, we showed that genomic DNA was stable at pH 5.1 and the pH-dependent shut down of the DNA uptake process was reversible. These cells regained DNA uptake capacity of 59.8 ± 3.2%.

3. Discussion

We used a single cell DNA uptake assay to monitor DNA uptake during natural transformation in *C. jejuni*. As known for *H. pylori*, this assay visualizes uptake of fluorescently labelled DNA into a DNase-resistant state, namely the periplasm [22,32], which is the first step of natural transformation. In *C. jejuni* the fraction of cells with active DNA uptake correlated with transformation rates (Table 1), was energy-dependent and limited to uptake of genomic DNA of *C. jejuni* with the methylated RATTY motif [26], suggesting

that the assay was an excellent tool to decipher parameters for regulation of competence development and of the DNA uptake process itself.

C. jejuni grows at temperatures above 30 °C [35]. In cattle, pigs and other warm-blooded animals, including the human being, the typical temperature is around 37 °C, while in birds *C. jejuni* thrives at an elevated temperature of 42 °C. *C. jejuni* is a fastidious bacterium with special needs for growth and survival [36]. pH was revealed as major regulating parameter for competence development and DNA uptake itself. Using the classical approach with a chloramphenicol resistance marker, it was previously reported that natural transformation of *C. jejuni* efficiently occurred above pH 7 [23]. Wilson and colleagues described an effect of CO_2 on transformation rates [24]. Using different carbonate concentrations or CO_2 levels, with highly controlled pH values we could reveal that CO_2 or carbonate levels did not affect competence in *C. jejuni* but that pH $\geq$ 7 was a prerequisite for efficient natural transformation activity. At pH 5 competence development and DNA uptake activity in *C. jejuni* was completely shut down.

Under non-growing conditions, i.e., at atmospheric conditions, *C. jejuni* ceased competence development even at permissive pH without losing viability. This is in contrast to *H. pylori* where oxidative stress was a stimulating factor for competence development [22]. However, when we measured the regulation of functional DNA uptake complexes, competent bacteria even showed slightly enhanced DNA uptake under aerobic conditions (Figure S1). In *C. coli* natural transformation of point mutation resistances was also not reduced in an aerobic atmosphere, after the cells had been grown to exponential phase under microaerobic conditions [37]. Now that our study enabled us to dissect competence development and the DNA uptake process in *C. jejuni*, the data suggest that outside the host *Campylobacter* would not be able to develop competence. However, if competent cells are shedded from the host, these bacteria might still be able to undergo genetic exchange.

At pH 7.5, and under microaerobic conditions a transformation rate of $1.3 \times 10^{-4} \pm 2.3 \times 10^{-4}$ and around 50% cells with active DNA uptake were observed. Hence, one out of 4000 cells with active DNA uptake successfully recombined the genomic *rpsL* resistance marker into the chromosome and was able to grow on streptomycin-containing agar plates. Several steps of sub-cultivation in fresh medium did not further enhance competence development, confirming that bacteria stay bimodal and do not switch completely into the competent state. Variation of pH and incubation at aerobic atmosphere drastically influenced both the fraction of competent *C. jejuni* and the number of transformants. This demonstrated that the single cell assay was a suitable tool for direct monitoring of natural transformation capacity. It also implicated that regulation of natural transformation in *C. jejuni* primarily occurred at the level of regulation of the first step of DNA uptake, as observed for *H. pylori* [21,22]. Consecutive steps of natural transformation, like DNA uptake into the cytoplasm or homologous recombination appeared to be quite stable under our tested conditions, however, limiting resistance marker expression.

Growth but not cell division seemed to be important for competence development (Figures 4 and 5). In stationary growth phase, DNA uptake capacity was slightly reduced. Absence of CO_2 did support growth of *C. jejuni* under our conditions. The inoculum of 3×10^{-7} and 2.5×10^{-5}, corresponding to only 1500 to 1.25×10^5 bacteria per mL (assuming that OD_{600} of 0.2 corresponds to 10^9 cell counts per ml [38]) guaranteed that respiration of *C. jejuni* was insufficient to significantly elevate the CO_2 concentration in the medium. Growth at zero CO_2 or at limiting growth temperature of 32 °C led to prolonged generation times and potential elongated cell morphology, but did not drastically affect competence development. Hence, neither the frequency of cell division nor the length of the generation time seemed to be signals for competence development. This was consistent with the finding that localization of DNA uptake in *C. jejuni*, as observed in *H. pylori* [22], did not strictly occur at the cell poles, as was previously shown for *B. subtilis* [39,40].

For colonization of the avian gut, *C. jejuni* has to first passage through the esophagus into the crop. The pH in the crop is largely influenced by the ingested food. It has been mostly measured to range between 4 and 6, with maximal values reported below 7 [41]. The

pH decreases to around 3 to 4 in the gizzard due to gastric juice secretion. Our data suggest that *C. jejuni* is non-competent within passage through these parts of the avian digestive system. Once reaching the intestine, pH rises to 6–7.5, with maximal pH values of 7.9 [41] and competence might rapidly develop. Thus, the intestine is suggested to be the location with extensive genetic exchange between different *Campylobacter* spp. strains harboring DNA with the methylated DNA uptake motif. Shed competent *C. jejuni* are still able to take up DNA but will lose competence with time outside the host due to the inhibitory effect of atmospheric oxygen. We propose that *C. jejuni* increases genetic diversity at its multiplication site in order to pre-adapt to unfavorable conditions outside the host and/or for efficient host switch.

4. Materials and Methods

4.1. Strains and Growth Conditions

C. jejuni field strain BfR-CA-14430, isolated from chicken and previously sequenced [42] and reference strain *C. jejuni* 81-176 were used. Strains were stored at −80 °C in cryocultures (MAST Group Ltd., Bootle, UK). Cells were cultured either on Columbia blood agar plates (ColBA, Oxoid, Thermo Fisher Scientific Inc., Waltham, MA, USA), supplemented with 5% defibrinated sheep blood (Oxoid, Thermo Fisher Scientific Inc, Waltham, MA, USA), in brain heart infusion (BHI, Oxoid, Thermo Fisher Scientific Inc, Waltham, MA, USA) or in Bolton broth basis without selective supplements (Oxoid, Thermo Fisher Scientific, Waltham, MA, USA). The cultivation on ColBA was performed at 37 °C under microaerobic conditions (5% O_2, 10% CO_2, rest N_2) using a microaerobic incubator (Binder, Tuttlingen, Germany). For the transformation experiments, selection of transformants was performed on ColBA supplemented with 20 µg/mL of streptomycin (Sigma-Aldrich, Steinheim, Germany).

Liquid cultures were incubated in gas replacement jars (Oxoid Anaerobia System, Thermo Fisher Scientific Inc., Waltham, MA, USA), filled with the appropriate gas mixtures (Air Liquide, Paris, France) under shaking at 140 rpm at 37 °C. All the gas mixtures had 3.5% H_2 and 6% O_2 and only differed in the concentration of CO_2 (and respective N_2). Four gas cylinders were used: 0% CO_2, 1% CO_2, 7% CO_2 and 50% CO_2. Jars were evacuated to −70 kPa using a vacuum pump (KNF Neuberger GmbH, Freiburg im Breisgau, Germany) and the appropriate gas was filled into the jar. Evacuation to −70 kPa and refilling led to 70% exchange of atmosphere. The refilling process was done twice. To achieve 15% CO_2, the jar was first filled with the gas mixture containing 50% CO_2 and subsequently with 7% CO_2 (1st filling: 0.7 × 50% CO_2 = 35% CO_2; 2nd filling: 0.7 × 7% CO_2 + 0.3 × 35% CO_2 = 15.4%). For 35% CO_2, the jar was filled with 0% CO_2 in the first refilling process and, thereafter, with 50% CO_2 (1st filling: only O_2 was decreased and H_2 increased; 2nd filling: 0.7 × 50% CO_2 = 35%).

pH of media also depends on the amount of dissolved CO_2 and, thus, on the amount of gaseous CO_2. Hence, opening the jar will decrease CO_2, thereby, increasing pH. We checked that during the first 10 min after opening the jar the pH only marginally increased (Figure S5) and that handling of the bacterial suspensions and pH measurements were acceptable within this time period.

4.2. DNA Uptake Assay

Genomic DNA of *C. jejuni* was extracted using the PureLink Genomic DNA Kit (Thermo Fisher Scientific Inc, Waltham, MA, USA) according to manufacturer's instructions. Non-methylated λ-DNA (Thermo Fisher Scientific Inc., Waltham, MA, USA) and extracted genomic *C. jejuni* DNA were covalently labelled with fluorescein in a 1:1 (volume:weight) ratio of Label IT reagent to nucleic acid using the Mirus Label IT Fluorescein kit (Mirus Bio LLC, Madison, WI, USA).

Cells were streaked out from a −80 °C stock culture and sub-cultured on ColBA at 37 °C in an incubator (Binder, Tuttlingen, Germany) under microaerobic conditions (5% O_2, 10% CO_2, rest N_2) for 18 ± 4 h. Cells from ColBA were resuspended in BHI or Bolton broth

at an initial optical density at 600 nm (OD_{600}) of 0.3 and pre-cultured for 7 ± 2 h hours using gas jars. Subsequently, cells were sub-cultured in fresh BHI or Bolton if indicated pH was adjusted with sodium hydroxide (NaOH) or hydrochloric acid (HCl). An appropriate initial OD_{600} to obtain a maximal OD_{600} of 0.5 for "exponential phase" cultures and an OD_{600} between 1 and 1.5 for "stationary phase" cultures, after 18 ± 4 h was used. Over all conditions the initial OD_{600} ranged between 0.007 and 1×10^{-6}. Generation times varied between 1 h and 5 h. This assay setup in which cells were grown in medium with pH change before 18 ± 4 h growth period was called "long-term pH". For the assay setup "short-term pH" cells were grown for 18 ± 4 h, then the jar was opened, pH was measured (Mettler Toledo, Columbus, OH, USA), and adjusted by adding a suitable volume of sodium hydroxide (NaOH) or hydrochloric acid (HCl). For condition "fresh medium", cultures were 3 to 5-fold diluted with fresh Bolton broth in order to reach an OD_{600} of 0.05. Indicated pH values are pH ± 0.1. Cells were incubated for another 3 h under microaerobic conditions (3.5% H_2, 6% O_2, 7% CO_2, rest N_2) or if indicated under aerobic conditions at 37 °C and shaking at 140 rpm. Afterwards cells were harvested by centrifugation for 5 min at 16,000× *g* and resuspended in 100 µL BHI. If indicated, pH was changed with NaOH or HCl. For deviation of pH between aerobic and microaerobic conditions before and after growth see Figure S2.

For the DNA uptake assay, 1 µL labelled gDNA of *C. jejuni* (100 ng/µL) and as a control the same amount of labelled λ-DNA (Thermo Fisher Scientific Inc., Waltham, MA, USA) were added to the cells. Incubation was performed for 30 min at 37 °C under microaerobic (Figures 6, S3 and S4) or aerobic conditions (Figures 1, 2, 4 and 5). The cell suspension was centrifuged for 5 min at 16,000× *g*, resuspended in 15 µL BHI (or Bolton broth) and incubated for 5 min at 37 °C after addition of 1 µL DNase (10 U) (Roche, Rotkreuz, Schweiz). If appropriate cells were stained by adding 0.4 µL 1 mg/mL SynaptoRed C2 (Sigma-Aldrich, Steinheim, Germany) to the cell suspension. Cells were immobilized on 1.5% agarose surface. For evaluation of competence status cells were analyzed using the fluorescence microscope Zeiss Axio Observer Z1 with differential phase contrast (DIC), a metal halide light source (HXP120C) and a plan apochromatic 63 ×/1.4 objective. For detection of fluorescein-labelled DNA a filter set with excitation at 470 ± 20 nm and emission at 525 ± 25 nm and for detection of SynaptoRed staining a filter set with excitation at 550 ± 12 nm and emission at 605 ± 35 nm was used. Images were taken with a 12-bit monochromatic AxioCam MRm camera. Cells harboring at least one fluorescent focus were considered competent. For each condition at least 750 cells were counted.

To analyze if DNA uptake requires proton motive force, *C. jejuni* BfR-CA-14430 was preincubated for 10 min with 250 µM CCCP before DNA uptake was performed by incubation for 30 min at 37 °C.

4.3. Transformation Assay

Cells were grown as described for the DNA uptake assay. Cells were incubated for 1 h under microaerobic conditions (5% O_2, 10% CO_2, rest N_2) at 37 °C with 100 ng of *C. jejuni* gDNA of strain BfR-CA-14430-strep containing the point mutation A128G in *rpsL*, conferring streptomycin resistance. Then, cells were centrifuged for 5 min at 16,000× *g* and 1 µL DNase [10 U/µL] was added. For chromosomal integration and expression of the streptomycin resistance marker, the suspension was incubated for another 3 h under microaerobic conditions (5% O_2, 10% CO_2, rest N_2) at 37 °C and a serial dilution of the cells was plated onto ColBA for CFU determination as well as on ColBA containing 20 µg/mL streptomycin for quantification of transformants. Agar plates were incubated for 2 days under microaerobic atmosphere (5% O_2, 10% CO_2, rest N_2) at 37 °C. Transformation rate was calculated as the ratio of transformants per CFU.

5. Conclusions

The single cell assay enabled us to dissect competence development and DNA uptake in *C. jejuni*. Natural transformation in *C. jejuni* was strongly regulated by pH and oxidative

stress was an inhibitory factor for competence development but not DNA uptake itself. At slightly acidic conditions, natural transformation activity was shut down. The data suggest that *C. jejuni* most probably increases genetic diversity at its multiplication site in the host intestine in order to prepare the pathogen for survival in the environment and for host switch.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/ijms221810169/s1, Figure S1: DNA uptake is favored under aerobic compared to microaerobic conditions, Figure S2: pH variation of pH-adjusted growth medium before and after *C. jejuni* growth into exponential phase, Figure S3: pH-dependent competence development in *C. jejuni* BfR-CA-14430 is independent of CO_2 concentration, Figure S4: Competence development depends on pH but not on sodium hydrogen carbonate, Figure S5: pH change of Bolton broth as a function of time after opening the jar, Table S1: Generation time and final pH of BfR-CA-14430 grown under different conditions.

Author Contributions: Conceptualization, K.S. and J.C.G.; methodology and investigation, J.C.G.; formal analysis, J.C.G. and K.S.; writing, J.C.G. and K.S. All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by the German Federal Ministry of Education and Research (BMBF), zoonoses research consortium PAC-CAMPY, project IP3/01KI1725B/01KI2007B.

Data Availability Statement: Data is available in the manuscript and in the Supplementary Materials.

Acknowledgments: We thank our colleagues at the NRL for *Campylobacter* for sustained support.

Conflicts of Interest: The authors declare no conflict of interest.

References

1. EFSA. The European Union One Health 2019 Zoonoses Report. *EFSA J.* **2021**, *19*, e06406.
2. Shange, N.; Gouws, P.; Hoffman, L.C. *Campylobacter* and *Arcobacter* species in food-producing animals: Prevalence at primary production and during slaughter. *World J. Microbiol. Biotechnol.* **2019**, *35*, 146. [CrossRef]
3. Whiley, H.; van den Akker, B.; Giglio, S.; Bentham, R. The role of environmental reservoirs in human campylobacteriosis. *Int. J. Environ. Res. Public Health* **2013**, *10*, 5886–5907. [CrossRef] [PubMed]
4. Waldenström, J.; Axelsson-Olsson, D.; Olsen, B.; Hasselquist, D.; Griekspoor, P.; Jansson, L.; Teneberg, S.; Svensson, L.; Ellström, P. *Campylobacter jejuni* colonization in wild birds: Results from an infection experiment. *PLoS ONE* **2010**, *5*, e9082. [CrossRef] [PubMed]
5. Rosner, B.M.; Schielke, A.; Didelot, X.; Kops, F.; Breidenbach, J.; Willrich, N.; Gölz, G.; Alter, T.; Stingl, K.; Josenhans, C.; et al. A combined case-control and molecular source attribution study of human *Campylobacter* infections in Germany, 2011–2014. *Sci. Rep.* **2017**, *7*, 5139. [CrossRef] [PubMed]
6. EFSA. Panel on Biological Hazards (BIOHAZ). Scientific opinion on *Campylobacter* in broiler meat production: Control options and performance objectives and/or targets at different stages of the food chain. *EFSA J.* **2011**, *9*, 2105. [CrossRef]
7. Doyle, M.P.; Roman, D.J. Growth and survival of *Campylobacter fetus* subsp. *jejuni* as a function of temperature and pH. *J. Food Prot.* **1981**, *44*, 596–601. [PubMed]
8. Kim, S.H.; Chelliah, R.; Ramakrishnan, S.R.; Perumal, A.S.; Bang, W.S.; Rubab, M.; Daliri, E.B.; Barathikannan, K.; Elahi, F.; Park, E.; et al. Review on stress tolerance in *Campylobacter jejuni*. *Front. Cell. Infect. Microbiol.* **2020**, *10*, 596570. [CrossRef] [PubMed]
9. Baffone, W.; Casaroli, A.; Citterio, B.; Pierfelici, L.; Campana, R.; Vittoria, E.; Guaglianone, E.; Donelli, G. *Campylobacter jejuni* loss of culturability in aqueous microcosms and ability to resuscitate in a mouse model. *Int. J. Food Microbiol.* **2006**, *107*, 83–91. [CrossRef]
10. Crofts, A.A.; Poly, F.M.; Ewing, C.P.; Kuroiwa, J.M.; Rimmer, J.E.; Harro, C.; Sack, D.; Talaat, K.R.; Porter, C.K.; Gutierrez, R.L.; et al. *Campylobacter jejuni* transcriptional and genetic adaptation during human infection. *Nat. Microbiol.* **2018**, *3*, 494–502. [CrossRef]
11. Aksomaitiene, J.; Novoslavskij, A.; Kudirkiene, E.; Gabinaitiene, A.; Malakauskas, M. Whole genome sequence-based prediction of resistance determinants in high-level multidrug-resistant *Campylobacter jejuni* isolates in Lithuania. *Microorganisms* **2020**, *9*, 66. [CrossRef] [PubMed]
12. Chu, H.Y.; Sprouffske, K.; Wagner, A. Assessing the benefits of horizontal gene transfer by laboratory evolution and genome sequencing. *BMC Evol. Biol.* **2018**, *18*, 54. [CrossRef] [PubMed]
13. Samarth, D.P.; Kwon, Y.M. Horizontal genetic exchange of chromosomally encoded markers between *Campylobacter jejuni* cells. *PLoS ONE* **2020**, *15*, e0241058. [CrossRef] [PubMed]
14. Golz, J.C.; Epping, L.; Knüver, M.T.; Borowiak, M.; Hartkopf, F.; Deneke, C.; Malorny, B.; Semmler, T.; Stingl, K. Whole genome sequencing reveals extended natural transformation in *Campylobacter* impacting diagnostics and the pathogens adaptive potential. *Sci. Rep.* **2020**, *10*, 3686. [CrossRef] [PubMed]

15. Golz, J.C.; Stingl, K. Natural competence and horizontal gene transfer in *Campylobacter*. *Curr. Top Microbiol. Immunol.* **2021**, *431*, 265–292. [PubMed]
16. Attaiech, L.; Boughammoura, A.; Brochier-Armanet, C.; Allatif, O.; Peillard-Fiorente, F.; Edwards, R.A.; Omar, A.R.; MacMillan, A.M.; Glover, M.; Charpentier, X. Silencing of natural transformation by an RNA chaperone and a multitarget small RNA. *Proc. Natl. Acad. Sci. USA* **2016**, *113*, 8813–8818. [CrossRef] [PubMed]
17. Engelmoer, D.J.; Donaldson, I.; Rozen, D.E. Conservative sex and the benefits of transformation in *Streptococcus pneumoniae*. *PLoS Pathog.* **2013**, *9*, e1003758. [CrossRef]
18. Attaiech, L.; Charpentier, X. Silently transformable: The many ways bacteria conceal their built-in capacity of genetic exchange. *Curr. Genet.* **2017**, *63*, 451–455. [CrossRef]
19. Dubnau, D.; Blokesch, M. Mechanisms of DNA uptake by naturally competent bacteria. *Annu. Rev. Genet.* **2019**, *53*, 217–237. [CrossRef]
20. Johnston, C.; Martin, B.; Fichant, G.; Polard, P.; Claverys, J.P. Bacterial transformation: Distribution, shared mechanisms and divergent control. *Nat. Rev. Microbiol.* **2014**, *12*, 181–196. [CrossRef]
21. Corbinais, C.; Mathieu, A.; Damke, P.P.; Kortulewski, T.; Busso, D.; Prado-Acosta, M.; Radicella, J.P.; Marsin, S. ComB proteins expression levels determine *Helicobacter pylori* competence capacity. *Sci. Rep.* **2017**, *7*, 41495. [CrossRef]
22. Krüger, N.J.; Knüver, M.T.; Zawilak-Pawlik, A.; Appel, B.; Stingl, K. Genetic diversity as consequence of a microaerobic and neutrophilic lifestyle. *PLoS Pathog.* **2016**, *12*, e1005626. [CrossRef]
23. Vegge, C.S.; Brondsted, L.; Ligowska-Marzeta, M.; Ingmer, H. Natural transformation of *Campylobacter jejuni* occurs beyond limits of growth. *PLoS ONE* **2012**, *7*, e45467.
24. Wilson, D.L.; Bell, J.A.; Young, V.B.; Wilder, S.R.; Mansfield, L.S.; Linz, J.E. Variation of the natural transformation frequency of *Campylobacter jejuni* in liquid shake culture. *Microbiology* **2003**, *149 Pt 12*, 3603–3615. [CrossRef]
25. Wang, Y.; Taylor, D.E. Natural transformation in *Campylobacter* species. *J. Bacteriol.* **1990**, *172*, 949–955. [CrossRef]
26. Beauchamp, J.M.; Leveque, R.M.; Dawid, S.; DiRita, V.J. Methylation-dependent DNA discrimination in natural transformation of *Campylobacter jejuni*. *Proc. Natl. Acad. Sci. USA* **2017**, *114*, E8053–E8061. [CrossRef]
27. Jeon, B.; Zhang, Q. Cj0011c, a periplasmic single- and double-stranded DNA-binding protein, contributes to natural transformation in *Campylobacter jejuni*. *J. Bacteriol.* **2007**, *189*, 7399–7407. [CrossRef] [PubMed]
28. Jeon, B.; Muraoka, W.; Sahin, O.; Zhang, Q. Role of Cj1211 in natural transformation and transfer of antibiotic resistance determinants in *Campylobacter jejuni*. *Antimicrob. Agents Chemother.* **2008**, *52*, 2699–2708. [CrossRef] [PubMed]
29. Wiesner, R.S.; Hendrixson, D.R.; DiRita, V.J. Natural transformation of *Campylobacter jejuni* requires components of a type II secretion system. *J. Bacteriol.* **2003**, *185*, 5408–5418. [CrossRef]
30. Beauchamp, J.M.; Erfurt, R.S.; DiRita, V.J. Characterization and localization of the *Campylobacter jejuni* transformation system proteins CtsE, CtsP, and CtsX. *J. Bacteriol.* **2015**, *197*, 636–645. [CrossRef] [PubMed]
31. Maier, B.; Chen, I.; Dubnau, D.; Sheetz, M.P. DNA transport into *Bacillus subtilis* requires proton motive force to generate large molecular forces. *Nat. Struct. Mol. Biol.* **2004**, *11*, 643–649. [CrossRef] [PubMed]
32. Stingl, K.; Müller, S.; Scheidgen-Kleyboldt, G.; Clausen, M.; Maier, B. Composite system mediates two-step DNA uptake into *Helicobacter pylori*. *Proc. Natl. Acad. Sci. USA* **2010**, *107*, 1184–1189. [CrossRef]
33. Cohen, E.J.; Nakane, D.; Kabata, Y.; Hendrixson, D.R.; Nishizaka, T.; Beeby, M. *Campylobacter jejuni* motility integrates specialized cell shape, flagellar filament, and motor, to coordinate action of its opposed flagella. *PLoS Pathog.* **2020**, *16*, e1008620. [CrossRef]
34. Blair, D.F. Flagellar movement driven by proton translocation. *FEBS Lett.* **2003**, *545*, 86–95. [CrossRef]
35. Hazeleger, W.C.; Wouters, J.A.; Rombouts, F.M.; Abee, T. Physiological activity of *Campylobacter jejuni* far below the minimal growth temperature. *Appl. Environ. Microbiol.* **1998**, *64*, 3917–3922. [CrossRef]
36. Biesta-Peters, E.G.; Jongenburger, I.; de Boer, E.; Jacobs-Reitsma, W.F. ISO_10272-1:2017, Microbiology of the food chain—Horizontal method for detection and enumeration of *Campylobacter* spp.—Part 1: Detection method. *Int. J. Food Microbiol.* **2019**, *288*, 39–46. [CrossRef] [PubMed]
37. Kim, J.S.; Kim, J.W.; Kathariou, S. Differential effects of temperature on natural transformation to erythromycin and nalidixic acid resistance in *Campylobacter coli*. *Appl. Environ. Microbiol.* **2008**, *74*, 6121–6125. [CrossRef] [PubMed]
38. Krüger, N.J.; Buhler, C.; Iwobi, A.N.; Huber, I.; Ellerbroek, L.; Appel, B.; Stingl, K. "Limits of control"—crucial parameters for a reliable quantification of viable *Campylobacter* by real-time PCR. *PLoS ONE* **2014**, *9*, e88108.
39. Hahn, J.; Maier, B.; Haijema, B.J.; Sheetz, M.; Dubnau, D. Transformation proteins and DNA uptake localize to the cell poles in *Bacillus subtilis*. *Cell* **2005**, *122*, 59–71. [CrossRef]
40. Kaufenstein, M.; van der Laan, M.; Graumann, P.L. The three-layered DNA uptake machinery at the cell pole in competent *Bacillus subtilis* cells is a stable complex. *J. Bacteriol.* **2011**, *193*, 1633–1642. [CrossRef] [PubMed]
41. Svihus, B. Effect of digestive tract conditions, feed processing and ingredients on response to NSP enzymes. In *Enzymes in farm animal nutrition*, 2nd ed.; Bedford, M.R., Partridge, G.G., Eds.; CAB International: Wallingford, UK, 2011; pp. 129–159. [CrossRef]
42. Epping, L.; Golz, J.C.; Knüver, M.T.; Huber, C.; Thürmer, A.; Wieler, L.H.; Stingl, K.; Semmler, T. Comparison of different technologies for the decipherment of the whole genome sequence of *Campylobacter jejuni* BfR-CA-14430. *Gut Pathog.* **2019**, *11*, 59. [CrossRef] [PubMed]

6 Discussion

Campylobacter is genetically diverse whereas the main driving force is thought to be horizontal gene transfer (Wilson et al. 2009; Sheppard et al. 2008). This enlarges the adaptive potential of *Campylobacter* and could be a reason for the great persistence. Levels of natural transformation have shown to vary but the exact parameters stimulating or inhibiting competence development are only poorly understood (Wilson et al. 2003; Vegge et al. 2012). Therefore, we developed an assay to monitor competence development with fluorescently labelled DNA on single cell level. We used this assay to test different parameters for their effect on competence development and DNA uptake in *C. jejuni.* This included pH, temperature, carbonic levels or the substance curcumin which was suggested as a potential treatment option for campylobacteriosis. We used the *Campylobacter* field strain BfR-CA-14430 recently isolated from chicken and sequenced this strain for characterization with different sequencing methods including short-read and long-read techniques and compared different assembly tools. Since we further observed *Campylobacter* strains with ambiguous results in species differentiation, we analyzed these strains by sequencing to find out which species they were and to determine if genetic exchange had taken place accounting for the ambiguous typing results.

6.1 Choice and characterization of a reference strain for the PAC-CAMPY consortium

For better comparability of results within the PAC-CAMPY consortium which includes different projects, with the overall aim to prevent and combat campylobacter infections, we choose the strain BfR-CA-14430 (Epping et al. 2019). This strain had been isolated from chicken meat in 2016 and served as a fresh field isolate. Since no genome data were available we sequenced this strain. There are different sequencing methods available as well as various assembly tools. Using sequence data from the short-read Illumina MiSeq in combination with SMRT long-read sequencing by PacBio or MinION the quality could be improved regarding mismatches as well as insertions and deletions. Most likely BfR-CA-14430 has a chromosome size of 1,645,980 bp and harbors a plasmid of 41,1772 bp. The chromosome is thought to have 1665 coding sequences and the plasmid is predicted to code for 46 genes. The genome size of BfR-CA-14430 has nearly the same size as type strain NCTC11168 with 1,641,481 bp and the number of predicted proteins vary by only 11 (Parkhill et al. 2000). The GC content from BfR-CA-14430 and NCTC11168 is also very similar with 30.4 and 30.6 % respectively (Epping et al. 2019; Parkhill et al. 2000). BfR-CA-14430 carries the beta-lactam resistance gene $bla_{\text{OXA-61}}$, which is very common among *C. jejuni* strains (Griggs et al. 2009). Furthermore, it carries a point mutation in *gyrA* leading to the amino acid substitution T86I in the gyrase subunit A which conveys resistance to (fluoro-)quinolones (Leekitcharoenphon et al. 2018). This is also very

typical as resistance against ciprofloxacin, a fluoroquinolone, is the most frequently reported antimicrobial resistance in *C. jejuni* isolated from humans in 2019 (EFSA 2021b).

6.2 Identification of parameters triggering competence development using a single cell assay

To identify parameters triggering competence development in *C. jejuni* we developed a single cell based assay and visualized DNA uptake using fluorescently labeled DNA (Golz and Stingl 2021). Therefore, we grew cells in a liquid shaking culture. This has the advantage that oxygen, CO_2, nutrients and other components are equally distributed which is important since the physiological state of competence might be distorted by fluctuating growth conditions. During growth phase in which competence development might occur no DNA was added to the cells. Only after growth period bacteria cells were incubated with fluorescently labeled DNA for 30 min. That means the growth phase in which competence might develop was separated from the DNA uptake process of already competent cells. This assay which allows for the resolution if factors have an effect on competence development or DNA uptake has successfully been used for *H. pylori* (Krüger et al. 2016). Additionally, the periplasmic localization of fluorescently labelled DNA has previously been shown for *H. pylori* (Stingl et al. 2010). The uptake into the periplasm is the first step of natural transformation where DNA is protected from external DNases. To test if the single cell uptake assay was a suitable tool to draw a conclusion under which conditions the whole process of natural transformation occurs, we performed experiments using this assay and a classical transformation assay with an antibiotic resistance marker in parallel (Golz and Stingl 2021). The fraction of cells with fluorescence signal indicating DNA uptake correlated with transformation rates obtained by an antibiotic resistance marker. In *C. jejuni* as observed for *H. pylori* DNA uptake occurred in an energy-dependent manner (Stingl et al. 2010; Golz and Stingl 2021). In contrast to *H. pylori, Campylobacter* only took up DNA which was methylated at RAATTY sites as shown by Beauchamp and colleagues (Beauchamp et al. 2017; Golz and Stingl 2021).

Using the single cell DNA uptake assay we identified pH as a major regulation factor of competence and DNA uptake in *C. jejuni*. At pH 5 competence development as well as DNA uptake were abolished (Golz and Stingl 2021). Between pH 6.5 and 7.5 competence development increased with rising pH. Vegge and colleagues already showed that natural transformation occurred above pH 7 by using a classical transformation approach with selection for antibiotic resistance (Vegge et al. 2012). It was further suggested that natural transformation was influenced by CO_2 levels but a pH effect could not be ruled out since pH at higher CO_2 levels was lower than under lower CO_2 levels (Wilson et al. 2003). By controlling pH levels when changing gaseous CO_2 concentrations or carbonate levels we showed that pH influenced competence development but not CO_2 levels (Golz and Stingl 2021). Furthermore,

oxidative stress abolished competence development in *C. jejuni* which is in contrast to *H. pylori* (Golz and Stingl 2021; Krüger et al. 2016). DNA uptake was slightly enhanced under aerobic conditions in *C. jejuni* suggesting that genetic exchange could occur outside of the host (Golz and Stingl 2021). *C. coli* was also shown to do not reduce genetic exchange in response to higher oxygen levels after inoculation at microaerobic conditions (Kim et al. 2008). This was observed by using the classical approach with selection of a resistance marker.

In the host *C. jejuni* will mainly reside at 37°C to 42°C, as these are the body temperatures of mammals and birds. We have shown that competence development occurred at 37°C as well as at 42°C (Golz and Stingl 2021). Even at 32°C where generation times were prolonged and cells were elongated no significant alteration in the development of competence was observed. Therefore, it is likely that *C. jejuni* undergoes natural transformation when residing in mammalian or avian host as long as the pathogen experiences a neutral to slightly alkaline pH and reduced oxygen levels. Since in the avian crop as well as in the gizzard the pH is acidic it is likely that this is not the place where genetic exchange occurs (Svihus 2011). Once *C. jejuni* reaches the intestine and pH increases to a slightly alkaline pH competence might develop and natural transformation could occur. Since *C. jejuni* resides in the intestine there could be a lot of opportunity for extensive genetic exchange. Oxygen levels of an aerobic atmosphere abolished competence development but already assembled uptake complexes still took up DNA. Therefore, it is conceivable that competent cells which were shed out from the host are able to undergo genetic exchange for a defined time-period until the uptake complexes disassemble. This could be a strategy to prime for new environmental conditions for example for a host switch. We have recently shown that there are *C. coli* strains which incorporated *C. jejuni* gene variants or fragments which might be adaptions to harsh environmental conditions impacting routine diagnostic typing (Golz et al. 2020).

Using the single cell assay under standard conditions including growth at pH 7.5, it turned out that nearly 50 % of *C. jejuni* cells were competent (Golz and Stingl 2021). As shown for *B. subtilis* only a subpopulation undergoes the competence state (Hamoen et al. 2003). Even though the percentage of competent cells in *C. jejuni* is higher than in *B. subtilis* where only 10-20% were reported to be competent it seems as if also for *Campylobacter* it is beneficial to restrict natural transformation to a sub-population (Hamoen et al. 2003). By using the classical transformation approach with the antibiotic resistance maker streptomycin in parallel to the uptake assay it turned out that one out of 4000 cells with active DNA uptake undergoes the whole process of natural transformation. This ratio is way smaller than in *H. pylori* were up to one out of three cells with active DNA uptake was transformed which was detected by the resistance marker (Krüger et al. 2016).

The change of growth conditions with an influence on competence development monitored by the single cell assay like pH or aerobic stress altered the transformation frequency nearly to the same extent (Golz and Stingl 2021). This further indicates that the single cell assay is a suitable tool to monitor competence development. On top of that, it suggests that regulation of natural transformation in *C. jejuni* like in *H. pylori* occurred at the first steps of DNA uptake rather than at subsequent steps like import into the cytoplasm, homologous recombination or expression of the resistance marker (Golz and Stingl 2021; Corbinais et al. 2017; Krüger et al. 2016). In contrast to *H. pylori*, in *C. jejuni* refreshing the medium before changing to slightly alkaline pH resulted in an elevated competence rate (Krüger et al. 2016; Golz and Stingl 2021). Since in fresh medium the quenching capacity might be higher than in medium after growth the oxidative stress for the cells might be reduced. In *C. jejuni* in contrast to *H. pylori* oxidative stress inhibited competence development. This could be an explanation why refreshing the medium in *C. jejuni* and *H. pylori* had an opposite effect on competence development. Furthermore, in *C. jejuni* competence development was most efficient in exponential phase (Golz and Stingl 2021). In stationary phase cells were not able to switch into the competent state but if cells have already switched they partly retained this status and were able to undergo genetic exchange. Vegge and colleagues also observed that natural transformation was most efficient in exponential phase using a classical transformation approach (Vegge et al. 2012). In the experiment to evaluate if carbonate plays a role in competence development we grew *C. jejuni* in an atmosphere without carbon dioxide (Golz and Stingl 2021). It turned out that at least in the presence of 3.5 % H_2 *C. jejuni* was able to grow without CO_2 in a liquid shaking culture. Compared to standard CO_2 levels only slightly reduced doubling times were observed. At the beginning of the growth period only few bacteria were inoculated, ranging from 1500 to 1.25×10^5, guaranteeing that production of CO_2 from the cells themselves was neglectable, which would not be the case upon high cell densities. This hints that *C. jejuni* is not a capnophilic bacterium standing in the need of elevated carbon dioxide levels as has been reported (Macé et al. 2015).

Using a biphasic system with a kanamycin marker it was shown that *Aliarcobacter butzleri*, a close relative of *C. jejuni*, was transformable at 20°C, 30°C and 37°C under aerobic, microaerobic as well as anaerobic atmosphere (Bonifácio et al. 2021). Whereas highest transformation levels were observed at 30°C and a microaerobic atmosphere oxygen stress levels seem to have no huge impact on natural transformation in *A. butzleri*. As for *C. jejuni* natural transformation was more efficient with cells in exponential phase than in stationary phase. Interestingly, *A. butzleri* was not transformable by *C. coli* genomic DNA carrying a gentamycin resistance gene but it was transformable by PCR products (Bonifácio et al. 2021). Since *A. butzleri* has no *ctsM* homolog we propose that *C. jejuni* which cannot take up DNA that is unmethylated at RAATTY-site is unable to take up *A. butzleri* DNA. Efficient

transformation occurs in *C. jejuni* as well as in *H. pylori* or *A. butzleri* after incubation with DNA for 10 min or 30 min (Golz and Stingl 2021; Krüger et al. 2016; Bonifácio et al. 2021). Longer incubation times with DNA further increased natural transformation efficiency in *A. butzleri* (Bonifácio et al. 2021).

6.3 Curcumin plays neither a role in competence development nor in DNA uptake in *C. jejuni*

Competence development has been shown to be triggered by internal as well as external factors. For example, in *V. cholera* chitin induces competence (Meibom et al. 2005). Curcumin is part of the herb *Curcuma longa* and is used to make curry powder (Kocaadam and Sanlier 2017). It is known as a traditional medicine. Curcumin was tested for its effect to reduce epithelial barrier dysfunction caused by *C. jejuni* (Lobo de Sá et al. 2019). The pathogen mediates an immune response resulting in epithelial barrier dysfunction. This can result in redistribution of tight junction, apoptosis induction, and inflammatory response. In cell culture experiments it was shown that curcumin could reverse these effects. This means that curcumin could protect against barrier dysfunction and might be considered as a therapy option for campylobacteriosis or as a protective agent.

We used the single cell DNA uptake assay to test if curcumin plays a role in competence development (Lobo de Sá et al. 2019). In the presence of 50 µM curcumin during growth, which was the same concentration as used for eukaryotic cell culture experiments, we did not observe an alteration of competence development. Starting with competent cells we found out that the presence of curcumin during incubation with DNA had also no effect on the DNA uptake process. Therefore, we conclude that curcumin plays no role in natural transformation of *C. jejuni*.

6.4 *C. coli* / *C. jejuni* hybrid strains with impact on species differentiation

Detection of *mapA*, an outer membrane protein which plays a role in chicken colonization as well as *ceuE* which is important for iron acquisition are two targets for routine species differentiation according to Mayr and Best (Johnson et al. 2014; Best et al. 2003; Mayr et al. 2010). For the correct species differentiation primers have to be able to anneal to the target DNA. Primers were designed on the basis of the published typical sequences of *C. coli* and *C. jejuni* which differ from each other. If genetic exchange of the target DNA occurs an unambiguous species differentiation might not be possible. In total 37 isolates from the strain collection of the German National Reference Laboratory for *Campylobacter* showed ambiguous PCR results for species differentiation in *mapA* and *ceuE* (Golz et al. 2020). Using next-generation sequencing and subsequent k-mer analysis we identified *C. coli* strains with introgression of up to 15 % of the genome by *C. jejuni* sequences. A total of 21 strains showed

introgression of more than 10% and were named “hybrid” strains, 16 strains were introgressed by less than 10% and were called “half-hybrids”. Species differentiation according to Chaban which uses *cpn60* as a target failed in hybrid strains (Chaban et al. 2009). Also, MLST typing was impaired since introgression occurred in six out of seven housekeeping genes. An introgression in the housekeeping gene *aspA*, of two *C. coli* strains has previously been reported (Chan et al. 2008). This shows that typing methods applied in routine monitoring programs have their limits and an amendment of methods is required for a reliable species differentiation.

Interestingly, even though there were only few isolates from eggs in total this isolation source was dramatically overrepresented for isolation of hybrid strains. We propose that *Campylobacter* has to cope with different types of stress while drying on an egg shell. These could include osmotic stress, oxidative stress, starvation stress, cold stress. Generally, in feces *Campylobacter* loses its cultivability after 5-6 days (Ahmed et al. 2013; Bui et al. 2012). Interestingly, the hybrids shared a common set of genes with *C. jejuni* introgression. This included genes with functions in oxidative or nitrosative stress response like *katA*, Cj1386, *mrsB* (cj1112c), and *cgb* (Flint and Stintzi 2015; Atack and Kelly 2008; Elvers et al. 2004). Furthermore, genes with roles in general stress response were introgressed like *clpA*, *cpn10*, and *cpn60*. There is also a method for species differentiation which uses *cpn60* as a target (Chaban et al. 2009). So, an introgression in this gene might also lead to a wrong species differentiation. Additionally, genes with functions in DNA metabolism and repair, motility, cell wall and capsule biosynthesis showed *C. jejuni* introgressions. This might hint that the hybrid strains are well adapted to harsh environmental conditions.

Sheppard and colleagues observed that there is convergence between *C. coli* and *C. jejuni* (Sheppard et al. 2008). As long as both species share the same niche it could happen that they exchange genetic information. For *C. jejuni* we showed that competence development and accordingly natural transformation could occur at neutral to slightly alkaline pH and reduced oxygen levels at temperatures prevailing in mammals and avian hosts (Golz and Stingl 2021). Since DNA uptake of already competent cells still occurred under atmospheric oxygen levels genetic exchange could take place on an egg shell. It has been shown that *C. coli* belonging to ST-828 and ST-1150 which reside in the agricultural niche have incorporated substantial genetic material of *C. jejuni* (Sheppard et al. 2013). The *C. coli* core genome of ST-1150 showed introgression of up to 23%, ST-828 showed 10-11%. This shows that genetic exchange between *C. coli* and *C. jejuni* happens in nature.

For functional adaptations alterations in the amino acid chain are a way that protein structures change. Proteins of *C. jejuni* and *C. coli* differ on average in about 40 amino acids since the difference on nucleotide level is 15 % (Sheppard et al. 2013). An introgression covering 20%

of the gene is thought to result in changes of amino acids. This was confirmed by BLAST analysis showing that *C. coli* harbors *C. jejuni* proteins. Functional consequences of the changed proteins in hybrid strains remains to be elucidated.

6.5 Conclusions

Even though *Campylobacter* is a fastidious organism it is widely distributed and very successful in colonizing different host as well as in spreading in a flock (Natsos et al. 2020; Rawson et al. 2020). Recombination events for example by natural transformation seems to play a crucial role in the genetic diversity of the pathogen (Wilson et al. 2009). Factors triggering competence development are suggested to largely occur in the host where *Campylobacter* resides. This potentially offers the opportunity for extensive genetic exchange. The identification of *C. coli* / *C. jejuni* hybrid strains, which showed ambiguous results in routine species differentiation emphasizes that *Campylobacter* is able to undergo extensive genetic exchange which potentially enlarges the fitness of the pathogen and at the same time challenges current routine species differentiation techniques.

7 References

Ahmed MF, Schulz J, Hartung J (2013) Survival of *Campylobacter jejuni* in naturally and artificially contaminated laying hen feces. Poult Sci 92 (2):364-369. doi:10.3382/ps.2012-02496

Albano M, Hahn J, Dubnau D (1987) Expression of competence genes in *Bacillus subtilis*. J Bacteriol 169 (7):3110-3117. doi:10.1128/jb.169.7.3110-3117.1987

Assalkhou R, Balasingham S, Collins RF, Frye SA, Davidsen T, Benam AV, Bjoras M, Derrick JP, Tonjum T (2007) The outer membrane secretin PilQ from *Neisseria meningitidis* binds DNA. Microbiology 153 (Pt 5):1593-1603. doi:10.1099/mic.0.2006/004200-0

Atack JM, Kelly DJ (2008) Contribution of the stereospecific methionine sulphoxide reductases MsrA and MsrB to oxidative and nitrosative stress resistance in the food-borne pathogen *Campylobacter jejuni*. Microbiology (Reading) 154 (Pt 8):2219-2230. doi:10.1099/mic.0.2008/019711-0

Avery OT, MacLeod CM, McCarty M (1944) Studies on the chemical nature of the substance inducing transformation of Pneumococcal types : induction of transformation by a desoxyribonucleic acid fraction isolated from Pneumococcus type III. J Exp Med 79 (2):137-158. doi:10.1084/jem.79.2.137

Baffone W, Casaroli A, Citterio B, Pierfelici L, Campana R, Vittoria E, Guaglianone E, Donelli G (2006) *Campylobacter jejuni* loss of culturability in aqueous microcosms and ability to resuscitate in a mouse model. Int J Food Microbiol 107 (1):83-91. doi:10.1016/j.ijfoodmicro.2005.08.015

Beauchamp JM, Erfurt RS, DiRita VJ (2015) Characterization and localization of the *Campylobacter jejuni* transformation system proteins CtsE, CtsP, and CtsX. J Bacteriol 197 (3):636-645. doi:10.1128/JB.02434-14

Beauchamp JM, Leveque RM, Dawid S, DiRita VJ (2017) Methylation-dependent DNA discrimination in natural transformation of *Campylobacter jejuni*. Proc Natl Acad Sci U S A 114 (38):E8053-E8061. doi:10.1073/pnas.1703331114

Best EL, Powell EJ, Swift C, Grant KA, Frost JA (2003) Applicability of a rapid duplex real-time PCR assay for speciation of *Campylobacter jejuni* and *Campylobacter coli* directly from culture plates. FEMS Microbiology Letters 229 (2):237-241. doi:10.1016/s0378-1097(03)00845-0

Biswas GD, Sox T, Blackman E, Sparling PF (1977) Factors affecting genetic transformation of *Neisseria gonorrhoeae*. J Bacteriol 129 (2):983-992. doi:10.1128/JB.129.2.983-992.1977

Bonifácio M, Mateus C, Alves AR, Maldonado E, Duarte AP, Domingues F, Oleastro M, Ferreira S (2021) Natural transformation as a mechanism of horizontal gene transfer in *Aliarcobacter butzleri*. Pathogens 10 (7). doi:10.3390/pathogens10070909

Bui XT, Wolff A, Madsen M, Bang DD (2012) Reverse transcriptase real-time PCR for detection and quantification of viable *Campylobacter jejuni* directly from poultry faecal samples. Res Microbiol 163 (1):64-72. doi:10.1016/j.resmic.2011.10.007

Campoccia D, Montanaro L, Arciola CR (2021) Extracellular DNA (eDNA). A major ubiquitous element of the bacterial biofilm architecture. Int J Mol Sci 22 (16). doi:10.3390/ijms22169100

Chaban B, Musil KM, Himsworth CG, Hill JE (2009) Development of *cpn60*-based real-time quantitative PCR assays for the detection of 14 *Campylobacter* species and application to screening of canine fecal samples. Appl Environ Microbiol 75 (10):3055-3061. doi:10.1128/AEM.00101-09

Chaisowwong W, Kusumoto A, Hashimoto M, Harada T, Maklon K, Kawamoto K (2012) Physiological characterization of *Campylobacter jejuni* under cold stresses conditions: its potential for public threat. J Vet Med Sci 74 (1):43-50. doi:10.1292/jvms.11-0305

Chan K, Elhanafi D, Kathariou S (2008) Genomic evidence for interspecies acquisition of chromosomal DNA from *Campylobacter jejuni* by *Campylobacter coli* strains of a turkey-associated clonal group (cluster II). Foodborne Pathog Dis 5 (4):387-398. doi:10.1089/fpd.2008.0113

Claverys JP, Prudhomme M, Martin B (2006) Induction of competence regulons as a general response to stress in gram-positive bacteria. Annu Rev Microbiol 60:451-475. doi:10.1146/annurev.micro.60.080805.142139

Cody AJ, Bray JE, Jolley KA, McCarthy ND, Maiden MCJ (2017) Core genome multilocus sequence typing scheme for stable, comparative analyses of *Campylobacter jejuni* and *C. coli* human disease isolates. J Clin Microbiol 55 (7):2086-2097. doi:10.1128/JCM.00080-17

Corbinais C, Mathieu A, Damke PP, Kortulewski T, Busso D, Prado-Acosta M, Radicella JP, Marsin S (2017) ComB proteins expression levels determine *Helicobacter pylori* competence capacity. Sci Rep 7:41495. doi:10.1038/srep41495

Corry JEL, Atabay HI, Forsythe SJ, Mansfield LP (2003) Culture media for the isolation of *Campylobacters*, *Helicobacters* and *Arcobacters*. In: Corry JEL, Curtis GDW, Baird RM (eds) Progress in Industrial Microbiology, vol 37. Elsevier, pp 271-316. doi:https://doi.org/10.1016/S0079-6352(03)80021-8

Costa D, Iraola G (2019) Pathogenomics of emerging *Campylobacter* species. Clinical microbiology reviews 32 (4). doi:10.1128/cmr.00072-18

Danner DB, Deich RA, Sisco KL, Smith HO (1980) An eleven-base-pair sequence determines the specificity of DNA uptake in *Haemophilus* transformation. Gene 11 (3-4):311-318. doi:10.1016/0378-1119(80)90071-2

de Boer P, Wagenaar JA, Achterberg RP, van Putten JP, Schouls LM, Duim B (2002) Generation of *Campylobacter jejuni* genetic diversity in vivo. Mol Microbiol 44 (2):351-359. doi:10.1046/j.1365-2958.2002.02930.x

Debruyne L, Gevers D, Vandamme P (2008) Taxonomy of the family *Campylobacteraceae*. In: Campylobacter. pp 1-25. doi:https://doi.org/10.1128/9781555815554.ch1

Debruyne L, Gevers DV, Peter (2008) Taxonomy of the family *Campylobacteraceae*. In: *Campylobacter*. ASM Press, Washington DC, pp 1-25

Dingle KE, McCarthy ND, Cody AJ, Peto TE, Maiden MC (2008) Extended sequence typing of *Campylobacter* spp., United Kingdom. Emerg Infect Dis 14 (10):1620-1622. doi:10.3201/eid1410.071109

Dingle KE, Van Den Braak N, Colles FM, Price LJ, Woodward DL, Rodgers FG, Endtz HP, Van Belkum A, Maiden MC (2001) Sequence typing confirms that *Campylobacter jejuni* strains associated with Guillain-Barré and Miller-Fisher syndromes are of diverse genetic lineage, serotype, and flagella type. J Clin Microbiol 39 (9):3346-3349. doi:10.1128/JCM.39.9.3346-3349.2001

Drake SL, Koomey M (1995) The product of the *pilQ* gene is essential for the biogenesis of type IV pili in *Neisseria gonorrhoeae*. Mol Microbiol 18 (5):975-986. doi:10.1111/j.1365-2958.1995.18050975.x

Dubnau D, Blokesch M (2019) Mechanisms of DNA uptake by naturally competent bacteria. Annu Rev Genet 53:217-237. doi:10.1146/annurev-genet-112618-043641

EFSA (2019) The European Union One Health 2018 zoonoses report. EFSA J 17 (12):e05926. doi:10.2903/j.efsa.2019.5926

EFSA (2021a) The European Union One Health 2019 zoonoses report. EFSA Journal 19 (2):e06406

EFSA (2021b) The European Union summary report on antimicrobial resistance in zoonotic and indicator bacteria from humans, animals and food in 2018/2019. EFSA Journal 19 (4)

Elkins C, Thomas CE, Seifert HS, Sparling PF (1991) Species-specific uptake of DNA by gonococci is mediated by a 10-base-pair sequence. J Bacteriol 173 (12):3911-3913. doi:10.1128/jb.173.12.3911-3913.1991

Ellison CK, Dalia TN, Vidal Ceballos A, Wang JC, Biais N, Brun YV, Dalia AB (2018) Retraction of DNA-bound type IV competence pili initiates DNA uptake during natural transformation in *Vibrio cholerae*. Nat Microbiol 3 (7):773-780. doi:10.1038/s41564-018-0174-y

Elvers KT, Wu G, Gilberthorpe NJ, Poole RK, Park SF (2004) Role of an inducible single-domain hemoglobin in mediating resistance to nitric oxide and nitrosative stress in

Campylobacter jejuni and *Campylobacter coli*. J Bacteriol 186 (16):5332-5341. doi:10.1128/JB.186.16.5332-5341.2004

Epping L, Antao EM, Semmler T (2021) Population biology and comparative genomics of *Campylobacter* species. Curr Top Microbiol Immunol 431:59-78. doi:10.1007/978-3-030-65481-8_3

Epping L, Golz JC, Knüver MT, Huber C, Thürmer A, Wieler LH, Stingl K, Semmler T (2019) Comparison of different technologies for the decipherment of the whole genome sequence of *Campylobacter jejuni* BfR-CA-14430. Gut Pathog 11:59. doi:10.1186/s13099-019-0340-7

Euzéby JP (1997) List of bacterial names with standing in nomenclature: a folder available on the internet. Int J Syst Bacteriol 47 (2):590-592. doi:10.1099/00207713-47-2-590

Facciolà A, Riso R, Avventuroso E, Visalli G, Delia SA, Laganà P (2017) *Campylobacter*: from microbiology to prevention. Journal of preventive medicine and hygiene 58 (2):E79-e92

Feng J, Ma L, Nie J, Konkel ME, Lu X (2018) Environmental stress-induced bacterial lysis and extracellular DNA release contribute to *Campylobacter jejuni* biofilm formation. Appl Environ Microbiol 84 (5). doi:10.1128/AEM.02068-17

Finkel SE, Kolter R (2001) DNA as a nutrient: novel role for bacterial competence gene homologs. J Bacteriol 183 (21):6288-6293. doi:10.1128/JB.183.21.6288-6293.2001

Flint A, Stintzi A (2015) Cj1386, an atypical hemin-binding protein, mediates hemin trafficking to KatA in *Campylobacter jejuni*. J Bacteriol 197 (5):1002-1011. doi:10.1128/JB.02346-14

Frirdich E, Biboy J, Pryjma M, Lee J, Huynh S, Parker CT, Girardin SE, Vollmer W, Gaynor EC (2019) The *Campylobacter jejuni* helical to coccoid transition involves changes to peptidoglycan and the ability to elicit an immune response. Mol Microbiol 112 (1):280-301. doi:10.1111/mmi.14269

Gamba P, Jonker MJ, Hamoen LW (2015) A novel feedback loop that controls bimodal expression of genetic competence. PLoS Genet 11 (6):e1005047. doi:10.1371/journal.pgen.1005047

Gangel H, Hepp C, Müller S, Oldewurtel ER, Aas FE, Koomey M, Maier B (2014) Concerted spatio-temporal dynamics of imported DNA and ComE DNA uptake protein during gonococcal transformation. PLoS Pathog 10 (4):e1004043. doi:10.1371/journal.ppat.1004043

Garrity GM, Bell JA, Lilburn T (2005) Class V. Epsilonproteobacteria class. nov. In: Bergey's Manual® of Systematic Bacteriology. Springer, pp 1145-1194. doi:10.1007/978-0-387-29298-4_4

Golz JC, Epping L, Knüver MT, Borowiak M, Hartkopf F, Deneke C, Malorny B, Semmler T, Stingl K (2020) Whole genome sequencing reveals extended natural transformation in *Campylobacter* impacting diagnostics and the pathogens adaptive potential. Sci Rep 10 (1):3686. doi:10.1038/s41598-020-60320-y

Golz JC, Stingl K (2021) "Take it or leave it"-Factors regulating competence development and DNA uptake in *Campylobacter jejuni*. Int J Mol Sci 22 (18). doi:10.3390/ijms221810169

Goodman SD, Scocca JJ (1988) Identification and arrangement of the DNA sequence recognized in specific transformation of *Neisseria gonorrhoeae*. Proc Natl Acad Sci U S A 85 (18):6982-6986. doi:10.1073/pnas.85.18.6982

Goodwin CS, Armstrong JA, Chilvers T, Peters M, Collins MD, Sly L, McConnell W, Harper WES (1989) Transfer of *Campylobacter pylori* and *Campylobacter mustelae* to *Helicobacter* gen. nov. as *Helicobacter pylori* comb. nov. and *Helicobacter mustelae* comb. nov., respectively. International Journal of Systematic and Evolutionary Microbiology 39 (4):397-405

Griffith F (1928) The significance of pneumococcal types. J Hyg (Lond) 27 (2):113-159. doi:10.1017/s0022172400031879

Griggs DJ, Peake L, Johnson MM, Ghori S, Mott A, Piddock LJ (2009) Beta-lactamase-mediated beta-lactam resistance in *Campylobacter* species: prevalence of Cj0299 (bla OXA-61) and evidence for a novel beta-lactamase in *C. jejuni*. Antimicrobial agents and chemotherapy 53 (8):3357-3364. doi:10.1128/AAC.01655-08

Hahn J, DeSantis M, Dubnau D (2021) Mechanisms of transforming DNA uptake to the periplasm of *Bacillus subtilis*. mBio 12 (3):e0106121. doi:10.1128/mBio.01061-21

Hamilton HL, Dominguez NM, Schwartz KJ, Hackett KT, Dillard JP (2005) *Neisseria gonorrhoeae* secretes chromosomal DNA via a novel type IV secretion system. Mol Microbiol 55 (6):1704-1721. doi:10.1111/j.1365-2958.2005.04521.x

Hamoen LW, Venema G, Kuipers OP (2003) Controlling competence in *Bacillus subtilis*: shared use of regulators. Microbiology 149 (Pt 1):9-17. doi:10.1099/mic.0.26003-0

Hannu T, Mattila L, Rautelin H, Pelkonen P, Lahdenne P, Siitonen A, Leirisalo-Repo M (2002) *Campylobacter*-triggered reactive arthritis: a population-based study. Rheumatology (Oxford) 41 (3):312-318. doi:10.1093/rheumatology/41.3.312

Hepp C, Maier B (2016) Kinetics of DNA uptake during transformation provide evidence for a translocation ratchet mechanism. Proc Natl Acad Sci U S A 113 (44):12467-12472. doi:10.1073/pnas.1608110113

Herriott RM, Meyer EY, Vogt M, Modan M (1970) Defined medium for growth of *Haemophilus influenzae*. J Bacteriol 101 (2):513-516. doi:10.1128/jb.101.2.513-516.1970

Hofreuter D, Odenbreit S, Haas R (2001) Natural transformation competence in *Helicobacter pylori* is mediated by the basic components of a type IV secretion system. Mol Microbiol 41 (2):379-391. doi:10.1046/j.1365-2958.2001.02502.x

Hull DM, Harrell E, van Vliet AHM, Correa M, Thakur S (2021) Antimicrobial resistance and interspecies gene transfer in *Campylobacter coli* and *Campylobacter jejuni* isolated from food animals, poultry processing, and retail meat in North Carolina, 2018-2019. PLoS One 16 (2):e0246571. doi:10.1371/journal.pone.0246571

Igwaran A, Okoh AI (2019) Human campylobacteriosis: A public health concern of global importance. Heliyon 5 (11):e02814. doi:10.1016/j.heliyon.2019.e02814

ISO_10272-2:2017 (2019) Microbiology of the food chain-horizontal method for detection and enumeration of *Campylobacter* spp.-part 2: colony-count technique. International journal of food microbiology 288:32-38

Jeon B, Muraoka W, Sahin O, Zhang Q (2008) Role of Cj1211 in natural transformation and transfer of antibiotic resistance determinants in *Campylobacter jejuni*. Antimicrobial agents and chemotherapy 52 (8):2699-2708. doi:10.1128/aac.01607-07

Jeon B, Zhang Q (2007) Cj0011c, a periplasmic single- and double-stranded DNA-binding protein, contributes to natural transformation in *Campylobacter jejuni*. J Bacteriol 189 (20):7399-7407. doi:10.1128/jb.01012-07

Johnson JG, Livny J, Dirita VJ (2014) High-throughput sequencing of *Campylobacter jejuni* insertion mutant libraries reveals *mapA* as a fitness factor for chicken colonization. J Bacteriol 196 (11):1958-1967. doi:10.1128/JB.01395-13

Johnston C, Martin B, Fichant G, Polard P, Claverys JP (2014) Bacterial transformation: distribution, shared mechanisms and divergent control. Nat Rev Microbiol 12 (3):181-196. doi:10.1038/nrmicro3199

Johnston C, Martin B, Granadel C, Polard P, Claverys JP (2013) Programmed protection of foreign DNA from restriction allows pathogenicity island exchange during pneumococcal transformation. PLoS Pathog 9 (2):e1003178. doi:10.1371/journal.ppat.1003178

Kim JS, Kim JW, Kathariou S (2008) Differential effects of temperature on natural transformation to erythromycin and nalidixic acid resistance in *Campylobacter coli*. Appl Environ Microbiol 74 (19):6121-6125. doi:10.1128/aem.01075-08

Kim SH, Chelliah R, Ramakrishnan SR, Perumal AS, Bang WS, Rubab M, Daliri EB, Barathikannan K, Elahi F, Park E, Jo HY, Hwang SB, Oh DH (2020) Review on Stress Tolerance in *Campylobacter jejuni*. Front Cell Infect Microbiol 10:596570. doi:10.3389/fcimb.2020.596570

Kocaadam B, Sanlier N (2017) Curcumin, an active component of turmeric (*Curcuma longa*), and its effects on health. Crit Rev Food Sci Nutr 57 (13):2889-2895. doi:10.1080/10408398.2015.1077195

Krüger NJ, Knüver MT, Zawilak-Pawlik A, Appel B, Stingl K (2016) Genetic diversity as consequence of a microaerobic and neutrophilic lifestyle. PLoS Pathog 12 (5):e1005626. doi:10.1371/journal.ppat.1005626

Leekitcharoenphon P, Garcia-Graells C, Botteldoorn N, Dierick K, Kempf I, Olkkola S, Rossi M, Nykäsenoja S, Malorny B, Stingl K (2018) Comparative genomics of quinolone-resistant and susceptible *Campylobacter jejuni* of poultry origin from major poultry producing European countries (GENCAMP). EFSA Supporting Publications 15 (5):1398E

Llarena AK, Ribeiro-Gonçalves BF, Nuno Silva D, Halkilahti J, Machado MP, Da Silva MS, Jaakkonen A, Isidro J, Hämäläinen C, Joenperä J (2018) INNUENDO: a cross-sectoral platform for the integration of genomics in the surveillance of food-borne pathogens. EFSA Supporting Publications 15 (11):1498E

Lobo de Sá FD, Butkevych E, Nattramilarasu PK, Fromm A, Mousavi S, Moos V, Golz JC, Stingl K, Kittler S, Seinige D, Kehrenberg C, Heimesaat MM, Bereswill S, Schulzke JD, Bücker R (2019) Curcumin mitigates immune-induced epithelial barrier dysfunction by *Campylobacter jejuni*. Int J Mol Sci 20 (19). doi:10.3390/ijms20194830

Lv R, Wang K, Feng J, Heeney DD, Liu D, Lu X (2019) Detection and quantification of viable but non-culturable *Campylobacter jejuni*. Front Microbiol 10:2920. doi:10.3389/fmicb.2019.02920

Macé S, Haddad N, Zagorec M, Tresse O (2015) Influence of measurement and control of microaerobic gaseous atmospheres in methods for *Campylobacter* growth studies. Food Microbiol 52:169-176. doi:10.1016/j.fm.2015.07.014

Magajna B, Schraft H (2015) Evaluation of propidium monoazide and quantitative PCR to quantify viable *Campylobacter jejuni* biofilm and planktonic cells in log phase and in a viable but nonculturable state. J Food Prot 78 (7):1303-1311. doi:10.4315/0362-028X.JFP-14-583

Matthey N, Blokesch M (2016) The DNA-Uptake process of naturally competent *Vibrio cholerae*. Trends Microbiol 24 (2):98-110. doi:10.1016/j.tim.2015.10.008

Mayr AM, Lick S, Bauer J, Thärigen D, Busch U, Huber I (2010) Rapid detection and differentiation of *Campylobacter jejuni*, *Campylobacter coli*, and *Campylobacter lari* in food, using multiplex real-time PCR. J Food Prot 73 (2):241-250. doi:10.4315/0362-028x-73.2.241

Mazurier S, van de Giessen A, Heuvelman K, Wernars K (1992) RAPD analysis of *Campylobacter* isolates: DNA fingerprinting without the need to purify DNA. Lett Appl Microbiol 14 (6):260-262. doi:10.1111/j.1472-765x.1992.tb00700.x

McCarthy N, Giesecke J (2001) Incidence of Guillain-Barré syndrome following infection with *Campylobacter jejuni*. Am J Epidemiol 153 (6):610-614. doi:10.1093/aje/153.6.610

Meibom KL, Blokesch M, Dolganov NA, Wu CY, Schoolnik GK (2005) Chitin induces natural competence in *Vibrio cholerae*. Science 310 (5755):1824-1827. doi:10.1126/science.1120096

Meinersmann R, Helsel L, Fields P, Hiett K (1997) Discrimination of *Campylobacter jejuni* isolates by fla gene sequencing. Journal of Clinical Microbiology 35 (11):2810-2814

Mell JC, Hall IM, Redfield RJ (2012) Defining the DNA uptake specificity of naturally competent *Haemophilus influenzae* cells. Nucleic Acids Res 40 (17):8536-8549. doi:10.1093/nar/gks640

Mughini Gras L, Smid JH, Wagenaar JA, de Boer AG, Havelaar AH, Friesema IH, French NP, Busani L, van Pelt W (2012) Risk factors for campylobacteriosis of chicken, ruminant, and environmental origin: a combined case-control and source attribution analysis. PLoS One 7 (8):e42599. doi:10.1371/journal.pone.0042599

Natsos G, Mouttotou NK, Magiorkinis E, Ioannidis A, Rodi-Burriel A, Chatzipanagiotou S, Koutoulis KC (2020) Prevalence of and risk factors for *Campylobacter* spp. colonization of broiler chicken flocks in Greece. Foodborne Pathog Dis 17 (11):679-686. doi:10.1089/fpd.2020.2795

Nennig M, Llarena AK, Herold M, Mossong J, Penny C, Losch S, Tresse O, Ragimbeau C (2020) Investigating major recurring *Campylobacter jejuni* lineages in Luxembourg using four core or whole genome sequencing typing schemes. Front Cell Infect Microbiol 10:608020. doi:10.3389/fcimb.2020.608020

Obergfell KP, Seifert HS (2016) The pilin N-terminal domain maintains *Neisseria gonorrhoeae* transformation competence during pilus phase variation. PLoS Genet 12 (5):e1006069. doi:10.1371/journal.pgen.1006069

Oh E, McMullen L, Jeon B (2015) Impact of oxidative stress defense on bacterial survival and morphological change in *Campylobacter jejuni* under aerobic conditions. Front Microbiol 6:295. doi:10.3389/fmicb.2015.00295

Parker CT, Quinones B, Miller WG, Horn ST, Mandrell RE (2006) Comparative genomic analysis of *Campylobacter jejuni* strains reveals diversity due to genomic elements similar to those present in *C. jejuni* strain RM1221. J Clin Microbiol 44 (11):4125-4135. doi:10.1128/JCM.01231-06

Parkhill J, Wren BW, Mungall K, Ketley JM, Churcher C, Basham D, Chillingworth T, Davies RM, Feltwell T, Holroyd S, Jagels K, Karlyshev AV, Moule S, Pallen MJ, Penn CW, Quail MA, Rajandream MA, Rutherford KM, van Vliet AH, Whitehead S, Barrell BG (2000) The genome sequence of the food-borne pathogen *Campylobacter jejuni* reveals hypervariable sequences. Nature 403 (6770):665-668. doi:10.1038/35001088

Parte AC, Sardà Carbasse J, Meier-Kolthoff JP, Reimer LC, Göker M (2020) List of prokaryotic names with standing in nomenclature (LPSN) moves to the DSMZ. Int J Syst Evol Microbiol 70 (11):5607-5612. doi:10.1099/ijsem.0.004332

Penner J, Hennessy J, Congi R (1983) Serotyping of *Campylobacter jejuni* and *Campylobacter coli* on the basis of thermostable antigens. European journal of clinical microbiology 2 (4):378-383

Pope JE, Krizova A, Garg AX, Thiessen-Philbrook H, Ouimet JM (2007) *Campylobacter* reactive arthritis: a systematic review. Semin Arthritis Rheum 37 (1):48-55. doi:10.1016/j.semarthrit.2006.12.006

Rawson T, Paton RS, Colles FM, Maiden MCJ, Dawkins MS, Bonsall MB (2020) A mathematical modeling approach to uncover factors influencing the spread of *Campylobacter* in a flock of broiler-breeder chickens. Front Microbiol 11:576646. doi:10.3389/fmicb.2020.576646

Reuter M, Periago PM, Mulholland F, Brown HL, van Vliet AH (2015) A PAS domain-containing regulator controls flagella-flagella interactions in *Campylobacter jejuni*. Front Microbiol 6:770. doi:10.3389/fmicb.2015.00770

Rosner BM, Gassowski M, Albrecht S, Stark K (2021) Investigating the *Campylobacter* enteritis winter peak in Germany, 2018/2019. Sci Rep 11 (1):22902. doi:10.1038/s41598-021-02423-8

Rosner BM, Schielke A, Didelot X, Kops F, Breidenbach J, Willrich N, Golz G, Alter T, Stingl K, Josenhans C, Suerbaum S, Stark K (2017) A combined case-control and molecular source attribution study of human *Campylobacter* infections in Germany, 2011-2014. Sci Rep 7 (1):5139. doi:10.1038/s41598-017-05227-x

Rossi M, Silva M, Ribeiro-Gonçalves B, Silva D, Machado M, Oleastro M (2018) INNUENDO whole genome and core genome MLST schemas and datasets for *Campylobacter jejuni*. Zenodo doi 10

Sebald M, Veron M (1963) [Base DNA content and classification of *Vibrios*]. Ann Inst Pasteur (Paris) 105:897-910

Seitz P, Blokesch M (2013) DNA-uptake machinery of naturally competent *Vibrio cholerae*. Proc Natl Acad Sci U S A 110 (44):17987-17992. doi:10.1073/pnas.1315647110

Sheppard SK, Colles FM, McCarthy ND, Strachan NJ, Ogden ID, Forbes KJ, Dallas JF, Maiden MC (2011) Niche segregation and genetic structure of *Campylobacter jejuni* populations from wild and agricultural host species. Mol Ecol 20 (16):3484-3490. doi:10.1111/j.1365-294X.2011.05179.x

Sheppard SK, Didelot X, Jolley KA, Darling AE, Pascoe B, Meric G, Kelly DJ, Cody A, Colles FM, Strachan NJ, Ogden ID, Forbes K, French NP, Carter P, Miller WG, McCarthy ND, Owen R, Litrup E, Egholm M, Affourtit JP, Bentley SD, Parkhill J, Maiden MC, Falush D (2013) Progressive genome-wide introgression in agricultural *Campylobacter coli*. Mol Ecol 22 (4):1051-1064. doi:10.1111/mec.12162

Sheppard SK, McCarthy ND, Falush D, Maiden MC (2008) Convergence of *Campylobacter* species: implications for bacterial evolution. Science 320 (5873):237-239. doi:10.1126/science.1155532

Skarp-de Haan CP, Culebro A, Schott T, Revez J, Schweda EK, Hänninen ML, Rossi M (2014) Comparative genomics of unintrogressed *Campylobacter coli* clades 2 and 3. BMC Genomics 15:129. doi:10.1186/1471-2164-15-129

Sparling PF (1966) Genetic transformation of *Neisseria gonorrhoeae* to streptomycin resistance. J Bacteriol 92 (5):1364-1371. doi:10.1128/jb.92.5.1364-1371.1966

Stingl K, Müller S, Scheidgen-Kleyboldt G, Clausen M, Maier B (2010) Composite system mediates two-step DNA uptake into *Helicobacter pylori*. Proc Natl Acad Sci U S A 107 (3):1184-1189. doi:10.1073/pnas.0909955107

Svensson SL, Pryjma M, Gaynor EC (2014) Flagella-mediated adhesion and extracellular DNA release contribute to biofilm formation and stress tolerance of *Campylobacter jejuni*. PLoS One 9 (8):e106063. doi:10.1371/journal.pone.0106063

Svihus B (2011) Effect of digestive tract conditions, feed processing and ingredients on response to NSP enzymes. In: Bedford M, Partridge G (eds) Enzymes in farm animal nutrition, vol 2. 2nd edn. CAB International, UK, Wallingford, pp 129-159

Ternhag A, Törner A, Svensson A, Giesecke J, Ekdahl K (2005) Mortality following *Campylobacter* infection: a registry-based linkage study. BMC Infect Dis 5:70. doi:10.1186/1471-2334-5-70

Vandamme P, On SL (2001) Recommendations of the subcommittee on the taxonomy of *Campylobacter* and related bacteria. Int J Syst Evol Microbiol 51 (Pt 2):719-721. doi:10.1099/00207713-51-2-719

Vegge CS, Brondsted L, Ligowska-Marzeta M, Ingmer H (2012) Natural transformation of *Campylobacter jejuni* occurs beyond limits of growth. PLoS One 7 (9):e45467. doi:10.1371/journal.pone.0045467

Waldenström J, Axelsson-Olsson D, Olsen B, Hasselquist D, Griekspoor P, Jansson L, Teneberg S, Svensson L, Ellström P (2010) *Campylobacter jejuni* colonization in wild birds: results from an infection experiment. PLoS One 5 (2):e9082. doi:10.1371/journal.pone.0009082

Wang Y, Taylor DE (1990) Natural transformation in *Campylobacter* species. J Bacteriol 172 (2):949-955. doi:10.1128/jb.172.2.949-955.1990

Wholey WY, Kochan TJ, Storck DN, Dawid S (2016) Coordinated bacteriocin expression and competence in *Streptococcus pneumoniae* contributes to genetic adaptation through neighbor predation. PLoS Pathog 12 (2):e1005413. doi:10.1371/journal.ppat.1005413

Wiesner RS, Hendrixson DR, DiRita VJ (2003) Natural transformation of *Campylobacter jejuni* requires components of a type II secretion system. J Bacteriol 185 (18):5408-5418. doi:10.1128/jb.185.18.5408-5418.2003

Wijdicks EF, Klein CJ (2017) Guillain-Barré syndrome. Mayo Clin Proc 92 (3):467-479. doi:10.1016/j.mayocp.2016.12.002

Wilson DJ, Gabriel E, Leatherbarrow AJ, Cheesbrough J, Gee S, Bolton E, Fox A, Fearnhead P, Hart CA, Diggle PJ (2008) Tracing the source of campylobacteriosis. PLoS Genet 4 (9):e1000203. doi:10.1371/journal.pgen.1000203

Wilson DJ, Gabriel E, Leatherbarrow AJ, Cheesbrough J, Gee S, Bolton E, Fox A, Hart CA, Diggle PJ, Fearnhead P (2009) Rapid evolution and the importance of recombination to the gastroenteric pathogen *Campylobacter jejuni*. Mol Biol Evol 26 (2):385-397. doi:10.1093/molbev/msn264

Wilson DL, Bell JA, Young VB, Wilder SR, Mansfield LS, Linz JE (2003) Variation of the natural transformation frequency of *Campylobacter jejuni* in liquid shake culture. Microbiology 149 (Pt 12):3603-3615. doi:10.1099/mic.0.26531-0

Wulsten IF, Galeev A, Stingl K (2020) Underestimated survival of *Campylobacter* in raw milk highlighted by viability real-time PCR and growth recovery. Front Microbiol 11:1107. doi:10.3389/fmicb.2020.01107

Yan W, Chang N, Taylor DE (1991) Pulsed-field gel electrophoresis of *Campylobacter jejuni* and *Campylobacter coli* genomic DNA and its epidemiologic application. J Infect Dis 163 (5):1068-1072. doi:10.1093/infdis/163.5.1068

Yuki N, Taki T, Inagaki F, Kasama T, Takahashi M, Saito K, Handa S, Miyatake T (1993) A bacterium lipopolysaccharide that elicits Guillain-Barré syndrome has a GM1 ganglioside-like structure. J Exp Med 178 (5):1771-1775. doi:10.1084/jem.178.5.1771

Zautner AE, Goldschmidt AM, Thurmer A, Schuldes J, Bader O, Lugert R, Gross U, Stingl K, Salinas G, Lingner T (2015) SMRT sequencing of the *Campylobacter coli* BfR-CA-9557 genome sequence reveals unique methylation motifs. BMC Genomics 16:1088. doi:10.1186/s12864-015-2317-3

Zhang XS, Blaser MJ (2012) Natural transformation of an engineered *Helicobacter pylori* strain deficient in type II restriction endonucleases. J Bacteriol 194 (13):3407-3416. doi:10.1128/JB.00113-12

8 Appendix

8.1 List of Abbreviations

cgMLST	core genome MLST
cts	*Campylobacter* transformation system
dsDNA	double-stranded DNA
MLST	multilocus sequence typing
SMRT	single molecule real-time
ssDNA	single-stranded DNA
VBNC	viable but non-culturable
wgMLST	whole genome MLST

8.2 Supplementary material of own publications

8.2.1 Publication 1: Curcumin mitigates immune-induced epithelial barrier dysfunction by *Campylobacter jejuni*.

Appendix A

Acute Campylobacteriosis Contains Activated Signaling Pathways for which Curcumin Has Counter-Regulatory Properties in IPA Analysis

From acute infected *C. jejuni* patients a RNA-Sequencing (RNA-Seq) analysis with concomitant ingenuity pathway analysis (IPA, Qiagen Silicon Valley) was performed. Once gene expression modifications were revealed, a bioinformatic prediction about possible inhibitors of the changed gene regulation could be carried out [6]. The hypothesis is, that upstream regulators, which have an inhibited activation pattern, may re-activate in *Campylobacter* infection, when the substance is applied during infection. Consequently, inhibited upstream regulators could be protective or therapeutic approaches in *Campylobacter* infection by activation of the corresponding downstream pathways. Different potential candidates were screened for barrier-protective and anti-inflammatory properties in *C. jejuni* infection. One promising predicted regulator candidate that might counter-regulate the *C. jejuni*-induced downstream pathways was curcumin. Curcumin showed a significant effect on downstream target genes, with a *p*-value of $2.06E^{-5}$ and an activation *z*-score of -3.489, and might therefore be another promising barrier-protecting or potential therapeutic substance in campylobacteriosis (Table S1). The *C. jejuni*-induced target genes in the dataset that could be counter-regulated by curcumin belong mainly to pro-inflammatory pathways, such as TNF-α or IL-1β. Another promising and studied candidate against *C. jejuni* infections is calcitriol (active vitamin D). Vitamin D shows in the RNA-Seq analysis with concomitant IPA analysis from patients in contrast to curcumin an even higher significance value (overlap *p*-value of $8.97E^{-25}$, z-score -6.25; with negative expression direction) [6].

Table S1. Curcumin is an upstream regulator in *C. jejuni*-infected human mucosa identified by IPA

Upstream Regulator	Predicted Activation	Activation Z-Score	*p*-Value of Overlap	Target Molecules in Dataset
curcumin	inhibited	-3.489	$2.06E^{-5}$	ABCB1,ABCC1,ABCG1,ADAMTS4,ADIPOQ,APOE,ATOX1,AXIN1,BIRC3,BIRC5,CCNB1,CD44,CD80,CD86,CDK1,CDK4,CR1,CRP,CSNK1A1,CTGF,CXCL1,CXCL3,CXCL8,CXCR3,CXCR4,CYP2E1,CYP3A4,DDIT3,EDN1,EGFR,EIF3H,ERBB2,ETS1,FOS,FTL,GCLM,GFAP,GRIN2B,GRK6,HIF1A,HSP90B1,HSPA8,ICAM1,IFNG,IL17A,IL1B,IL6,JUN,LPL,LSP1,MMP1,MMP14,MMP3,MMP9,MTHFD1,NAMPT,NOS2,OLR1,PLAU,PPARGC1A,PRAP1,PRPS2,SELE,SERPINE1,SOCS1,SOCS3,SOD1,STAT3,TFAM,TFRC,TLR2,TLR4,TNF,TOP1,TOP2A,UBE2E2,UCP2,VEGFA,ZMYND8

1

ubcellular Tight Junction Protein Distribution in Co-Cultures in Confocal Laser-Scanning Microscopy

Curcumin alone showed no influence on the subcellular tight junction distribution of claudin-4 and claudin-8 in comparison to the untreated control (Figure S2).

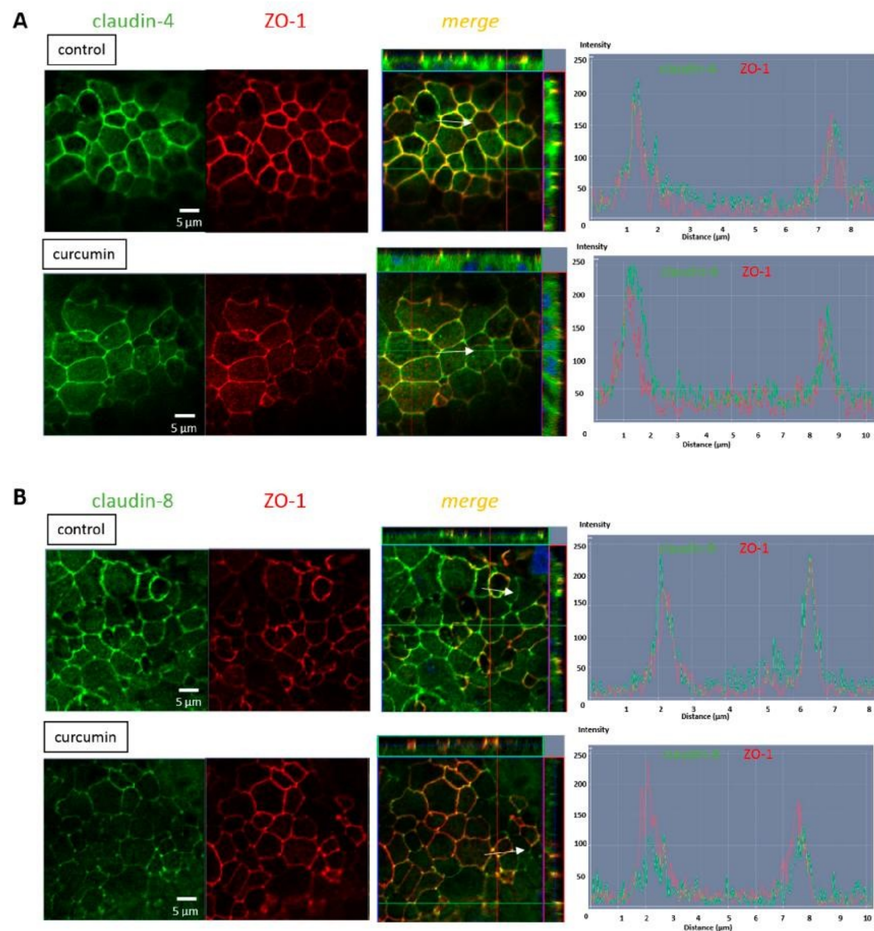


Figure S2. Tight junction distribution in control conditions after treatment without or with 50 µM curcumin. Representative confocal laser-scanning microscopy pictures of HT-29/B6-GR/MR after co-culturing together with immune cells. (**A**) Claudin-4 (green) and zonula occludens protein-1 (ZO-1, red), and (**B**) claudin-8 (green) and ZO-1 (red). Nuclei are stained in blue with 4´-6-diamidino-2-phenylindole dihydrochloride (DAPI).

2

8.2.2 Publication 2: Comparison of different technologies for the decipherment of the whole genome sequence of *Campylobacter jejuni* BfR-CA-14430.

Table S1: Campylobacter jejuni Genomes used to build the Prokka Database

Genbank Accsession	Assembly Accession
GCA_000009085.1	ASM908v1
GCA_000011865.1	ASM1186v1
GCA_000015525.1	ASM1552v1
GCA_000017485.1	ASM1748v1
GCA_000017905.1	ASM1790v1
GCA_000025425.1	ASM2542v1
GCA_000148705.1	ASM14870v1
GCA_000171795.2	ASM17179v2
GCA_000184085.1	ASM18408v1
GCA_000184205.1	ASM18420v1
GCA_000302555.4	ASM30255v4
GCA_000304375.1	AINO
GCA_000430385.1	ASM43038v1
GCA_000466065.2	ASM46606v2
GCA_000466075.2	ASM46607v2
GCA_000466105.2	ASM46610v2
GCA_000468915.2	ASM46891v2
GCA_000493495.1	TS
GCA_000632435.1	ASM63243v1
GCA_000737085.1	ASM73708v1
GCA_000772225.1	ASM77222v1
GCA_000807355.1	ASM80735v1
GCA_000830775.1	ASM83077v1
GCA_000830805.1	ASM83080v1
GCA_000830825.1	ASM83082v1
GCA_000830845.1	ASM83084v1
GCA_000830865.1	ASM83086v1
GCA_000835285.1	ASM83528v1
GCA_000835305.1	ASM83530v1
GCA_000835345.1	ASM83534v1
GCA_000835365.1	ASM83536v1
GCA_000934305.1	ASM93430v1
GCA_001299565.1	ASM129956v1
GCA_001299595.1	ASM129959v1
GCA_001314285.1	ASM131428v1
GCA_001412295.1	ASM141229v1
GCA_001457695.1	NCTC11351
GCA_001506185.1	ASM150618v1
GCA_001506205.1	ASM150620v1
GCA_001506225.1	ASM150622v1
GCA_001506245.1	ASM150624v1
GCA_001506265.1	ASM150626v1
GCA_001506285.1	ASM150628v1
GCA_001506305.1	ASM150630v1
GCA_001506325.1	ASM150632v1
GCA_001506345.1	ASM150634v1
GCA_001506365.1	ASM150636v1
GCA_001506385.1	ASM150638v1

GCA_001506405.1	ASM150640v1
GCA_001506425.1	ASM150642v1
GCA_001506445.1	ASM150644v1
GCA_001506465.1	ASM150646v1
GCA_001506485.1	ASM150648v1
GCA_001506505.1	ASM150650v1
GCA_001506525.1	ASM150652v1
GCA_001506545.1	ASM150654v1
GCA_001506565.1	ASM150656v1
GCA_001506585.1	ASM150658v1
GCA_001506605.1	ASM150660v1
GCA_001506625.1	ASM150662v1
GCA_001506645.1	ASM150664v1
GCA_001506665.1	ASM150666v1
GCA_001506685.1	ASM150668v1
GCA_001506705.1	ASM150670v1
GCA_001506725.1	ASM150672v1
GCA_001506745.1	ASM150674v1
GCA_001506765.1	ASM150676v1
GCA_001506785.1	ASM150678v1
GCA_001506805.1	ASM150680v1
GCA_001506825.1	ASM150682v1
GCA_001506845.1	ASM150684v1
GCA_001506865.1	ASM150686v1
GCA_001506885.1	ASM150688v1
GCA_001506905.1	ASM150690v1
GCA_001506925.1	ASM150692v1
GCA_001506945.1	ASM150694v1
GCA_001506965.1	ASM150696v1
GCA_001506985.1	ASM150698v1
GCA_001507005.1	ASM150700v1
GCA_001507025.1	ASM150702v1
GCA_001507045.1	ASM150704v1
GCA_001507065.1	ASM150706v1
GCA_001507085.1	ASM150708v1
GCA_001507105.1	ASM150710v1
GCA_001507125.1	ASM150712v1
GCA_001507145.1	ASM150714v1
GCA_001507165.1	ASM150716v1
GCA_001507185.1	ASM150718v1
GCA_001507205.1	ASM150720v1
GCA_001507225.1	ASM150722v1
GCA_001507245.1	ASM150724v1
GCA_001507265.1	ASM150726v1
GCA_001563565.1	ASM156356v1
GCA_001587015.1	ASM158701v1
GCA_001587035.1	ASM158703v1
GCA_001686905.1	ASM168690v1
GCA_001717625.1	ASM171762v1
GCA_001721945.1	ASM172194v1

GCA_001721965.1	ASM172196v1
GCA_001721985.1	ASM172198v1
GCA_001767215.1	ASM176721v1
GCA_001865395.1	ASM186539v1
GCA_001865415.1	ASM186541v1
GCA_001865435.1	ASM186543v1
GCA_001865595.1	ASM186559v1
GCA_001865615.1	ASM186561v1
GCA_001870085.1	ASM187008v1
GCA_001870105.1	ASM187010v1
GCA_001951235.1	ASM195123v1
GCA_001951255.1	ASM195125v1
GCA_001951275.1	ASM195127v1
GCA_001951295.1	ASM195129v1
GCA_001951315.1	ASM195131v1
GCA_001951335.1	ASM195133v1
GCA_002024325.1	ASM202432v1
GCA_002028305.1	ASM202830v1
GCA_002101355.1	ASM210135v1
GCA_002209005.1	ASM220900v1
GCA_002209025.1	ASM220902v1
GCA_002209045.1	ASM220904v1
GCA_002209065.1	ASM220906v1
GCA_002214785.1	ASM221478v1
GCA_002224325.1	ASM222432v1
GCA_002224385.1	ASM222438v1
GCA_002234455.1	ASM223445v1
GCA_002238355.1	ASM223835v1
GCA_002238375.1	ASM223837v1
GCA_002407125.1	ASM240712v1
GCA_002587105.1	ASM258710v1
GCA_002587225.1	ASM258722v1
GCA_002983725.1	ASM298372v1
GCA_003030185.1	ASM303018v1
GCA_900474415.1	39111_H02
GCA_900475265.1	43024_F01

8.2.3 Publication 3: Whole genome sequencing reveals extended natural transformation in *Campylobacter* impacting diagnostics and the pathogens adaptive potential.

Supplementary Figures

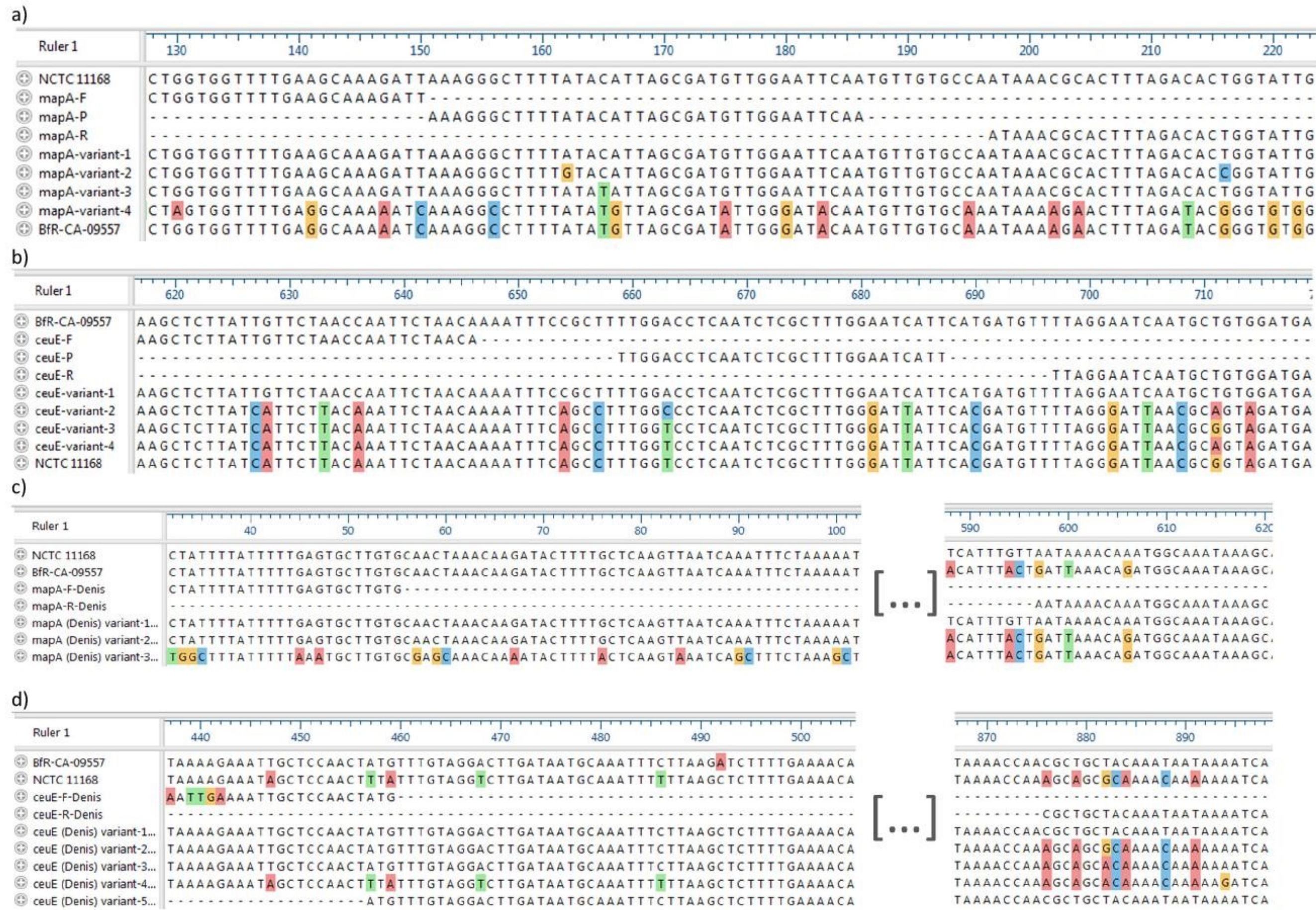
Whole genome sequencing reveals extended natural transformation in *Campylobacter* impacting diagnostics and the pathogens adaptive potential

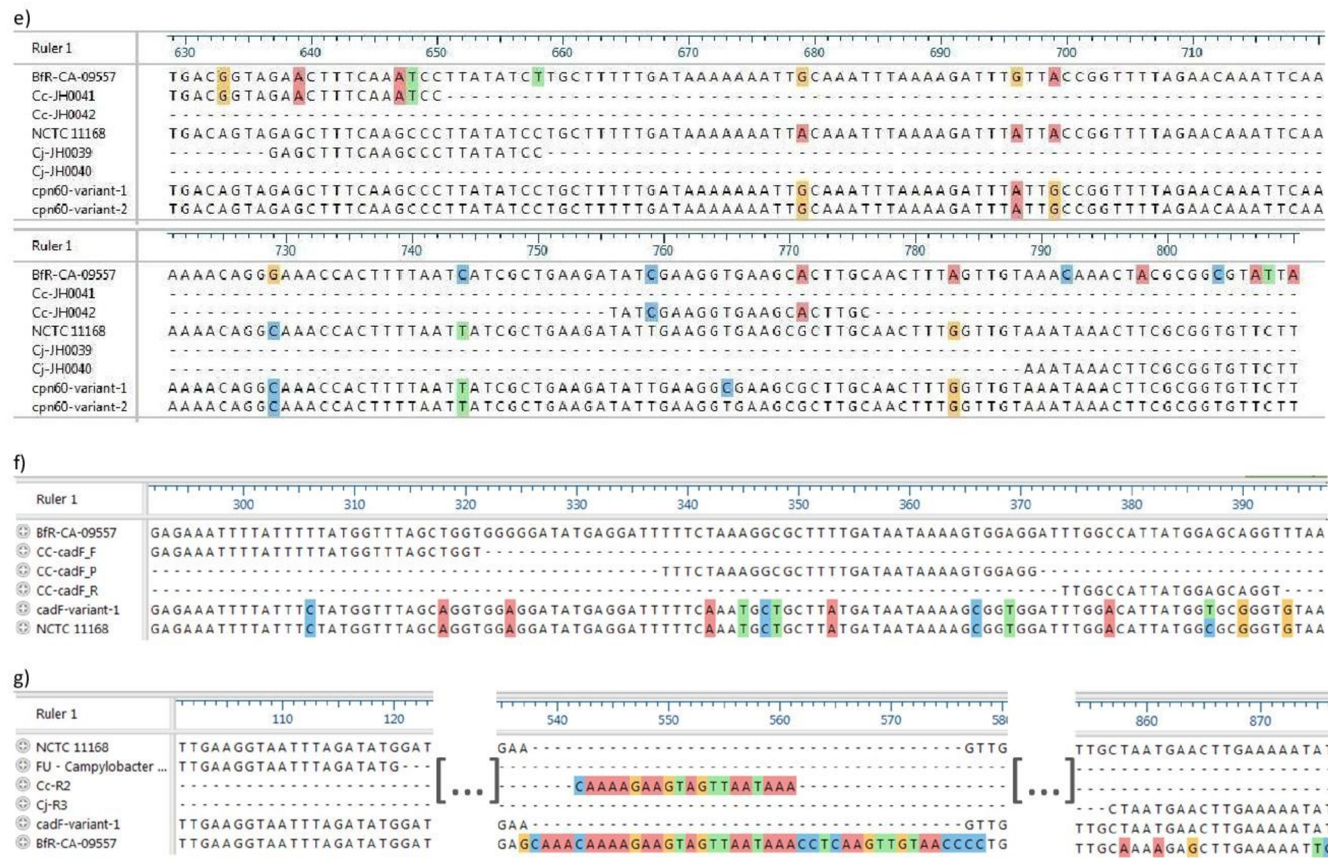
Running title: WGS analysis of *Campylobacter* hybrid strains

Julia C. Golz, Lennard Epping, Marie-Theres Knüver, Maria Borowiak, Felix Hartkopf, Carlus Deneke, Burkhard Malorny, Torsten Semmler, Kerstin Stingl

Suppl. Fig. S1. Alignment of target sequences from hybrid and half hybrid strains used for species differentiation in various published PCR assays. Primers and probes are aligned to target sequences of reference strains *C. jejuni* NCTC 11168 and *C. coli* BfR-CA-09557. Hybrids and half-hybrids with an introgression of ≥ 1 k-mer in the target gene were analyzed. Identified variants in the respective strains are detailed in Suppl. Tab. S1. Ruler 1, base location of target sequences of NCTC 11168 and in g) of BfR-CA-09557. a) *mapA* target according to [3,5]; b) *ceuE* target according to [3,5]; c) *mapA* target according to [25]; d) *ceuE* target according to [25]; e) *cpn60* target according to [24]; f) *cadF* target according to [29]; g) *cadF* target according to [28].

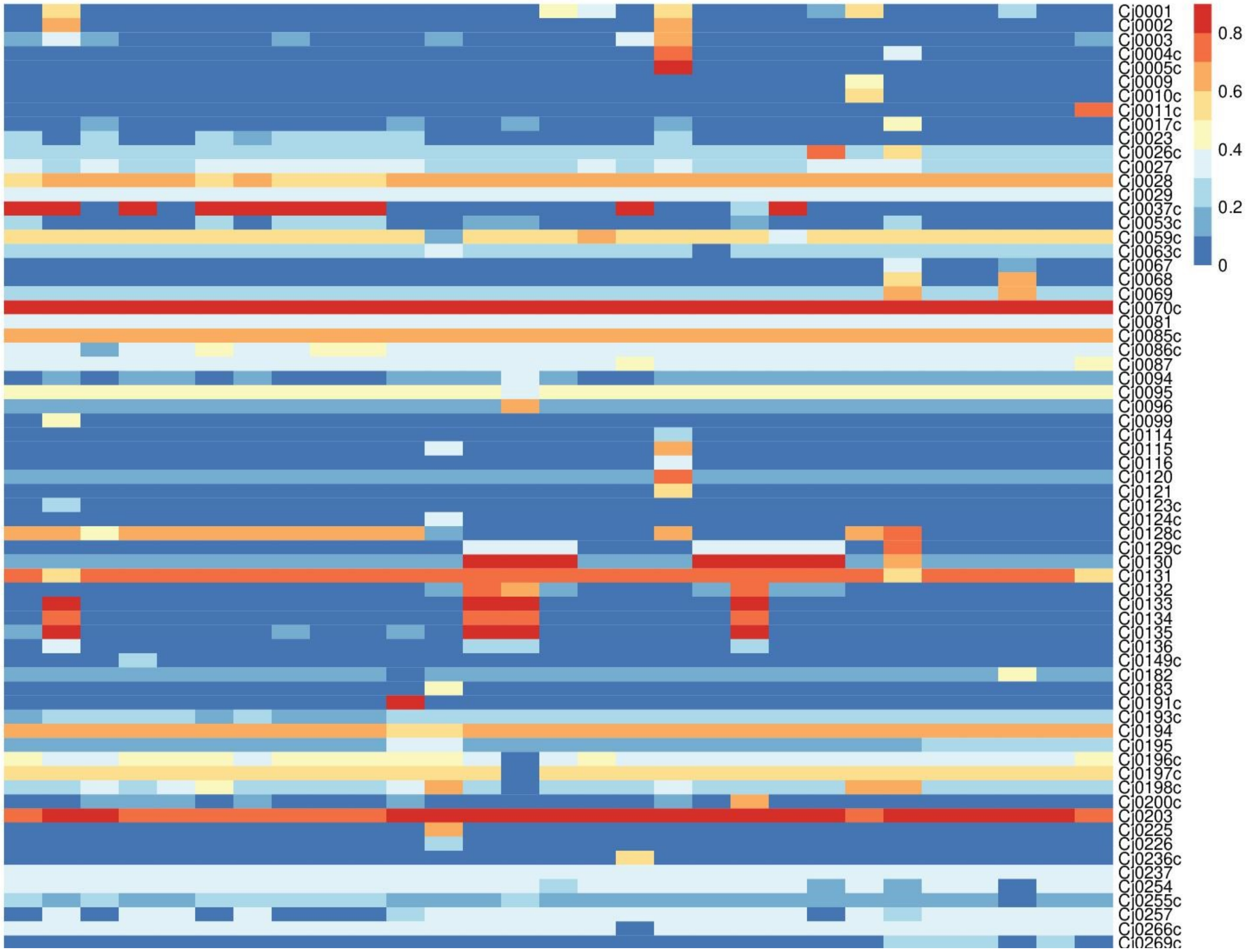
Suppl. Fig. S1

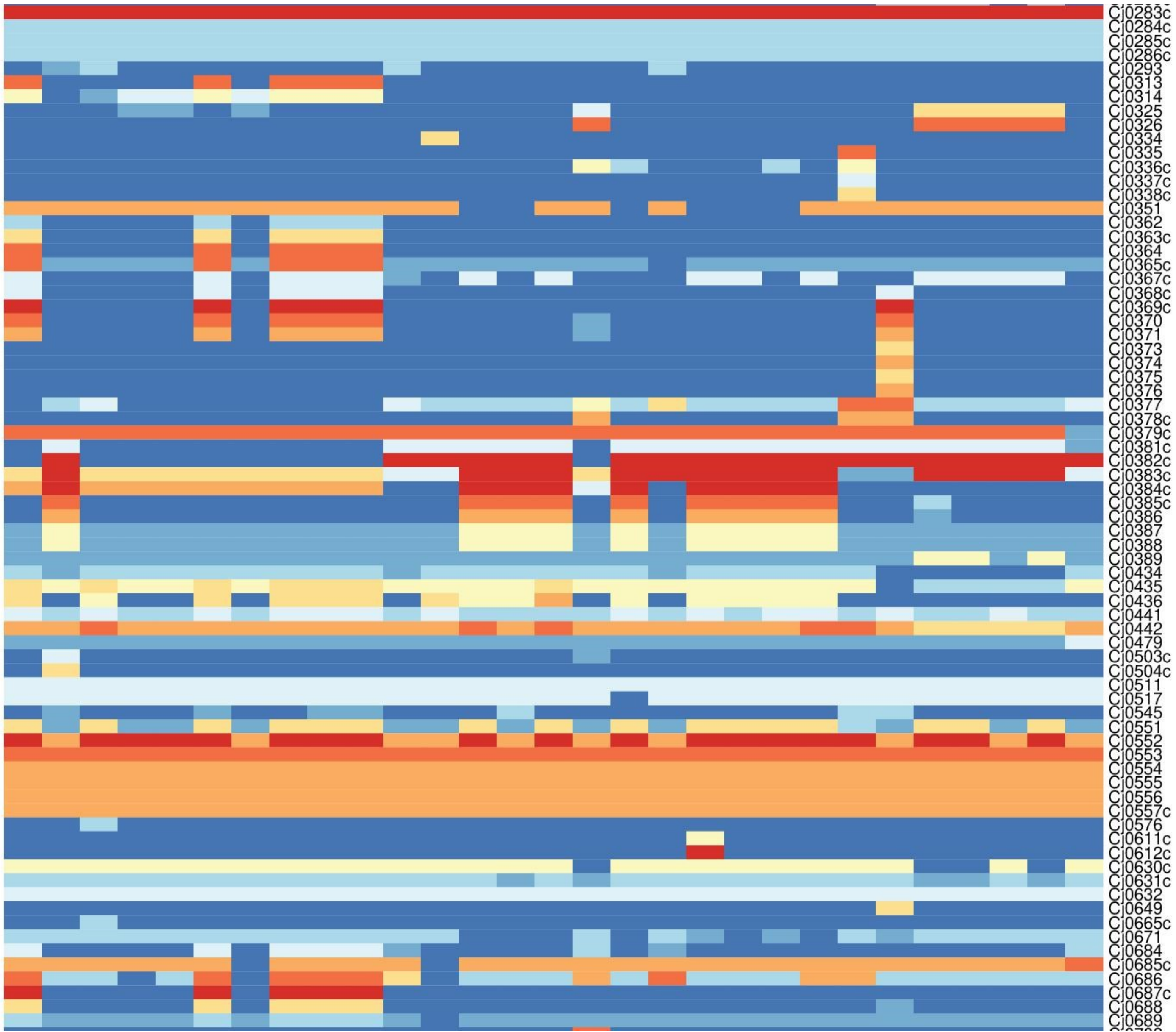




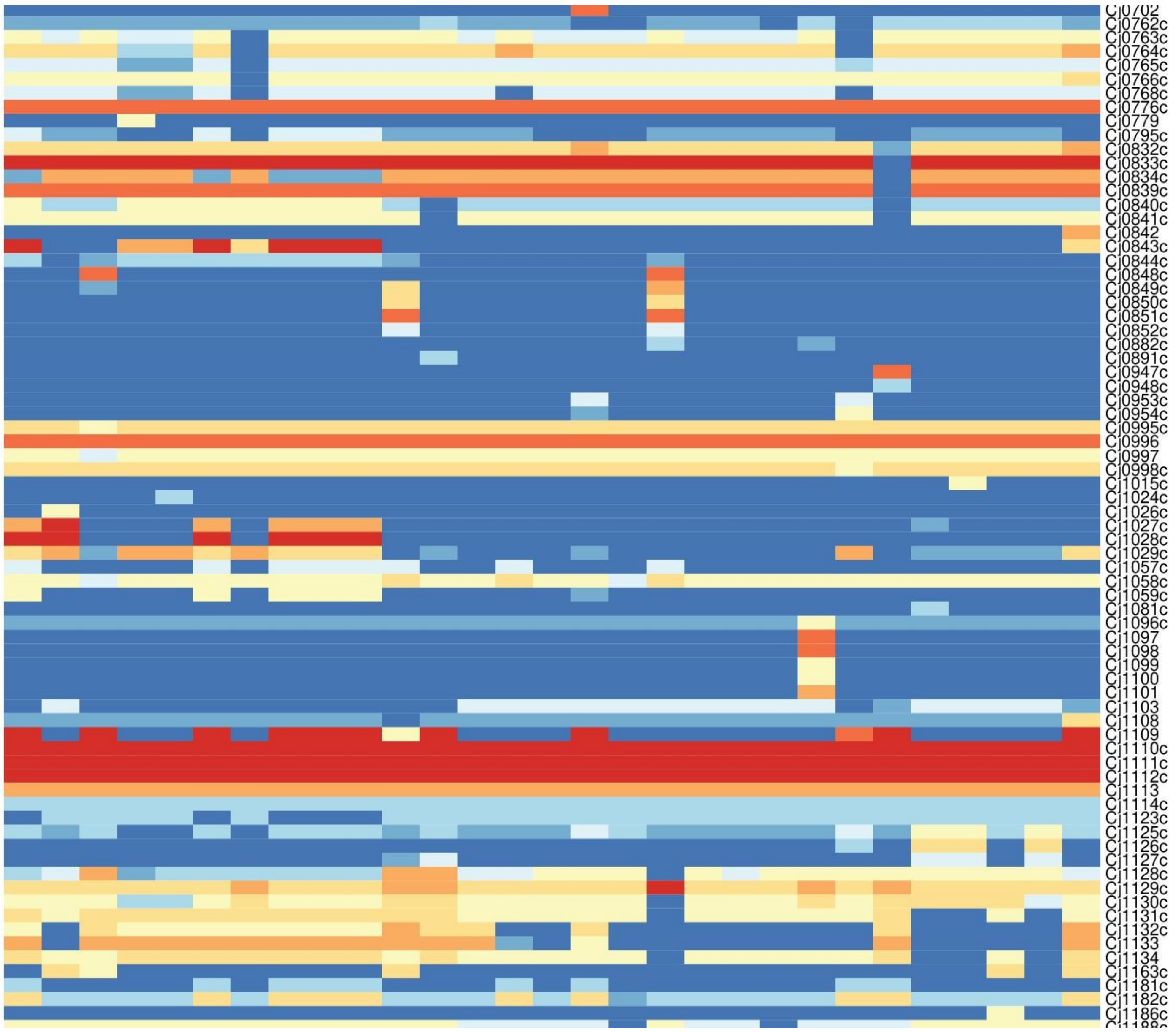
e)
Ruler 1
BfR-CA-09557
Cc-JH0041
Cc-JH0042
NCTC 11168
Cj-JH0039
Cj-JH0040
cpn60-variant-1
cpn60-variant-2
f)
Ruler 1
BfR-CA-09557
CC-cadF_F
CC-cadF_P
CC-cadF_R
cadF-variant-1
NCTC 11168
g)
Ruler 1
NCTC 11168
FU - Campylobacter ...
Cc-R2
Cj-R3
cadF-variant-1
BfR-CA-09557

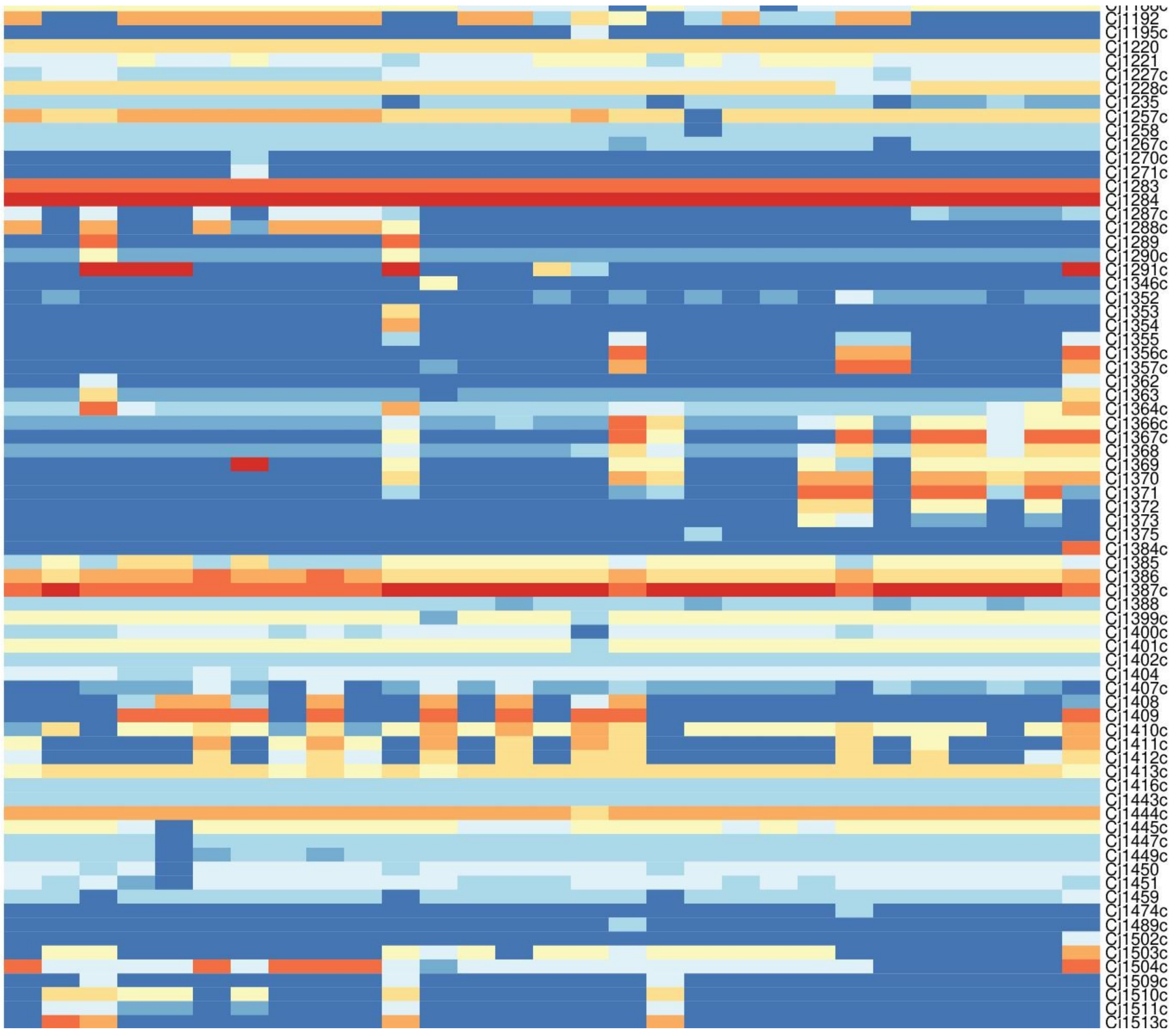
Suppl. Fig. S2. Heatmap visualization of genes with at least 20 % k-mer coverage of *C. jejuni* sequences identified in at least one of the 29 Cc/Cj hybrid strains (with >10 % *C. jejuni* introgression) X-axis, strains; y-axis, genes, sorted according to gene location in the reference *C. jejuni* NCTC 11168 sequence. Colours indicate coverage of gene length by *C. jejuni* sequence specific k-mers (16 bp) in % as detailed in the figure.

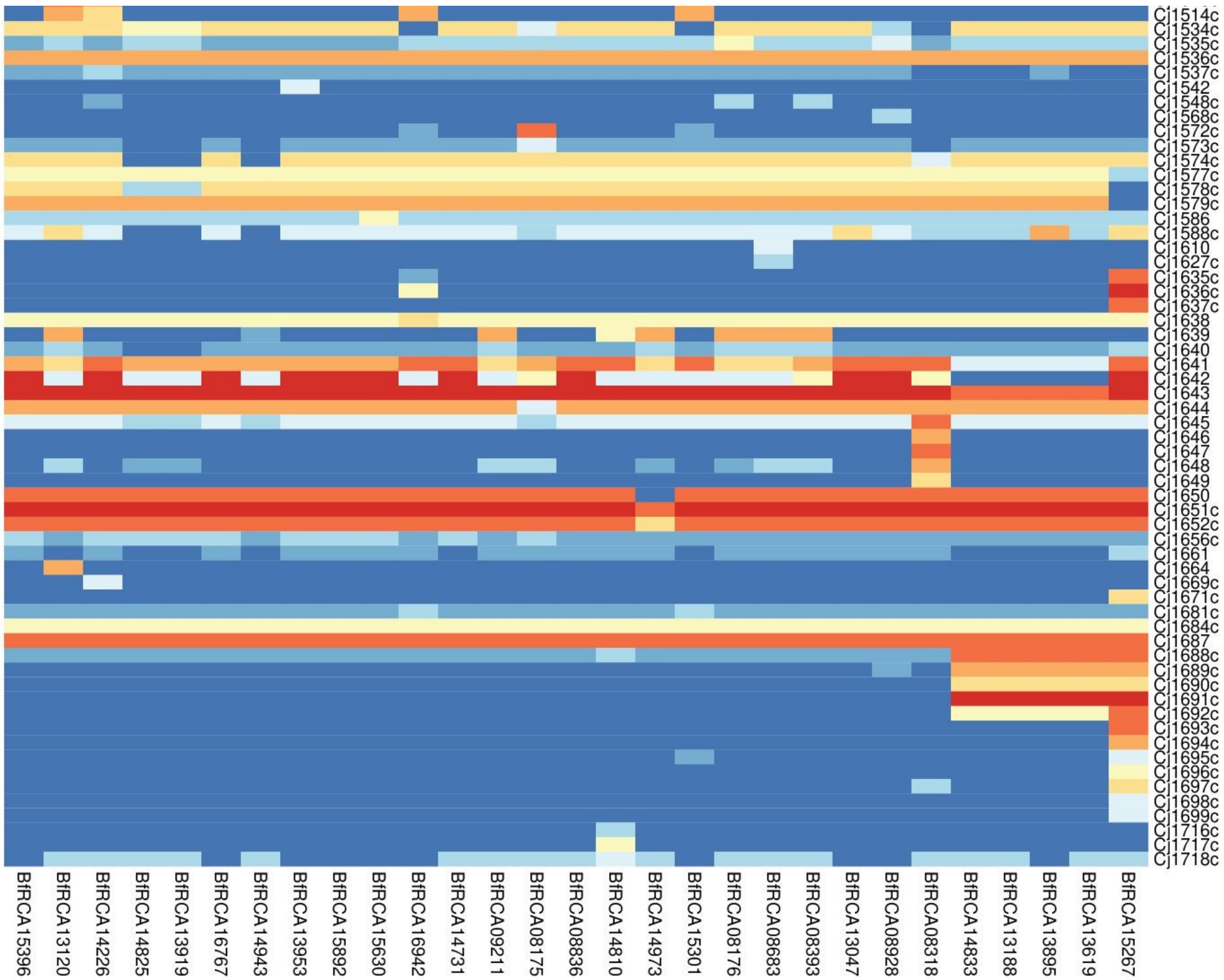

Cj0001
Cj0002
Cj0003
Cj0004c
Cj0005c
Cj0009
Cj0010c
Cj0011c
Cj0017c
Cj0023
Cj0026c
Cj0027
Cj0028
Cj0029
Cj0037c
Cj0053c
Cj0059c
Cj0063c
Cj0067
Cj0068
Cj0069
Cj0070c
Cj0081
Cj0085c
Cj0086c
Cj0087
Cj0094
Cj0095
Cj0096
Cj0099
Cj0114
Cj0115
Cj0116
Cj0120
Cj0121
Cj0123c
Cj0124c
Cj0128c
Cj0129c
Cj0130
Cj0131
Cj0132
Cj0133
Cj0134
Cj0135
Cj0136
Cj0149c
Cj0182
Cj0183
Cj0191c
Cj0193c
Cj0194
Cj0195
Cj0196c
Cj0197c
Cj0198c
Cj0200c
Cj0203
Cj0225
Cj0226
Cj0236c
Cj0237
Cj0254
Cj0255c
Cj0257
Cj0266c
Cj0269c
0.8
0.6
0.4
0.2
0



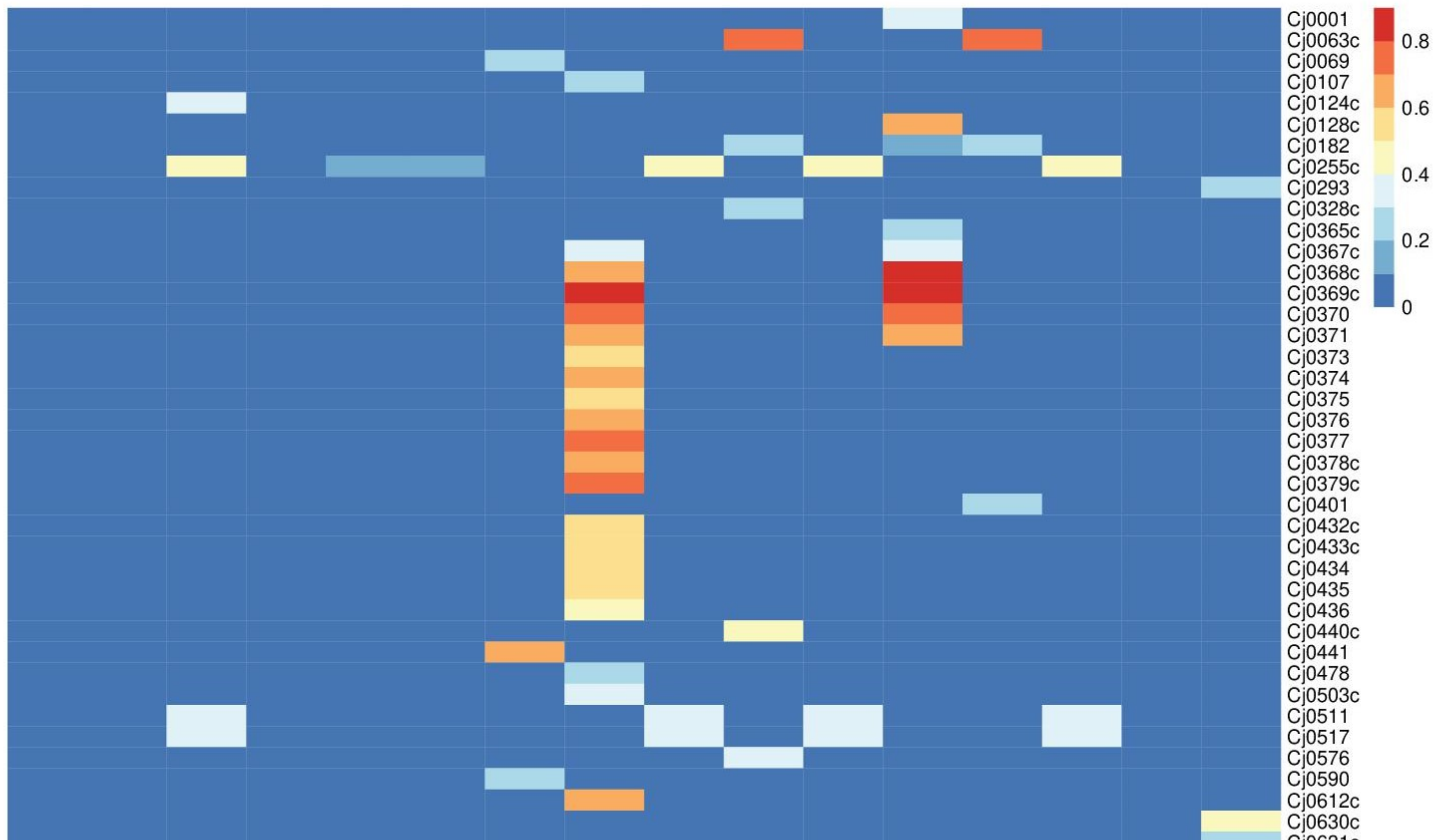
Cj0283c
Cj0284c
Cj0285c
Cj0286c
Cj0293
Cj0313
Cj0314
Cj0325
Cj0326
Cj0334
Cj0335
Cj0336c
Cj0337c
Cj0338c
Cj0351
Cj0362
Cj0363c
Cj0364
Cj0365c
Cj0367c
Cj0368c
Cj0369c
Cj0370
Cj0371
Cj0373
Cj0374
Cj0375
Cj0376
Cj0377
Cj0378c
Cj0379c
Cj0381c
Cj0382c
Cj0383c
Cj0384c
Cj0385c
Cj0386
Cj0387
Cj0388
Cj0389
Cj0434
Cj0435
Cj0436
Cj0441
Cj0442
Cj0479
Cj0503c
Cj0504c
Cj0511
Cj0517
Cj0545
Cj0551
Cj0552
Cj0553
Cj0554
Cj0555
Cj0556
Cj0557c
Cj0576
Cj0611c
Cj0612c
Cj0630c
Cj0631c
Cj0632
Cj0649
Cj0665c
Cj0671
Cj0684
Cj0685c
Cj0686
Cj0687c
Cj0688
Cj0689

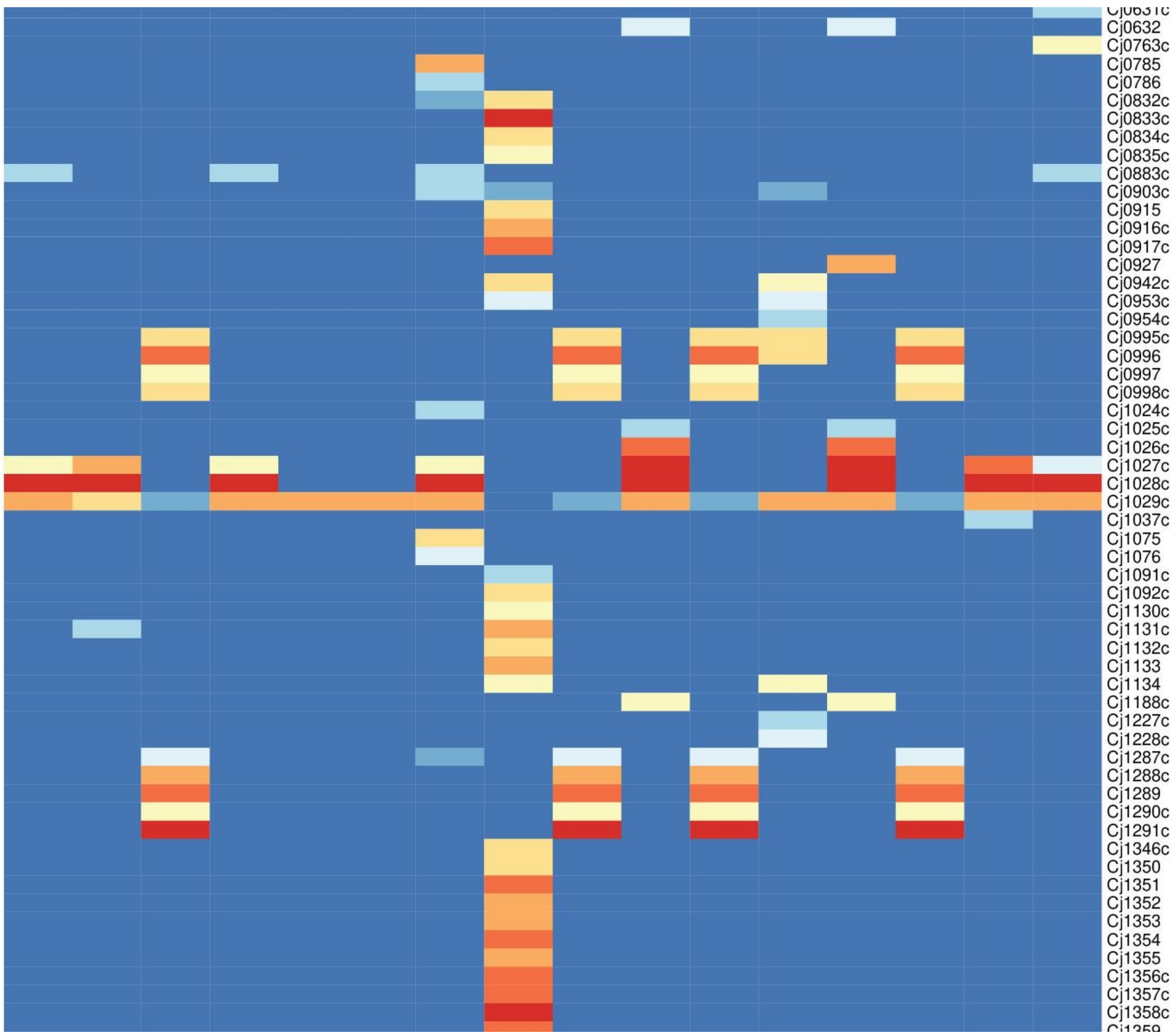

Cj0702
Cj0762c
Cj0763c
Cj0764c
Cj0765c
Cj0766c
Cj0768c
Cj0776c
Cj0779
Cj0795c
Cj0832c
Cj0833c
Cj0834c
Cj0839c
Cj0840c
Cj0841c
Cj0842
Cj0843c
Cj0844c
Cj0848c
Cj0849c
Cj0850c
Cj0851c
Cj0852c
Cj0882c
Cj0891c
Cj0947c
Cj0948c
Cj0953c
Cj0954c
Cj0995c
Cj0996
Cj0997
Cj0998c
Cj1015c
Cj1024c
Cj1026c
Cj1027c
Cj1028c
Cj1029c
Cj1057c
Cj1058c
Cj1059c
Cj1081c
Cj1096c
Cj1097
Cj1098
Cj1099
Cj1100
Cj1101
Cj1103
Cj1108
Cj1109
Cj1110c
Cj1111c
Cj1112c
Cj1113
Cj1114c
Cj1123c
Cj1125c
Cj1126c
Cj1127c
Cj1128c
Cj1129c
Cj1130c
Cj1131c
Cj1132c
Cj1133
Cj1134
Cj1163c
Cj1181c
Cj1182c
Cj1186c

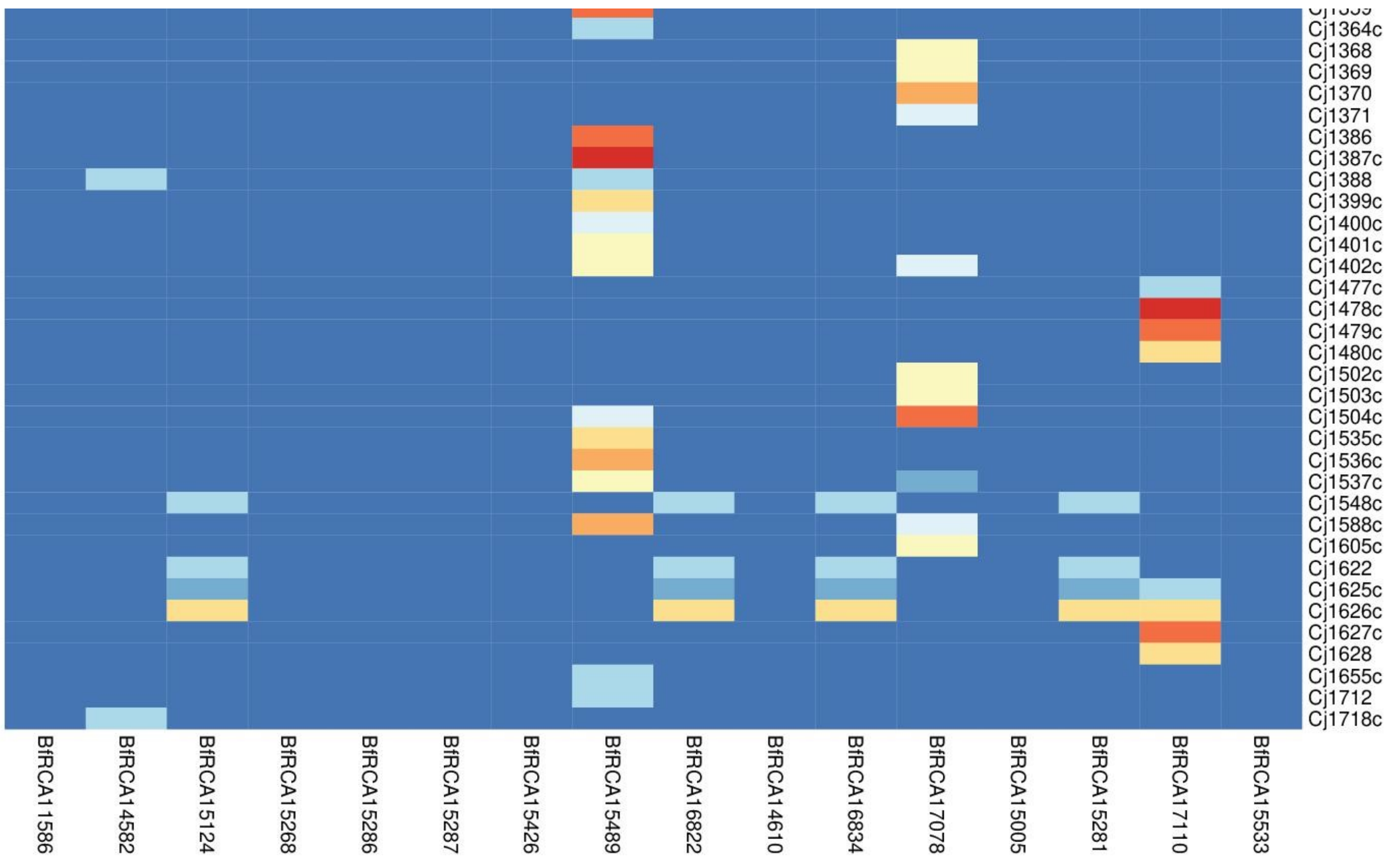

Cj1192
Cj1195c
Cj1220
Cj1221
Cj1227c
Cj1228c
Cj1235
Cj1257c
Cj1258
Cj1267c
Cj1270c
Cj1271c
Cj1283
Cj1284
Cj1287c
Cj1288c
Cj1289
Cj1290c
Cj1291c
Cj1346c
Cj1352
Cj1353
Cj1354
Cj1355
Cj1356c
Cj1357c
Cj1362
Cj1363
Cj1364c
Cj1366c
Cj1367c
Cj1368
Cj1369
Cj1370
Cj1371
Cj1372
Cj1373
Cj1375
Cj1384c
Cj1385
Cj1386
Cj1387c
Cj1388
Cj1399c
Cj1400c
Cj1401c
Cj1402c
Cj1404
Cj1407c
Cj1408
Cj1409
Cj1410c
Cj1411c
Cj1412c
Cj1413c
Cj1416c
Cj1443c
Cj1444c
Cj1445c
Cj1447c
Cj1449c
Cj1450
Cj1451
Cj1459
Cj1474c
Cj1489c
Cj1502c
Cj1503c
Cj1504c
Cj1509c
Cj1510c
Cj1511c
Cj1513c


Cj1514c
Cj1534c
Cj1535c
Cj1536c
Cj1537c
Cj1542
Cj1548c
Cj1568c
Cj1572c
Cj1573c
Cj1574c
Cj1577c
Cj1578c
Cj1579c
Cj1586
Cj1588c
Cj1610
Cj1627c
Cj1635c
Cj1636c
Cj1637c
Cj1638
Cj1639
Cj1640
Cj1641
Cj1642
Cj1643
Cj1644
Cj1645
Cj1646
Cj1647
Cj1648
Cj1649
Cj1650
Cj1651c
Cj1652c
Cj1656c
Cj1661
Cj1664
Cj1669c
Cj1671c
Cj1681c
Cj1684c
Cj1687
Cj1688c
Cj1689c
Cj1690c
Cj1691c
Cj1692c
Cj1693c
Cj1694c
Cj1695c
Cj1696c
Cj1697c
Cj1698c
Cj1699c
Cj1716c
Cj1717c
Cj1718c
BfRCA15396
BfRCA13120
BfRCA14226
BfRCA14825
BfRCA13919
BfRCA16767
BfRCA14943
BfRCA13953
BfRCA15892
BfRCA15630
BfRCA16942
BfRCA14731
BfRCA09211
BfRCA08175
BfRCA08836
BfRCA14810
BfRCA14973
BfRCA15301
BfRCA08176
BfRCA08683
BfRCA08393
BfRCA13047
BfRCA08928
BfRCA08318
BfRCA14833
BfRCA13188
BfRCA13895
BfRCA13619
BfRCA15267

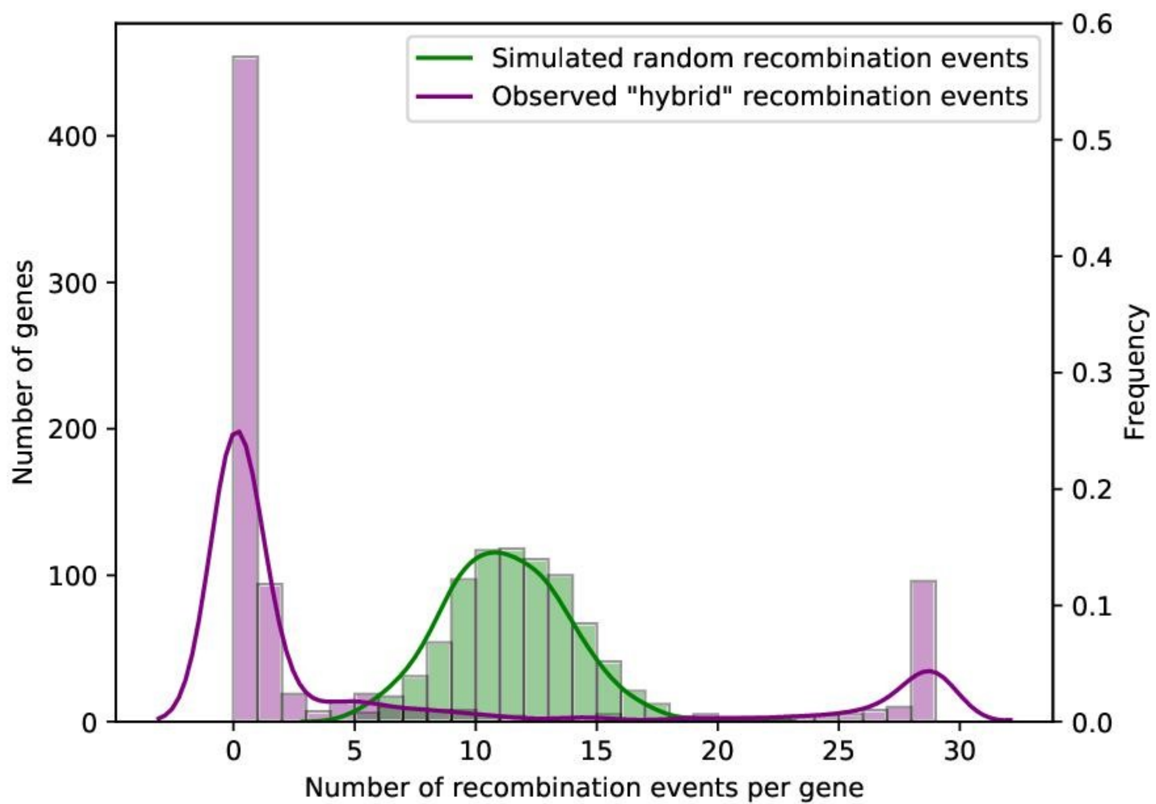
Suppl. Fig. S3. Heatmap visualization of genes with at least 20 % k-mer coverage of *C. jejuni* sequences identified in at least one of the 16 half hybrid strains (with <10 % *C. jejuni* introgression but with ambigious qPCR result). X-axis, strains; y-axis, genes, sorted according to gene location in the reference *C. jejuni* NCTC 11168 sequence. Colours indicate coverage of gene length by *C. jejuni* sequence specific k-mers (16 bp) in % as detailed in the figure.




Cj0632
Cj0763c
Cj0785
Cj0786
Cj0832c
Cj0833c
Cj0834c
Cj0835c
Cj0883c
Cj0903c
Cj0915
Cj0916c
Cj0917c
Cj0927
Cj0942c
Cj0953c
Cj0954c
Cj0995c
Cj0996
Cj0997
Cj0998c
Cj1024c
Cj1025c
Cj1026c
Cj1027c
Cj1028c
Cj1029c
Cj1037c
Cj1075
Cj1076
Cj1091c
Cj1092c
Cj1130c
Cj1131c
Cj1132c
Cj1133
Cj1134
Cj1188c
Cj1227c
Cj1228c
Cj1287c
Cj1288c
Cj1289
Cj1290c
Cj1291c
Cj1346c
Cj1350
Cj1351
Cj1352
Cj1353
Cj1354
Cj1355
Cj1356c
Cj1357c
Cj1358c


Cj1364c
Cj1368
Cj1369
Cj1370
Cj1371
Cj1386
Cj1387c
Cj1388
Cj1399c
Cj1400c
Cj1401c
Cj1402c
Cj1477c
Cj1478c
Cj1479c
Cj1480c
Cj1502c
Cj1503c
Cj1504c
Cj1535c
Cj1536c
Cj1537c
Cj1548c
Cj1588c
Cj1605c
Cj1622
Cj1625c
Cj1626c
Cj1627c
Cj1628
Cj1655c
Cj1712
Cj1718c
BfRCA11586
BfRCA14582
BfRCA15124
BfRCA15268
BfRCA15286
BfRCA15287
BfRCA15426
BfRCA15489
BfRCA16822
BfRCA14610
BfRCA16834
BfRCA17078
BfRCA15005
BfRCA15281
BfRCA17110
BfRCA15533

Suppl. Fig. S4. The distribution of simulated random recombination events in *C. coli* core genes (green) is significantly different from that of the observed recombination events in the hybrid strains (purple). This is indicative of a selection process (i. e. functional adaptation) after recombination.



Whole genome sequencing reveals extended natural transformation in *Campylobacter* impacting diagnostics and the pathogens adaptive potential
Running title: WGS analysis of *Campylobacter* hybrid strains

Julia C. Golz, Lennard Epping, Marie-Theres Knüver, Maria Borowiak, Felix Hartkopf, Carlus Deneke, Burkhard Malorny, Torsten Semmler, Kerstin Stingl

Suppl. Tab. S1. Strains included in the study including metadata.

Sheet1 "BfR-CA-hybrid strains", hybrid and half hybrid strains identified in the study; hybrid strain, defined as strain with >10 % *C. jejuni* content; half hybrid strain, defined as delivering ambiguous or false qPCR result according to [3,5] but harboring <10 % *C. jejuni* content according to k-mer analysis. Columns F-N, sequence variants for PCR targets detailed in Fig. S1 are shown for each strain; no introgression, ≤1 *C. jejuni* k-mer matched to the indicated gene; highlighted in grey, ambiguous or false species differentiation; column P, percentage of *C. jejuni* introgression according to Kmer Finder (CGE); columns Q-Y, MLST results from Ridom Seqsphere+; colours indicate different MLST ST-types consistent with Fig. 3; numbers in bold indicate new alleles or new MLST ST-types; columns Z-AF, proportion of gene length covered by *C. jejuni* k-mers; column AG, accession No. of sequences at NCBI.

Sheet2 "other strains", strains used for creating the k-mer databases.

Suppl. Tab. S1: Sheet1 “BfR-CA-hybrid strains”

Strain No.	Species	Sampling_date	Matrix catagory	qPCR result (Best/Mayr)	*mapA* seq (Best/Mayr)	*ceuE* seq (Best/Mayr)	result (Denis)	*mapA* seq (Denis)	*ceuE* seq (Denis)
BfR-CA-08175	Campylobacter coli	07.11.2009	eggs	coli	Cc variant-4	Cc variant-1	coli	Cc variant-3	Cc variant-1
BfR-CA-08176	Campylobacter coli	11.11.2009	eggs	coli	no introgression	Cc variant-1	coli	no introgression	Cc variant-1
BfR-CA-08318	Campylobacter coli	17.02.2010	eggs	none	no introgression	Cj variant-2	none	no introgression	Cj variant-3
BfR-CA-08393	Campylobacter coli	15.03.2010	eggs	coli	no introgression	Cc variant-1	none	no introgression	Cj variant-5
BfR-CA-08683	Campylobacter coli	19.07.2010	eggs	coli	no introgression	Cc variant-1	coli	no introgression	Cc variant-1
BfR-CA-08836	Campylobacter coli	16.08.2010	eggs	coli	no introgression	Cc variant-1	coli	no introgression	Cc variant-1
BfR-CA-08928	Campylobacter coli	13.10.2010	eggs	false jejuni	Cj variant-1	Cj variant-2	false jejuni	Cj variant-1	Cj variant-3
BfR-CA-09211	Campylobacter coli	03.12.2010	eggs	coli	no introgression	Cc variant-1	coli	no introgression	Cc variant-1
BfR-CA-11586	Campylobacter coli	02.09.2013	chicken meat	Cj/Cc mix	Cj variant-1	no introgression	Cj/Cc mix	Cj variant-1	no introgression
BfR-CA-13047	Campylobacter coli	12.11.2014	eggs	coli	no introgression	Cc variant-1	coli	no introgression	Cc variant-1
BfR-CA-13120	Campylobacter coli	17.11.2014	chicken meat	Cj/Cc mix	Cj variant-3	no introgression	Cj/Cc mix	Cj variant-1	no introgression
BfR-CA-13188	Campylobacter coli	07.02.2015	eggs	Cj/Cc mix	Cj variant-1	Cc variant-1	coli	Cc variant-2	Cc variant-1
BfR-CA-13619	Campylobacter coli	04.11.2015	eggs	Cj/Cc mix	Cj variant-1	Cc variant-1	coli	Cc variant-2	Cc variant-1
BfR-CA-13895	Campylobacter coli	24.02.2016	eggs	Cj/Cc mix	Cj variant-1	Cc variant-1	coli	Cc variant-2	Cc variant-1
BfR-CA-13919	Campylobacter coli	07.03.2016	turkey cecum	Cj/Cc mix	Cj variant-1	Cc variant-1	Cj/Cc mix	Cj variant-1	Cc variant-1
BfR-CA-13953	Campylobacter coli	07.03.2016	turkey cecum	Cj/Cc mix	Cj variant-2	Cc variant-1	Cj/Cc mix	Cj variant-1	Cc variant-1
BfR-CA-14226	Campylobacter coli	14.06.2016	turkey cecum	Cj/Cc mix	Cj variant-1	Cc variant-1	coli	Cc variant-2	Cc variant-1
BfR-CA-14582	Campylobacter coli	05.09.2016	chicken meat	Cj/Cc mix	Cj variant-2	no introgression	Cj/Cc mix	Cj variant-1	no introgression
BfR-CA-14610	Campylobacter coli	25.04.2016	turkey cecum	Cj/Cc mix	Cj variant-1	no introgression	Cj/Cc mix	Cj variant-1	no introgression
BfR-CA-14731	Campylobacter coli	06.10.2016	turkey meat	Cj/Cc mix	Cj variant-1	no introgression	coli	Cc variant-2	no introgression
BfR-CA-14810	Campylobacter coli	25.10.2016	eggs	Cj/Cc mix	Cj variant-1	Cc variant-1	coli	Cc variant-2	Cc variant-1
BfR-CA-14825	Campylobacter coli	07.11.2016	turkey cecum	Cj/Cc mix	Cj variant-1	Cc variant-1	Cj/Cc mix	Cj variant-1	Cc variant-1
BfR-CA-14833	Campylobacter coli	08.11.2016	eggs	Cj/Cc mix	Cj variant-1	Cc variant-1	coli	Cc variant-2	Cc variant-1
BfR-CA-14943	Campylobacter coli	12.12.2016	turkey cecum	Cj/Cc mix	Cj variant-1	Cc variant-1	Cj/Cc mix	Cj variant-1	Cc variant-1
BfR-CA-14973	Campylobacter coli	07.12.2016	eggs	none	no introgression	Cj variant-3	none	no introgression	Cj variant-2
BfR-CA-15005	Campylobacter coli	06.10.2016	turkey skin	Cj/Cc mix	Cj variant-1	no introgression	Cj/Cc mix	Cj variant-1	no introgression
BfR-CA-15124	Campylobacter coli	09.05.2017	chicken meat	Cj/Cc mix	Cj variant-1	no introgression	coli	Cc variant-2	no introgression
BfR-CA-15267	Campylobacter coli	18.08.2017	eggs	false jejuni	Cj variant-1	Cj variant-3	false jejuni	Cj variant-1	Cj variant-2
BfR-CA-15268	Campylobacter coli	24.08.2017	chicken meat	Cj/Cc mix	Cj variant-1	no introgression	Cj/Cc mix	Cj variant-1	no introgression
BfR-CA-15281	Campylobacter coli	28.08.2017	turkey meat	Cj/Cc mix	Cj variant-1	no introgression	coli	Cc variant-2	no introgression
BfR-CA-15286	Campylobacter coli	06.09.2017	chicken meat	Cj/Cc mix	Cj variant-1	no introgression	Cj/Cc mix	Cj variant-1	no introgression
BfR-CA-15287	Campylobacter coli	05.09.2017	chicken meat	Cj/Cc mix	Cj variant-1	no introgression	Cj/Cc mix	Cj variant-1	no introgression
BfR-CA-15301	Campylobacter coli	15.09.2017	eggs	coli	no introgression	Cc variant-1	coli	no introgression	Cc variant-1
BfR-CA-15396	Campylobacter coli	14.11.2017	chicken meat	Cj/Cc mix	Cj variant-2	Cc variant-1	Cj/Cc mix	Cj variant-1	Cc variant-1
BfR-CA-15426	Campylobacter coli	09.11.2017	chicken meat	Cj/Cc mix	Cj variant-1	no introgression	Cj/Cc mix	Cj variant-1	no introgression
BfR-CA-15489	Campylobacter coli	14.02.2018	turkey cecum	none	no introgression	Cj variant-4	none	no introgression	Cj variant-4
BfR-CA-15533	Campylobacter coli	06.03.2018	duck meat	Cj/Cc mix	Cj variant-1	no introgression	Cj/Cc mix	Cj variant-1	no introgression
BfR-CA-15630	Campylobacter coli	17.04.2018	turkey meat	Cj/Cc mix	Cj variant-2	Cc variant-1	Cj/Cc mix	Cj variant-1	Cc variant-1
BfR-CA-15892	Campylobacter coli	15.05.2018	turkey cecum	Cj/Cc mix	Cj variant-2	Cc variant-1	Cj/Cc mix	Cj variant-1	Cc variant-1
BfR-CA-16767	Campylobacter coli	18.09.2018	turkey cecum	Cj/Cc mix	Cj variant-2	Cc variant-1	Cj/Cc mix	Cj variant-1	Cc variant-1
BfR-CA-16822	Campylobacter coli	08.10.2018	turkey cecum	Cj/Cc mix	Cj variant-1	no introgression	coli	Cc variant-2	no introgression
BfR-CA-16834	Campylobacter coli	15.10.2018	turkey cecum	Cj/Cc mix	Cj variant-1	no introgression	coli	Cc variant-2	no introgression
BfR-CA-16942	Campylobacter coli	21.11.2018	turkey meat	none	no introgression	Cj variant-3	coli	no introgression	Cc variant-1
BfR-CA-17078	Campylobacter coli	27.11.2018	turkey cecum	Cj/Cc mix	Cj variant-1	no introgression	Cj/Cc mix	Cj variant-1	no introgression
BfR-CA-17110	Campylobacter coli	15.05.2018	turkey meat	Cj/Cc mix	Cj variant-1	no introgression	Cj/Cc mix	Cj variant-1	no introgression

result (*cpn60*)	*cpn60* seq	result (*cadF*)	*cadF* seq	WGS result	%Cj (CGE)	MLST ST	MLST CC	*aspA*	*glnA*	*gltA*	*glyA*	*pgm*	*tkt*
jejuni	Cj variant-1	coli	no introgression	hybrid	13.36	**10180**	?	114	195	103	140	188	43
jejuni	Cj variant-1	coli	no introgression	hybrid	13.13	7018	?	114	195	103	115	459	164
jejuni	Cj variant-1	coli	no introgression	hybrid	13.76	9102	?	103	110	30	140	459	3
jejuni	Cj variant-1	coli	no introgression	hybrid	13.2	**10181**	?	114	195	103	115	459	164
jejuni	Cj variant-1	coli	no introgression	hybrid	13.33	4148	?	114	195	103	140	459	164
jejuni	Cj variant-1	coli	no introgression	hybrid	12.77	4148	?	114	195	103	140	459	164
jejuni	Cj variant-1	coli	no introgression	hybrid	15.54	1487	ST-1150 complex	103	110	103	140	188	164
jejuni	Cj variant-1	coli	no introgression	hybrid	13.44	4148	?	114	195	103	140	459	164
coli	no introgression	coli	no introgression	half hybrid	1.46	1595	ST-828 complex	33	38	30	79	104	43
jejuni	Cj variant-1	coli	no introgression	hybrid	13.85	4148	?	114	195	103	140	459	164
jejuni	Cj variant-1	coli	no introgression	hybrid	13.87	**10182**	?	114	195	103	140	459	164
jejuni	Cj variant-1	coli	no introgression	hybrid	12.19	5439	?	114	406	103	140	459	164
jejuni	Cj variant-1	coli	no introgression	hybrid	12.3	5439	?	114	406	103	140	459	164
jejuni	Cj variant-1	coli	no introgression	hybrid	13.06	5439	?	114	406	103	140	459	164
jejuni	Cj variant-1	coli	no introgression	hybrid	12.32	4148	?	114	195	103	140	459	164
jejuni	Cj variant-1	coli	no introgression	hybrid	14.31	**10183**	?	114	110	30	140	104	625
jejuni	Cj variant-1	coli	no introgression	hybrid	13.96	**10184**	?	114	110	103	140	**1041**	164
coli	no introgression	coli	no introgression	half hybrid	1.99	830	ST-828 complex	33	39	30	79	104	47
coli	no introgression	coli	no introgression	half hybrid	1.55	1586	ST-828 complex	33	176	30	82	113	43
jejuni	Cj variant-1	coli	no introgression	hybrid	12.69	**10185**	?	265	195	103	140	113	164
jejuni	Cj variant-1	coli	no introgression	hybrid	13.66	**10186**	?	**541**	195	30	140	**1041**	164
jejuni	Cj variant-2	coli	no introgression	hybrid	12.13	4148	?	114	195	103	140	459	164
jejuni	Cj variant-1	coli	no introgression	hybrid	13.1	5439	?	114	406	103	140	459	164
jejuni	Cj variant-1	coli	no introgression	hybrid	13.85	4148	?	114	195	103	140	459	164
jejuni	Cj variant-1	coli	no introgression	hybrid	12.72	**10187**	?	2	195	103	140	459	164
coli	no introgression	coli	no introgression	half hybrid	1.17	1586	ST-828 complex	33	176	30	82	113	43
coli	no introgression	coli	no introgression	half hybrid	2.56	832	ST-828 complex	33	39	30	79	113	43
jejuni	Cj variant-1	coli	no introgression	hybrid	14.93	5903	ST-1150 complex	2	195	103	140	188	164
coli	no introgression	coli	no introgression	half hybrid	1.54	1595	ST-828 complex	33	38	30	79	104	43
coli	no introgression	coli	no introgression	half hybrid	2.2	832	ST-828 complex	33	39	30	79	113	43
coli	no introgression	coli	no introgression	half hybrid	0	9168	ST-828 complex	33	38	30	82	918	43
coli	no introgression	coli	no introgression	half hybrid	1.16	9168	ST-828 complex	33	38	30	82	918	43
jejuni	Cj variant-1	coli	no introgression	hybrid	13.69	**10188**	?	103	195	103	79	113	164
jejuni	Cj variant-1	coli	no introgression	hybrid	14.31	**10183**	?	114	110	30	140	104	625
coli	no introgression	coli	no introgression	half hybrid	1.01	1595	ST-828 complex	33	38	30	79	104	43
coli	no introgression	coli	no introgression	half hybrid	6.49	**10190**	?	33	39	30	79	104	35
coli	no introgression	coli	no introgression	half hybrid	1.11	1595	ST-828 complex	33	38	30	79	104	43
jejuni	Cj variant-1	coli	no introgression	hybrid	15.46	**10183**	?	114	110	30	140	104	625
jejuni	Cj variant-1	coli	no introgression	hybrid	14.35	**10183**	?	114	110	30	140	104	625
jejuni	Cj variant-1	coli	no introgression	hybrid	14.39	**10183**	?	114	110	30	140	104	625
coli	no introgression	coli	no introgression	half hybrid	2.22	832	ST-828 complex	33	39	30	79	113	43
coli	no introgression	coli	no introgression	half hybrid	3.48	832	ST-828 complex	33	39	30	79	113	43
jejuni	Cj variant-1	coli	no introgression	hybrid	15.17	**10194**	?	114	195	103	140	**1040**	164
coli	no introgression	coli	no introgression	half hybrid	4.78	**10195**	?	33	39	65	140	189	43
coli	no introgression	jejuni	Cj variant-1	half hybrid	1.53	1769	ST-828 complex	33	176	30	115	113	43

uncA	%k-mer in ***aspA***	%k-mer in ***glnA***	%k-mer in ***gltA***	%k-mer in ***glyA***	%k-mer in ***pgm***	%k-mer in ***tkt***	%k-mer in ***uncA***	**Accession-No.**
79	0.3837953	0.0405311	0.0527975	0.0128514	0.2102772	0.2553976	0.0239044	SAMN13577876
79	0.3837953	0.0405311	0.0527975	0.0128514	0.2102772	0.3101632	0.0239044	SAMN13577877
120	0.3923241	0.0405311	0.0464933	0	0	0.71406	0.0239044	SAMN13577878
669	0.3837953	0.0405311	0.0527975	0	0.2102772	0.3101632	0.0239044	SAMN13577879
79	0.3837953	0.0405311	0.0535855	0	0.2102772	0.3101632	0.0239044	SAMN13577880
79	0.3837953	0.0405311	0.0527975	0.0128514	0.2914131	0.373881	0.0239044	SAMN13577881
79	0.3909026	0.0405311	0.0614657	0	0.2102772	0.373881	0.0358566	SAMN13577882
79	0.3837953	0.0405311	0.0535855	0	0.2102772	0.3101632	0.0239044	SAMN13577883
17	0	0	0.0126084		0			SAMN13577884
79	0.3837953	0.0405311	0.0527975	0	0.2102772	0.373881	0.0239044	SAMN13577885
17	0.3837953	0.0405311	0.0488574	0	0.1967546	0.3101632	0	SAMN13577886
79	0.3837953	0.0216632	0.0527975	0	0	0.373881	0.0239044	SAMN13577887
79	0.3837953	0.0216632	0.0527975	0	0	0.373881	0.0239044	SAMN13577888
79	0.3837953	0.0216632	0.0527975	0.0128514	0	0.373881	0.0239044	SAMN13577889
79	0.3837953	0.0405311	0.0535855	0.0128514	0.2102772	0.2996314	0.0239044	SAMN13577890
79	0.3837953	0.0405311	0.0472813	0	0.2914131	0.3886256	0.0239044	SAMN13577891
342	0.3837953	0.0405311	0.0654058	0	0.2576065	0.373881	0.0239044	SAMN13577892
17	0	0	0		0			SAMN13577893
17	0	0	0		0			SAMN13577894
79	0.3816631	0.0405311	0.0535855	0	0.2102772	0.373881	0.0239044	SAMN13577895
79	0.3987207	0.0405311	0.0338849	0.0128514	0.2102772	0.373881	0.0239044	SAMN13577896
79	0.3837953	0.0405311	0.0535855	0	0.2102772	0.2996314	0.0239044	SAMN13577897
79	0.3837953	0.0216632	0.0527975	0.0128514	0	0.373881	0.0239044	SAMN13577898
79	0.3837953	0.0405311	0.0527975	0	0.2102772	0.2996314	0.0239044	SAMN13577899
79	0.4683724	0.0405311	0.0527975	0	0.2102772	0.3101632	0.0239044	SAMN13577900
17	0	0	0		0			SAMN13577901
17	0	0	0		0			SAMN13577902
79	0.4683724	0.0405311	0.0630418	0.0128514	0.2102772	0.3064771	0.1673307	SAMN13577903
17	0	0	0.0126084		0			SAMN13577904
17	0	0	0		0			SAMN13577905
17	0	0	0		0			SAMN13577906
17	0	0	0		0			SAMN13577907
120	0.3909026	0.0405311	0.0527975	0	0.1250845	0.373881	0.0239044	SAMN13577908
79	0.3837953	0.0405311	0.0472813	0	0.2576065	0.3886256	0.0239044	SAMN13577909
17	0	0	0.0126084		0			SAMN13577910
670	0.0625444	0.0307477	0.0267927		0.5949966			SAMN13577911
17	0	0	0.0126084		0			SAMN13577912
79	0.3837953	0.0405311	0.0472813	0	0.2576065	0.3886256	0.0239044	SAMN13577913
79	0.3837953	0.0405311	0.0472813	0.0128514	0.2576065	0.3886256	0.0239044	SAMN13577914
79	0.3837953	0.0405311	0.0472813	0.0128514	0.2576065	0.3886256	0.0239044	SAMN13577915
17	0	0	0		0			SAMN13577916
17	0	0	0		0			SAMN13577917
120	0.3837953	0.0405311	0.0535855	0.0128514	0.1250845	0.373881	0.0239044	SAMN13577918
17	0	0	0		0			SAMN13577919
17	0	0	0		0.0175794			SAMN13577920

Suppl. Tab. S1: Sheet2 “other strains”

ID	species
GCA_000146835.1_ASM14683v1_genomic	Campylobacter coli
GCA_000167415.1_ASM16741v1_genomic	Campylobacter coli
GCA_000505605.1_K3_genomic	Campylobacter coli
GCA_000505625.1_K7_genomic	Campylobacter coli
GCA_000531565.1_IPSID-1_genomic	Campylobacter coli
GCA_001225145.1_7092_1_57_genomic	Campylobacter coli
GCA_001228685.1_7092_1_27_genomic	Campylobacter coli
GCA_001228905.1_7038_3_46_genomic	Campylobacter coli
GCA_001228985.1_7213_3_54_genomic	Campylobacter coli
GCA_001234385.1_7092_1_13_genomic	Campylobacter coli
GCA_001235265.1_7038_3_62_genomic	Campylobacter coli
GCA_001235285.1_7092_1_29_genomic	Campylobacter coli
GCA_001236625.1_7065_7_40_genomic	Campylobacter coli
GCA_001236965.1_7213_3_16_genomic	Campylobacter coli
GCA_001291485.1_RC282_S32contigs.fa_genomic	Campylobacter coli
GCA_001291525.1_RC105_S13contigs.fa_genomic	Campylobacter coli
GCA_001291545.1_RC382_S38contigs.fa_genomic	Campylobacter coli
GCA_001291605.1_RC148_S18contigs.fa_genomic	Campylobacter coli
GCA_001291665.1_RC264_S27contigs.fa_genomic	Campylobacter coli
GCA_001291725.1_RC383_S39contigs.fa_genomic	Campylobacter coli
GCA_001291745.1_RC285_S34contigs.fa_genomic	Campylobacter coli
GCA_001291765.1_RC126_S16contigs.fa_genomic	Campylobacter coli
GCA_001291785.1_RC182_S22contigs.fa_genomic	Campylobacter coli
GCA_001291845.1_RC387_S41contigs.fa_genomic	Campylobacter coli
GCA_001291865.1_RC023_S5contigs.fa_genomic	Campylobacter coli
GCA_001291885.1_RC127_S17contigs.fa_genomic	Campylobacter coli
GCA_001291905.1_RC269_S28contigs.fa_genomic	Campylobacter coli
GCA_001291985.1_RC037_S7contigs.fa_genomic	Campylobacter coli
GCA_001292005.1_RC026_S6contigs.fa_genomic	Campylobacter coli
GCA_001292065.1_RC106_S14contigs.fa_genomic	Campylobacter coli
GCA_001292085.1_RC430_S42contigs.fa_genomic	Campylobacter coli
GCA_001292105.1_RC096_S11contigs.fa_genomic	Campylobacter coli
GCA_001292125.1_RC289_S35contigs.fa_genomic	Campylobacter coli
GCA_001292165.1_RC415_S43contigs.fa_genomic	Campylobacter coli
GCA_001292225.1_RC116_S15contigs.fa_genomic	Campylobacter coli
GCA_001292305.1_RC038_S8contigs.fa_genomic	Campylobacter coli
GCA_001292325.1_RC428_S45contigs.fa_genomic	Campylobacter coli
GCA_001292345.1_RC043_S10contigs.fa_genomic	Campylobacter coli
GCA_001292365.1_RC281_S31contigs.fa_genomic	Campylobacter coli
GCA_001292405.1_RC018_S4contigs.fa_genomic	Campylobacter coli
GCA_001292425.1_RC386_S40contigs.fa_genomic	Campylobacter coli
GCA_001292445.1_RC284_S33contigs.fa_genomic	Campylobacter coli
GCA_001292505.1_RC008_S1contigs.fa_genomic	Campylobacter coli
GCA_001305715.1_ASM130571v1_genomic	Campylobacter coli
GCA_001419355.1_Campylobacter_coli_CVM_41957_v1.0_genomic	Campylobacter coli
GCA_001491115.1_SWAN361_genomic	Campylobacter coli
GCA_001498315.1_H074360315_genomic	Campylobacter coli
GCA_001545275.1_ASM154527v1_genomic	Campylobacter coli
GCA_001545285.1_ASM154528v1_genomic	Campylobacter coli

GCA_001545295.1_ASM154529v1_genomic	Campylobacter coli
GCA_001545335.1_ASM154533v1_genomic	Campylobacter coli
GCA_001545355.1_ASM154535v1_genomic	Campylobacter coli
GCA_001545365.1_ASM154536v1_genomic	Campylobacter coli
GCA_001545415.1_ASM154541v1_genomic	Campylobacter coli
GCA_001545425.1_ASM154542v1_genomic	Campylobacter coli
GCA_001545435.1_ASM154543v1_genomic	Campylobacter coli
GCA_001545445.1_ASM154544v1_genomic	Campylobacter coli
GCA_001761965.1_ASM176196v1_genomic	Campylobacter coli
GCA_001761985.1_ASM176198v1_genomic	Campylobacter coli
GCA_001761995.1_ASM176199v1_genomic	Campylobacter coli
GCA_001762015.1_ASM176201v1_genomic	Campylobacter coli
GCA_001762045.1_ASM176204v1_genomic	Campylobacter coli
GCA_001762065.1_ASM176206v1_genomic	Campylobacter coli
GCA_001762085.1_ASM176208v1_genomic	Campylobacter coli
GCA_001762095.1_ASM176209v1_genomic	Campylobacter coli
GCA_001762225.1_BCW_6447_genomic	Campylobacter coli
GCA_001762245.1_BCW_6448_genomic	Campylobacter coli
GCA_001763285.1_ASM176328v1_genomic	Campylobacter coli
GCA_001763685.1_ASM176368v1_genomic	Campylobacter coli
GCA_001764015.1_ASM176401v1_genomic	Campylobacter coli
GCA_001765195.1_ASM176519v1_genomic	Campylobacter coli
GCA_002000205.1_ASM200020v1_genomic	Campylobacter coli
GCA_002178015.1_ASM217801v1_genomic	Campylobacter coli
GCA_002207945.1_ASM220794v1_genomic	Campylobacter coli
GCA_002207965.1_ASM220796v1_genomic	Campylobacter coli
GCF_000465235.1_ASM46523v1_genomic	Campylobacter coli
GCF_000494775.1_ASM49477v1_genomic	Campylobacter coli
GCF_000583755.1_ASM58375v1_genomic	Campylobacter coli
GCF_000583795.1_ASM58379v1_genomic	Campylobacter coli
GCF_000954195.1_ASM95419v1_genomic	Campylobacter coli
GCF_001417635.1_ASM141763v1_genomic	Campylobacter coli
GCF_001483845.1_ASM148384v1_genomic	Campylobacter coli
GCF_001639125.1_ASM163912v1_genomic	Campylobacter coli
GCF_001717605.1_ASM171760v1_genomic	Campylobacter coli
GCF_001865455.1_ASM186545v1_genomic	Campylobacter coli
GCF_001865475.1_ASM186547v1_genomic	Campylobacter coli
GCF_001865495.1_ASM186549v1_genomic	Campylobacter coli
GCF_001865515.1_ASM186551v1_genomic	Campylobacter coli
GCF_001865535.1_ASM186553v1_genomic	Campylobacter coli
GCF_001865555.1_ASM186555v1_genomic	Campylobacter coli
GCF_001936355.1_ASM193635v1_genomic	Campylobacter coli
GCF_002024185.1_ASM202418v1_genomic	Campylobacter coli
GCF_002407145.1_ASM240714v1_genomic	Campylobacter coli
GCF_002843985.1_ASM284398v1_genomic	Campylobacter coli
GCF_003030205.1_ASM303020v1_genomic	Campylobacter coli
GCF_000466065.2_ASM46606v2_genomic	Campylobacter jejuni
GCF_000466075.2_ASM46607v2_genomic	Campylobacter jejuni
GCF_002407125.1_ASM240712v1_genomic	Campylobacter jejuni
GCF_000009085.1_ASM908v1_genomic	Campylobacter jejuni

GCF_000011865.1_ASM1186v1_genomic	Campylobacter jejuni
GCF_000015525.1_ASM1552v1_genomic	Campylobacter jejuni
GCF_000017905.1_ASM1790v1_genomic	Campylobacter jejuni
GCF_000025425.1_ASM2542v1_genomic	Campylobacter jejuni
GCF_000148705.1_ASM14870v1_genomic	Campylobacter jejuni
GCF_000171795.2_ASM17179v2_genomic	Campylobacter jejuni
GCF_000184205.1_ASM18420v1_genomic	Campylobacter jejuni
GCF_000302555.5_ASM30255v4_genomic	Campylobacter jejuni
GCF_000304375.1_AINO_genomic	Campylobacter jejuni
GCF_000430385.1_ASM43038v1_genomic	Campylobacter jejuni
GCF_000466105.2_ASM46610v2_genomic	Campylobacter jejuni
GCF_000468915.2_ASM46891v2_genomic	Campylobacter jejuni
GCF_000493495.1_TS_genomic	Campylobacter jejuni
GCF_000737085.1_ASM73708v1_genomic	Campylobacter jejuni
GCF_000772225.1_ASM77222v1_genomic	Campylobacter jejuni
GCF_000830775.1_ASM83077v1_genomic	Campylobacter jejuni
GCF_000830805.1_ASM83080v1_genomic	Campylobacter jejuni
GCF_000830825.1_ASM83082v1_genomic	Campylobacter jejuni
GCF_000830845.1_ASM83084v1_genomic	Campylobacter jejuni
GCF_000830865.1_ASM83086v1_genomic	Campylobacter jejuni
GCF_000835285.1_ASM83528v1_genomic	Campylobacter jejuni
GCF_000835305.1_ASM83530v1_genomic	Campylobacter jejuni
GCF_000835345.1_ASM83534v1_genomic	Campylobacter jejuni
GCF_000835365.1_ASM83536v1_genomic	Campylobacter jejuni
GCF_000934305.1_ASM93430v1_genomic	Campylobacter jejuni
GCF_001299565.1_ASM129956v1_genomic	Campylobacter jejuni
GCF_001299595.1_ASM129959v1_genomic	Campylobacter jejuni
GCF_001314285.1_ASM131428v1_genomic	Campylobacter jejuni
GCF_001412295.1_ASM141229v1_genomic	Campylobacter jejuni
GCF_001457695.1_NCTC11351_genomic	Campylobacter jejuni
GCF_001506185.1_ASM150618v1_genomic	Campylobacter jejuni
GCF_001506205.1_ASM150620v1_genomic	Campylobacter jejuni
GCF_001506225.1_ASM150622v1_genomic	Campylobacter jejuni
GCF_001506245.1_ASM150624v1_genomic	Campylobacter jejuni
GCF_001506265.1_ASM150626v1_genomic	Campylobacter jejuni
GCF_001506285.1_ASM150628v1_genomic	Campylobacter jejuni
GCF_001506305.1_ASM150630v1_genomic	Campylobacter jejuni
GCF_001506345.1_ASM150634v1_genomic	Campylobacter jejuni
GCF_001506365.1_ASM150636v1_genomic	Campylobacter jejuni
GCF_001506385.1_ASM150638v1_genomic	Campylobacter jejuni
GCF_001506405.1_ASM150640v1_genomic	Campylobacter jejuni
GCF_001506425.1_ASM150642v1_genomic	Campylobacter jejuni
GCF_001506445.1_ASM150644v1_genomic	Campylobacter jejuni
GCF_001506465.1_ASM150646v1_genomic	Campylobacter jejuni
GCF_001506485.1_ASM150648v1_genomic	Campylobacter jejuni
GCF_001506505.1_ASM150650v1_genomic	Campylobacter jejuni
GCF_001506525.1_ASM150652v1_genomic	Campylobacter jejuni
GCF_001506545.1_ASM150654v1_genomic	Campylobacter jejuni
GCF_001506565.1_ASM150656v1_genomic	Campylobacter jejuni
GCF_001506585.1_ASM150658v1_genomic	Campylobacter jejuni

GCF_001506605.1_ASM150660v1_genomic	Campylobacter jejuni
GCF_001506625.1_ASM150662v1_genomic	Campylobacter jejuni
GCF_001506645.1_ASM150664v1_genomic	Campylobacter jejuni
GCF_001506665.1_ASM150666v1_genomic	Campylobacter jejuni
GCF_001506685.1_ASM150668v1_genomic	Campylobacter jejuni
GCF_001506705.1_ASM150670v1_genomic	Campylobacter jejuni
GCF_001506725.1_ASM150672v1_genomic	Campylobacter jejuni
GCF_001506745.1_ASM150674v1_genomic	Campylobacter jejuni
GCF_001506765.1_ASM150676v1_genomic	Campylobacter jejuni
GCF_001506785.1_ASM150678v1_genomic	Campylobacter jejuni
GCF_001506805.1_ASM150680v1_genomic	Campylobacter jejuni
GCF_001506825.1_ASM150682v1_genomic	Campylobacter jejuni
GCF_001506845.1_ASM150684v1_genomic	Campylobacter jejuni
GCF_001506865.1_ASM150686v1_genomic	Campylobacter jejuni
GCF_001506885.1_ASM150688v1_genomic	Campylobacter jejuni
GCF_001506905.1_ASM150690v1_genomic	Campylobacter jejuni
GCF_001506925.1_ASM150692v1_genomic	Campylobacter jejuni
GCF_001506945.1_ASM150694v1_genomic	Campylobacter jejuni
GCF_001506965.1_ASM150696v1_genomic	Campylobacter jejuni
GCF_001506985.1_ASM150698v1_genomic	Campylobacter jejuni
GCF_001507005.1_ASM150700v1_genomic	Campylobacter jejuni
GCF_001507025.1_ASM150702v1_genomic	Campylobacter jejuni
GCF_001507045.1_ASM150704v1_genomic	Campylobacter jejuni
GCF_001507065.1_ASM150706v1_genomic	Campylobacter jejuni
GCF_001507085.1_ASM150708v1_genomic	Campylobacter jejuni
GCF_001507105.1_ASM150710v1_genomic	Campylobacter jejuni
GCF_001507125.1_ASM150712v1_genomic	Campylobacter jejuni
GCF_001507145.1_ASM150714v1_genomic	Campylobacter jejuni
GCF_001507165.1_ASM150716v1_genomic	Campylobacter jejuni
GCF_001507185.1_ASM150718v1_genomic	Campylobacter jejuni
GCF_001507205.1_ASM150720v1_genomic	Campylobacter jejuni
GCF_001507225.1_ASM150722v1_genomic	Campylobacter jejuni
GCF_001507245.1_ASM150724v1_genomic	Campylobacter jejuni
GCF_001507265.1_ASM150726v1_genomic	Campylobacter jejuni
GCF_001563565.1_ASM156356v1_genomic	Campylobacter jejuni
GCF_001587015.1_ASM158701v1_genomic	Campylobacter jejuni
GCF_001587035.1_ASM158703v1_genomic	Campylobacter jejuni
GCF_001686905.1_ASM168690v1_genomic	Campylobacter jejuni
GCF_001717625.1_ASM171762v1_genomic	Campylobacter jejuni
GCF_001721945.1_ASM172194v1_genomic	Campylobacter jejuni
GCF_001721965.1_ASM172196v1_genomic	Campylobacter jejuni
GCF_001721985.1_ASM172198v1_genomic	Campylobacter jejuni
GCF_001767215.1_ASM176721v1_genomic	Campylobacter jejuni
GCF_001865395.1_ASM186539v1_genomic	Campylobacter jejuni
GCF_001865415.1_ASM186541v1_genomic	Campylobacter jejuni
GCF_001865435.1_ASM186543v1_genomic	Campylobacter jejuni
GCF_001865595.1_ASM186559v1_genomic	Campylobacter jejuni
GCF_001870085.1_ASM187008v1_genomic	Campylobacter jejuni
GCF_001870105.1_ASM187010v1_genomic	Campylobacter jejuni
GCF_001951235.1_ASM195123v1_genomic	Campylobacter jejuni

GCF_001951255.1_ASM195125v1_genomic	Campylobacter jejuni
GCF_001951275.1_ASM195127v1_genomic	Campylobacter jejuni
GCF_001951295.1_ASM195129v1_genomic	Campylobacter jejuni
GCF_001951315.1_ASM195131v1_genomic	Campylobacter jejuni
GCF_001951335.1_ASM195133v1_genomic	Campylobacter jejuni
GCF_002024325.1_ASM202432v1_genomic	Campylobacter jejuni
GCF_002028305.1_ASM202830v1_genomic	Campylobacter jejuni
GCF_002101355.1_ASM210135v1_genomic	Campylobacter jejuni
GCF_002209005.1_ASM220900v1_genomic	Campylobacter jejuni
GCF_002209025.1_ASM220902v1_genomic	Campylobacter jejuni
GCF_002209045.1_ASM220904v1_genomic	Campylobacter jejuni
GCF_002209065.1_ASM220906v1_genomic	Campylobacter jejuni
GCF_002214785.1_ASM221478v1_genomic	Campylobacter jejuni
GCF_002224325.1_ASM222432v1_genomic	Campylobacter jejuni
GCF_002224385.1_ASM222438v1_genomic	Campylobacter jejuni
GCF_002234455.1_ASM223445v1_genomic	Campylobacter jejuni
GCF_002238375.1_ASM223837v1_genomic	Campylobacter jejuni
GCF_002587105.1_ASM258710v1_genomic	Campylobacter jejuni
GCF_002587225.1_ASM258722v1_genomic	Campylobacter jejuni
GCF_003030185.1_ASM303018v1_genomic	Campylobacter jejuni
GCF_003060725.1_ASM306072v1_genomic	Campylobacter jejuni
GCF_003060745.1_ASM306074v1_genomic	Campylobacter jejuni
GCF_003060765.1_ASM306076v1_genomic	Campylobacter jejuni
GCF_003060785.1_ASM306078v1_genomic	Campylobacter jejuni
GCF_003368045.1_ASM336804v1_genomic	Campylobacter jejuni
GCF_003368065.1_ASM336806v1_genomic	Campylobacter jejuni
GCF_003368085.1_ASM336808v1_genomic	Campylobacter jejuni
GCF_003368105.1_ASM336810v1_genomic	Campylobacter jejuni
GCF_003368125.1_ASM336812v1_genomic	Campylobacter jejuni
GCF_003368145.1_ASM336814v1_genomic	Campylobacter jejuni
GCF_003368165.1_ASM336816v1_genomic	Campylobacter jejuni
GCF_003368185.1_ASM336818v1_genomic	Campylobacter jejuni
GCF_003368205.1_ASM336820v1_genomic	Campylobacter jejuni
GCF_003368225.1_ASM336822v1_genomic	Campylobacter jejuni
GCF_003368245.1_ASM336824v1_genomic	Campylobacter jejuni
GCF_003574945.1_ASM357494v1_genomic	Campylobacter jejuni
GCF_003950275.1_ASM395027v1_genomic	Campylobacter jejuni
GCF_003971585.1_ASM397158v1_genomic	Campylobacter jejuni
GCF_900475265.1_43024_F01_genomic	Campylobacter jejuni
GCF_900638165.1_56527_E01_genomic	Campylobacter jejuni
GCF_900638175.1_56527_F01_genomic	Campylobacter jejuni
GCF_900638185.1_56527_G01_genomic	Campylobacter jejuni
GCF_900638195.1_56553_F01_genomic	Campylobacter jejuni
GCF_900638205.1_56553_D01_genomic	Campylobacter jejuni
GCF_900638225.1_56553_E01_genomic	Campylobacter jejuni
GCF_900638235.1_56772_E02_genomic	Campylobacter jejuni
GCF_900638285.1_57043_B01_genomic	Campylobacter jejuni
GCF_900638365.1_57428_D01_genomic	Campylobacter jejuni
BfR-CA-11057	Campylobacter coli
BfR-CA-13264	Campylobacter coli

BfR-CA-13971	Campylobacter coli
BfR-CA-14216	Campylobacter coli
BfR-CA-14583	Campylobacter coli
BfR-CA-14709	Campylobacter coli
BfR-CA-14751	Campylobacter coli
BfR-CA-14815	Campylobacter coli
BfR-CA-15034	Campylobacter coli
BfR-CA-15062	Campylobacter coli
BfR-CA-15077	Campylobacter coli
BfR-CA-15371	Campylobacter coli
BfR-CA-15403	Campylobacter coli
BfR-CA-15629	Campylobacter coli
BfR-CA-15913	Campylobacter coli
BfR-CA-15969	Campylobacter coli
BfR-CA-15978	Campylobacter coli
DSM 4689	Campylobacter coli
BfRCA15282	Campylobacter jejuni
BfRCA15395	Campylobacter jejuni
BfRCA16737	Campylobacter jejuni

Whole genome sequencing reveals extended natural transformation in *Campylobacter* impacting diagnostics and the pathogens adaptive potential
Running title: WGS analysis of *Campylobacter* hybrid strains

Julia C. Golz, Lennard Epping, Marie-Theres Knüver, Maria Borowiak, Felix Hartkopf, Carlus Deneke, Burkhard Malorny, Torsten Semmler, Kerstin Stingl

Suppl. Tab. S2. Genes with at least 20 % *C. jejuni* introgression in at least 25 of the 29 *C. coli* hybrid strains and their potential function.

Sheet 1 "GeneList Cj introgression", Assignment to functional categories (column Z and overview on sheet 2) was derived by automatic annotation by EggNOG (columns X and Y) and integration of additional information of gene function for *Campylobacter* homologues (column Z). Numeric values are numbers of strains that share the percentage of *C. jejuni* k-mer introgression (column headers, 0.1-0.9 of the gene length) in the indicated genes, annotated according to NCTC 11168. Overlap of 104 genes was identified, independent of the k-mer size (16-mer, blue columns; 31-mer, green columns). 8 additional genes were found with 16-mers and 14 genes with 31-mers (total 126 genes). The American isolate RM4661 isolated from turkey carcass shares 100 genes with *C. jejuni* introgression of at least 0.2 gene length coverage (red columns, numeric values indicate percentage of coverage of gene length) and 6 further genes with at least 0.1 gene length coverage (in italics).

Sheet 2 "Overview func. cat.", overview of number of introgressed genes assigned to functional catagories

Suppl. Tab. S2: Sheet1 “GeneList Cj introgression”

Gene/Coverage 16-mer	0.1	0.2	0.3	0.4	0.5	0.6	0.7	0.8	0.9	Gene/Coverage 31-mer	0.1	0.2	0.3	0.4	0.5	0.6	0.7	0.8	0.9	Gene name (putative function)
Cj0026c	29	29	2	2	2	1	1	0	0											*thyX* (flavin dependent thymidylate synthase; catalyzes the formation of dTMP and tetrahydrofolate from dUMP and methylenetetrahydrofolate)
Cj0027	29	29	13	0	0	0	0	0	0	Cj0027	29	29	29	10	0	0	0	0	0	*pyrG* ; cytidine triphosphate synthetase; catalyzes the ATP-dependent amination of UTP to CTP with either L-glutamine or ammonia as the source of nitrogen
Cj0028	29	29	29	29	29	24	0	0	0	Cj0028	29	29	29	29	29	24	0	0	0	*recJ* (putative single-stranded-DNA-specific exonuclease)
Cj0029	29	29	29	0	0	0	0	0	0	Cj0029	29	29	7	0	0	0	0	0	0	*ansA* (cytoplasmic L-asparaginase)
Cj0059c	29	28	28	27	27	1	0	0	0	Cj0059c	29	28	27	26	1	0	0	0	0	*fliY* (one of three proteins for switching the direction of the flagellar motor)
Cj0063c	28	28	1	0	0	0	0	0	0	Cj0063c	28	28	1	0	0	0	0	0	0	ATP-binding protein
Cj0069	29	29	2	2	2	2	0	0	0											hypothetical protein
Cj0070c	29	29	29	29	29	29	29	29	0	Cj0070c	29	29	29	29	29	29	29	0	0	hypothetical protein
Cj0081	29	29	29	0	0	0	0	0	0	Cj0081	29	29	4	0	0	0	0	0	0	*cydA* (better *cioA*, cyanide-insensitive oxidase)
Cj0085c	29	29	29	29	29	29	0	0	0	Cj0085c	29	29	29	29	29	28	0	0	0	putative amino acid racemase
Cj0086c	29	28	28	3	0	0	0	0	0	Cj0086c	29	28	28	0	0	0	0	0	0	*ung* (uracil-DNA glycosylase, excises uracil residues from the DNA which can arise as a result of misincorporation of dUMP residues by DNA polymerase or due to deamination of cytosine)
Cj0087	29	29	29	2	0	0	0	0	0	Cj0087	29	29	29	28	2	0	0	0	0	*aspA* (aspartate ammonia-lyase)
Cj0095	29	29	29	28	0	0	0	0	0	Cj0095	29	29	29	29	0	0	0	0	0	*rpmA* (50S ribosomal protein L27, involved in the peptidyltransferase reaction during translation)
Cj0131	29	29	29	29	29	26	26	0	0	Cj0131	29	29	29	29	29	26	17	0	0	putative periplasmic protein
Cj0194	29	29	29	29	29	27	0	0	0	Cj0194	29	29	28	0	0	0	0	0	0	*folE* (GTP cyclohydrolase I, involved in the first step of tetrahydrofolate biosynthesis)
Cj0196c	28	28	28	11	0	0	0	0	0											*purF* (amidophosphoribosyltransferase, catalyzes first step of the de novo purine nucleotide biosynthetic pathway)
Cj0197c	28	28	28	28	28	0	0	0	0	Cj0197c	28	28	28	28	18	0	0	0	0	*dapB* (4-hydroxytetrahydrodipicolinate reductase)
Cj0198c	28	28	8	4	3	3	0	0	0	Cj0198c	28	28	4	3	3	0	0	0	0	recombination factor protein RarA (maintainance of genome stability)
Cj0203	29	29	29	29	29	29	29	19	0	Cj0203	29	29	29	29	29	20	2	0	0	putative transmembrane transport protein
Cj0237	29	29	29	0	0	0	0	0	0	Cj0237	29	29	28	0	0	0	0	0	0	*canB* (carbonic anhydrase); enables growth at low CO_2
Cj0254	28	26	25	0	0	0	0	0	0	Cj0254	29	26	25	5	0	0	0	0	0	hypothetical protein
Cj0266c	28	28	28	0	0	0	0	0	0											integral membrane protein
Cj0283c	29	29	29	29	29	29	29	29	29	Cj0283c	29	29	29	29	29	29	29	29	29	*cheW* (chemotaxis protein)
Cj0284c	29	29	0	0	0	0	0	0	0											*cheA* (chemotaxis histidine kinase)
Cj0285c	29	29	0	0	0	0	0	0	0	Cj0285c	29	29	0	0	0	0	0	0	0	*cheV* (chemotaxis protein)
Cj0286c	29	29	0	0	0	0	0	0	0	Cj0286c	29	29	0	0	0	0	0	0	0	hypothetical protein
Cj0379c	29	28	28	28	28	28	28	0	0	Cj0379c	28	28	28	28	28	28	7	0	0	hypothetical protein
Cj0383c	29	27	27	24	24	14	14	14	0	Cj0383c	29	27	27	24	24	24	14	14	9	*ribH* (6,7-dimethyl-8-ribityllumazine synthase), biosynthesis of riboflavin
										Cj0387	29	29	9	9	0	0	0	0	0	*aroK* (shikimate kinase, catalyzes the formation of shikimate 3-phosphate from shikimate in aromatic amino acid biosynthesis)

Cj0435	28	28	24	24	7	0	0	0	0											*fabG* (3-oxoacyl-ACP reductase, catalyzes the first of the two reduction steps in the elongation cycle of fatty acid synthesis)
Cj0441	29	29	13	0	0	0	0	0	0	Cj0441	29	26	0	0	0	0	0	0	0	*acpP* (acyl carrier protein, carries the fatty acid chain in fatty acid biosynthesis)
Cj0442	29	29	29	29	29	25	5	0	0	Cj0442	29	29	29	29	29	23	0	0	0	*fabF* (3-oxoacyl-[acyl-carrier-protein] synthase) (fatty acid biosynthesis)
Cj0511	29	29	29	0	0	0	0	0	0	Cj0511	29	29	0	0	0	0	0	0	0	protease
Cj0517	28	28	28	0	0	0	0	0	0	Cj0517	28	26	0	0	0	0	0	0	0	*crcB* (fluoride ion transporter, campher resistance)
Cj0552	29	29	29	29	29	29	19	19	0	Cj0552	29	29	29	29	29	20	19	18	0	putative membrane protein
Cj0553	29	29	29	29	29	29	29	0	0	Cj0553	29	29	29	29	29	19	0	0	0	integral membrane protein
Cj0554	29	29	29	29	29	29	0	0	0	Cj0554	29	29	29	29	29	0	0	0	0	hypothetical protein
Cj0555	29	29	29	29	29	29	0	0	0	Cj0555	29	29	29	29	29	29	0	0	0	putative integral membrane protein
Cj0556	29	29	29	29	29	29	0	0	0	Cj0556	29	29	29	29	0	0	0	0	0	amidohydrolase family protein
Cj0557c	29	29	29	29	29	29	0	0	0	Cj0557c	29	29	29	29	0	0	0	0	0	integral membrane protein
Cj0630c	25	25	25	25	0	0	0	0	0	Cj0630c	25	25	25	24	0	0	0	0	0	*holA* (DNA polymerase III subunit delta)
Cj0632	29	29	29	0	0	0	0	0	0	Cj0632	29	29	0	0	0	0	0	0	0	*ilvC* (ketol-acid reductoisomerase, valine and isoleucine biosynthesis)
Cj0685c	27	27	27	27	27	27	1	0	0	Cj0685c	27	27	27	27	27	27	5	0	0	*cipA* (invasion protein)
Cj0686	26	26	10	10	10	9	6	0	0											ispG (4-hydroxy-3-methylbut-2-en-1-yl diphosphate synthase (flavodoxin); involved in isoprenoid synthesis)
Cj0763c	27	27	27	17	0	0	0	0	0	Cj0763c	27	27	17	0	0	0	0	0	0	*cysE* (serine acetyltransferase)
Cj0764c	27	27	25	25	25	2	0	0	0	Cj0764c	27	27	25	18	0	0	0	0	0	*speA* (arginine decarboxylase)
Cj0765c	28	26	25	0	0	0	0	0	0	Cj0765c	28	26	25	0	0	0	0	0	0	*hisS* (histidine-tRNA ligase)
Cj0766c	28	28	28	28	1	0	0	0	0	Cj0766c	28	28	28	28	4	0	0	0	0	*tmk* (thymidylate kinase)
Cj0776c	29	29	29	29	29	29	29	0	0	Cj0776c	29	29	29	29	29	29	0	0	0	putative periplasmic protein
Cj0832c	29	28	28	28	28	2	0	0	0	Cj0832c	28	28	28	2	0	0	0	0	0	Na+/H+ antiporter family protein
Cj0833c	28	28	28	28	28	28	28	28	0	Cj0833c	28	28	28	28	28	28	26	0	0	oxidoreductase
Cj0839c	28	28	28	28	28	28	28	0	0	Cj0839c	28	28	28	28	28	28	28	28	0	hypothetical protein
Cj0840c	27	27	8	8	0	0	0	0	0											*fbp* (fructose 1,6 bisphosphatase)
Cj0841c	27	27	27	27	0	0	0	0	0	Cj0841c	27	27	27	27	1	0	0	0	0	*mobB* (molybdopterin-guanine dinucleotide biosynthesis protein)
Cj0995c	29	29	29	29	28	0	0	0	0	Cj0995c	29	29	29	0	0	0	0	0	0	*hemB* (delta-aminolevulinic acid dehydratase)
Cj0996	29	29	29	29	29	29	29	0	0	Cj0996	29	29	29	29	29	0	0	0	0	*ribA* (GTP cyclohydrolase II)
Cj0997	29	29	29	28	0	0	0	0	0	Cj0997	29	29	29	0	0	0	0	0	0	rRNA small subunit methyltransferase G
Cj0998c	29	29	29	29	28	0	0	0	0	Cj0998c	29	29	29	29	28	0	0	0	0	periplasmic protein
Cj1058c	29	29	29	27	3	0	0	0	0	Cj1058c	29	29	8	0	0	0	0	0	0	*guaB* (inosine 5'-monophosphate dehydrogenase)
										Cj1096c	29	28	1	1	1	0	0	0	0	*metK* (S-adenosylmethionine synthetase)
										Cj1108	28	28	1	1	1	0	0	0	0	*clpA* (Clp ATPase)
										Cj1109	25	25	12	12	12	11	11	11	11	*aat* (leucyl/phenylalanyl-tRNA--protein transferase)

Cj1110c	29	29	29	29	29	29	29	29	0	Cj1110c	29	29	29	29	29	29	29	29	4	Putative MCP-type signal transduction protein
Cj1111c	29	29	29	29	29	29	29	29	0	Cj1111c	29	29	29	29	29	29	29	0	0	integral membrane protein
Cj1112c	29	29	29	29	29	29	29	29	0	Cj1112c	29	29	29	29	29	29	29	28	0	mrsB; peptide-methionine (R)-S-oxide reductase
Cj1113	29	29	29	29	29	29	0	0	0	Cj1113	29	29	29	29	29	0	0	0	0	hypothetical protein
Cj1114c	29	29	0	0	0	0	0	0	0	Cj1114c	29	29	0	0	0	0	0	0	0	*pssA* (CDP-diacylglycerol--serine O-phosphatidyltransferase)
Cj1128c	28	27	20	15	3	3	0	0	0	Cj1128c	28	28	20	20	14	3	0	0	0	*pglI* (glycosylation)
Cj1129c	29	29	29	29	28	5	0	0	0	Cj1129c	29	29	29	29	5	0	0	0	0	*pglH* (GalNAc-alpha-(1->4)-GalNAc-alpha-(1->3)-diNAcBac-PP-undecaprenol alpha-1,4-N-acetyl-D-galactosaminyltransferase)
Cj1130c	28	28	26	25	8	0	0	0	0	Cj1130c	28	28	25	13	1	0	0	0	0	*pglK* (protein glycosylation K)
Cj1131c	25	25	25	25	12	0	0	0	0	Cj1131c	25	25	12	9	0	0	0	0	0	*gne* (UDP-GlcNAc/Glc 4-epimerase)
Cj1134	25	25	25	25	11	0	0	0	0	Cj1134	25	25	0	0	0	0	0	0	0	*htrB* (lipid A biosynthesis lauroyl acyltransferase); survival harsh environment
Cj1182c	29	28	10	10	10	0	0	0	0	Cj1182c	29	28	28	10	10	10	0	0	0	*rpsB* (30S ribosomal protein S2)
Cj1188c	27	27	27	18	0	0	0	0	0	Cj1188c	27	27	27	0	0	0	0	0	0	*gidA* (tRNA uridine 5-carboxymethylaminomethyl modification protein GidA; glucose-inhibited cell division protein A; involved in the 5-carboxymethylaminomethyl modification (mnm(5)s(2)U) of the wobble uridine base in some tRNAs)
Cj1220	29	29	29	29	29	0	0	0	0	Cj1220	29	29	29	29	29	29	29	0	0	10 kD chaperonin (cpn10); *groES*
Cj1221	29	29	27	9	0	0	0	0	0	Cj1221	29	29	27	1	0	0	0	0	0	*groEL* (cpn60)
Cj1227c	29	29	20	0	0	0	0	0	0	Cj1227c	29	29	0	0	0	0	0	0	0	two-component regulator (3' of *htrA*)
Cj1228c	29	29	29	27	27	0	0	0	0	Cj1228c	29	29	29	27	10	0	0	0	0	*htrA* (serine protease), virulence factor and HtrA may protect oxidatively damaged proteins; chaperone activity
Cj1257c	28	28	28	28	28	9	0	0	0	Cj1257c	28	28	28	28	9	0	0	0	0	efflux pump protein
Cj1258	28	28	0	0	0	0	0	0	0	Cj1258	28	28	0	0	0	0	0	0	0	phosphotyrosine protein phosphatase
Cj1267c	28	27	0	0	0	0	0	0	0											*hydA* (Ni/Fe-hydrogenase small subunit)
Cj1283	29	29	29	29	29	29	29	0	0	Cj1283	29	29	29	29	29	29	21	0	0	*ktrB* (putative K+ uptake protein)
Cj1284	29	29	29	29	29	29	29	29	0	Cj1284	29	29	29	29	29	29	0	0	0	*ktrA* (putative K+ uptake protein)
Cj1364c	29	29	8	4	3	3	1	0	0	Cj1364c	29	29	7	4	3	3	0	0	0	*fumC* (fumarate hydratase, class II family (does not require metal); functions in the TCA cycle)
Cj1385	29	29	22	20	3	0	0	0	0	Cj1385	29	29	22	21	3	3	0	0	0	*katA* (katalase)
Cj1386	29	29	29	29	29	12	2	0	0	Cj1386	29	29	29	29	29	29	29	12	2	Cj1386, an atypical hemin-binding protein, mediates hemin trafficking to KatA in *Campylobacter jejuni*
Cj1387c	29	29	29	29	29	29	29	17	0	Cj1387c	29	29	29	29	29	29	27	16	0	Cj1387c (YheO-like PAS6 domain linked to a helix-turn-helix domain) modulates post-translational modification of the flagella (Reuter et al., 2015 Front. Microbiol.)
Cj1388	29	25	0	0	0	0	0	0	0	Cj1388	29	29	0	0	0	0	0	0	0	endoribonuclease L-PSP (Endoribonuclease active on single-stranded mRNA. Inhibits protein synthesis by cleavage of mRNA)
Cj1399c	29	28	27	27	0	0	0	0	0	Cj1399c	29	28	27	0	0	0	0	0	0	*hydA2* (Ni/Fe-hydrogenase small subunit)
Cj1400c	28	28	22	0	0	0	0	0	0	Cj1400c	28	28	25	0	0	0	0	0	0	*fabI* (enoyl-ACP reductase; fatty acid biosynthesis)
Cj1401c	29	29	28	28	0	0	0	0	0	Cj1401c	28	28	0	0	0	0	0	0	0	*tpiA* (triosephosphate isomerase)
Cj1402c	29	29	0	0	0	0	0	0	0	Cj1402c	29	29	4	0	0	0	0	0	0	*pgk* (phosphoglycerate kinase)
Cj1404	29	29	26	0	0	0	0	0	0											*nadD* (nicotinate-nucleotide adenylyltransferase; central role in the synthesis of the redox cofactor NAD+)
Cj1413c	29	29	29	29	24	0	0	0	0	Cj1413c	29	29	29	29	24	9	0	0	0	*kpsS* (capsule polysaccharide modification protein)

Cj1416c	29	29	0	0	0	0	0	0	0	Cj1416c	29	29	0	0	0	0	0	0	0	sugar nucleotidyltransferase
Cj1443c	29	29	0	0	0	0	0	0	0											*kpsF* (D-arabinose 5-phosphate isomerase)
Cj1444c	29	29	29	29	29	28	0	0	0	Cj1444c	29	29	29	29	0	0	0	0	0	*kpsD* (capsule polysaccharide ABC transporter substrate-binding protein)
Cj1445c	28	28	28	22	0	0	0	0	0	Cj1445c	28	28	22	0	0	0	0	0	0	*kpsE* (capsule polysaccharide ABC transporter permease)
Cj1447c	28	28	0	0	0	0	0	0	0											*kpsT* (capsule polysaccharide ABC transporter ATP-binding protein)
Cj1449c	28	26	0	0	0	0	0	0	0											hypothetical protein
Cj1450	28	28	25	0	0	0	0	0	0	Cj1450	28	28	28	28	17	0	0	0	0	ATP/GTP-binding protein
Cj1451	28	27	20	0	0	0	0	0	0	Cj1451	28	25	16	0	0	0	0	0	0	*dut* (dUTPase, dNTP biosynthesis)
Cj1459	26	26	1	0	0	0	0	0	0	Cj1459	29	26	26	1	0	0	0	0	0	hypothetical protein
Cj1534c	26	26	25	24	22	0	0	0	0	Cj1534c	26	26	25	25	22	22	0	0	0	bacterioferritin (iron binding)
Cj1536c	29	29	29	29	29	29	0	0	0	Cj1536c	29	29	29	29	29	28	0	0	0	*galU* (UTP--glucose-1-phosphate uridylyltransferase)
Cj1574c	26	26	26	25	25	0	0	0	0	Cj1574c	26	26	25	0	0	0	0	0	0	hypothetical protein
										Cj1575c	29	29	0	0	0	0	0	0	0	hypothetical protein
Cj1577c	29	29	28	28	0	0	0	0	0	Cj1577c	29	29	28	28	0	0	0	0	0	*nuoC* (NADH-quinone oxidoreductase subunit C, catalyzes the transfer of electrons from NADH to ubiquinone)
Cj1578c	28	28	26	26	26	0	0	0	0	Cj1578c	28	28	26	26	0	0	0	0	0	*nuoB* (NADH-quinone oxidoreductase subunit B; The point of entry for the majority of electrons that traverse the respiratory chain eventually resulting in the reduction of oxygen)
Cj1579c	28	28	28	28	28	28	0	0	0	Cj1579c	28	28	28	28	28	28	0	0	0	*nuoA* (NADH dehydrogenase I chain A)
Cj1586	29	29	1	1	0	0	0	0	0											*cgb* (single domain hemoglobin)
Cj1588c	26	26	21	4	4	1	0	0	0	Cj1588c	26	26	26	5	4	4	3	0	0	Major facilitator transport protein for small solutes
Cj1638	29	29	29	29	1	0	0	0	0	Cj1638	29	29	29	12	0	0	0	0	0	*dnaG* (DNA primase)
										Cj1640	29	27	6	0	0	0	0	0	0	hypothetical protein
Cj1641	29	29	29	25	25	20	10	0	0	Cj1641	29	29	29	25	25	16	1	0	0	*murE* (peptidoglycane synthesis)
Cj1642	25	25	25	3	0	0	0	0	0	Cj1642	25	25	14	3	0	0	0	0	0	nucleoid-associated protein
Cj1643	29	29	29	29	29	29	29	25	0	Cj1643	29	29	29	29	29	29	25	0	0	putative periplasmic protein
Cj1644	29	29	29	28	28	28	0	0	0	Cj1644	29	29	28	28	1	0	0	0	0	*ispA* (geranyltranstransferase)
Cj1645	29	29	25	1	1	1	1	0	0	Cj1645	29	29	18	1	1	1	0	0	0	*tkt* (transketolase)
Cj1650	28	28	28	28	28	28	28	0	0	Cj1650	28	28	28	28	28	28	1	0	0	hypothetical protein
Cj1651c	29	29	29	29	29	29	29	28	0	Cj1651c	29	29	29	29	29	29	28	2	0	methionine aminopeptidase
Cj1652c	29	29	29	29	29	28	28	0	0	Cj1652c	29	29	29	29	29	29	28	0	0	glutamate racemase
										Cj1681c	29	29	0	0	0	0	0	0	0	*cysQ* (3'(2'),5'-bisphosphate nucleotidase CysQ)
Cj1684c	29	29	29	29	0	0	0	0	0	Cj1684c	29	29	29	29	0	0	0	0	0	transmembrane transport protein
Cj1687	29	29	29	29	29	29	29	0	0	Cj1687	29	29	29	29	29	29	0	0	0	putative efflux protein
										Cj1704c	29	29	0	0	0	0	0	0	0	*rplB* (50S ribosomal protein L2)

Gene/Coverage 16-mer	k-mer coverage in RM4661	EggNOG (4.5.1); catagory code	EggNOG (4.5.1); catagory text	Functional categories
Cj0026c	0.202	F	Nucleotide transport and metabolism	DNA metabolism and repair
Cj0027	0.203	F	Nucleotide transport and metabolism	DNA metabolism and repair
Cj0028	0.499	L	Replication, recombination and repair	DNA metabolism and repair
Cj0029	0.356	EJ	Amino acid transport and metabolism; Translation, ribosomal structure and biogenesis	Protein synthesis, Amino Acid metabolism
Cj0059c	0.586	N	Cell motility	cell motility
Cj0063c	0.292	D	Cell cycle control, cell division, chromosome partitioning	signal transduction
Cj0069	0.207	S	Function unknown	unknown
Cj0070c	0.887	S	Function unknown	unknown
Cj0081	0.316	C	Energy production and conversion	stress response (oxidative)
Cj0085c	0.687	M	Cell wall/membrane/envelope biogenesis	Cell wall/membrane/capsule
Cj0086c	0.329	L	Replication, recombination and repair	DNA metabolism and repair
Cj0087	0.263	E	Amino acid transport and metabolism	Protein synthesis, Amino Acid metabolism
		J	Translation, ribosomal structure and biogenesis	Protein synthesis, Amino Acid metabolism
Cj0131	0.733	M	Cell wall/membrane/envelope biogenesis	unknown
Cj0194	0.616	F	Nucleotide transport and metabolism	Metabolism of cofactors and vitamins
Cj0196c	0.354	F	Nucleotide transport and metabolism	DNA metabolism and repair
Cj0197c	0.587	E	Amino acid transport and metabolism	Protein synthesis, Amino Acid metabolism
Cj0198c	0.233	L	Replication, recombination and repair	DNA metabolism and repair
Cj0203	0.800	C	Energy production and conversion	membrane transport
Cj0237	0.388	P	Inorganic ion transport and metabolism	stress response (oxidative)
Cj0254	0.363	S	Function unknown	unknown
Cj0266c	0.341	S	Function unknown	membrane transport
Cj0283c	0.931	NT	Cell motility; Signal transduction mechanisms	cell motility
Cj0284c	0.235	T	Signal transduction mechanisms	cell motility
Cj0285c	0.282	T	Signal transduction mechanisms	cell motility
Cj0286c	0.275	S	Function unknown	unknown
Cj0379c	0.790	S	Function unknown	unknown
		H	Coenzyme transport and metabolism	Metabolism of cofactors and vitamins
Cj0387	*0.163*	F	Nucleotide transport and metabolism	Protein synthesis, Amino Acid metabolism

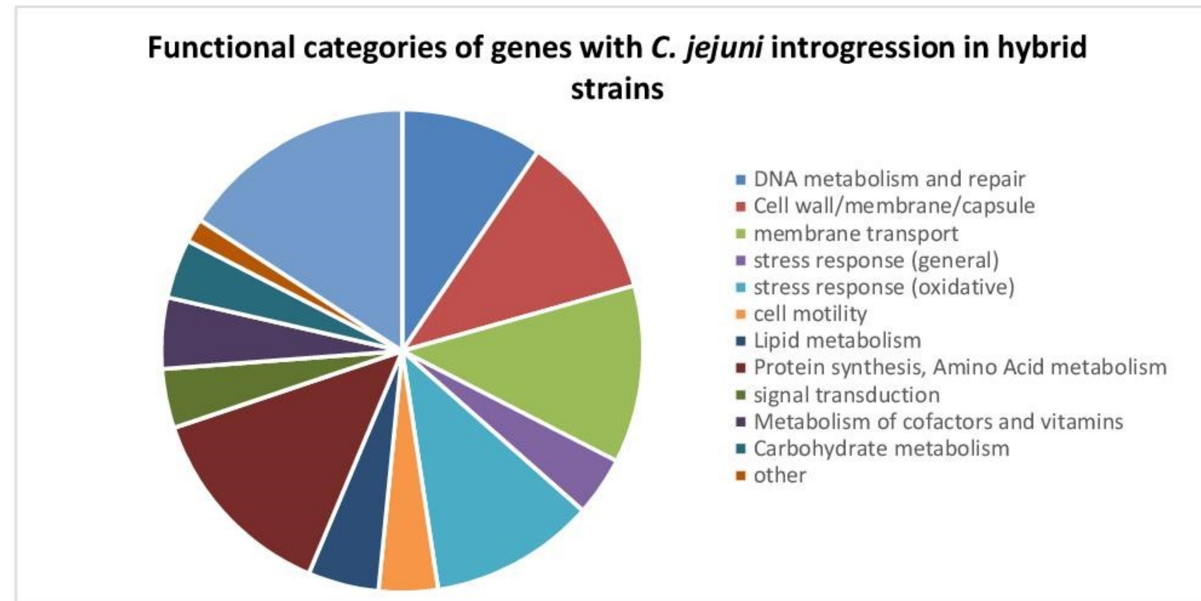
Cj0435	0.355	IQ	Lipid transport and metabolism; Secondary metabolites biosynthesis, transport, and catabolism	Lipid metabolism
Cj0441	0.282	IQ	Lipid transport and metabolism; Secondary metabolites biosynthesis, transport, and catabolism	Lipid metabolism
Cj0442	0.688	I	Lipid transport and metabolism	Lipid metabolism
Cj0511	0.267	M	Cell wall/membrane/envelope biogenesis	Protein synthesis, Amino Acid metabolism
Cj0517	0.322	D	Cell cycle control, cell division, chromosome partitioning	membrane transport
Cj0552	0.898	S	Function unknown	membrane transport
Cj0553	0.759	S	Function unknown	membrane transport
Cj0554	0.671	S	Function unknown	unknown
Cj0555	0.678	P	Inorganic ion transport and metabolism	membrane transport
Cj0556	0.639	S	Function unknown	unknown
Cj0557c	0.693	S	Function unknown	membrane transport
Cj0630c	0.458	L	Replication, recombination and repair	DNA metabolism and repair
Cj0632	0.322	H	Coenzyme transport and metabolism	Protein synthesis, Amino Acid metabolism
Cj0685c	0.709	S	Function unknown	other
Cj0686	0.771	I	Lipid transport and metabolism	Lipid metabolism
Cj0763c	0.429	E	Amino acid transport and metabolism	Protein synthesis, Amino Acid metabolism
Cj0764c	0.593	H	Coenzyme transport and metabolism	Protein synthesis, Amino Acid metabolism
Cj0765c	0.200	J	Translation, ribosomal structure and biogenesis	Protein synthesis, Amino Acid metabolism
Cj0766c	0.276	F	Nucleotide transport and metabolism	DNA metabolism and repair
Cj0776c	0.757	S	Function unknown	unknown
Cj0832c	0.358	C	Energy production and conversion	membrane transport
Cj0833c	0.808	S	Function unknown	stress response (oxidative)
Cj0839c	0.753	S	Function unknown	unknown
Cj0840c	0.418	G	Carbohydrate transport and metabolism	Carbohydrate metabolism
Cj0841c	0.431	H	Coenzyme transport and metabolism	Metabolism of cofactors and vitamins
Cj0995c	0.511	H	Coenzyme transport and metabolism	Metabolism of cofactors and vitamins
Cj0996	0.731	F	Nucleotide transport and metabolism	DNA metabolism and repair
Cj0997	0.399	J	Translation, ribosomal structure and biogenesis	Protein synthesis, Amino Acid metabolism
Cj0998c	0.538	S	Function unknown	unknown
Cj1058c	0.391	F	Nucleotide transport and metabolism	DNA metabolism and repair
		H	Coenzyme transport and metabolism	Metabolism of cofactors and vitamins
Cj1108	*0.173*	O	Post-translational modification, protein turnover, and chaperones	stress response (general)
Cj1109	0.813	O	Post-translational modification, protein turnover, and chaperones	Protein synthesis, Amino Acid metabolism

Cj1110c	0.847	NT	Cell motility; Signal transduction mechanisms	signal transduction
Cj1111c	0.805	U	Intracellular trafficking, secretion, and vesicular transport	membrane transport
Cj1112c	0.892	C	Energy production and conversion	stress response (oxidative)
Cj1113	0.664	S	Function unknown	unknown
Cj1114c	0.248	I	Lipid transport and metabolism	Lipid metabolism
Cj1128c	*0.122*	M	Cell wall/membrane/envelope biogenesis	Cell wall/membrane/capsule
Cj1129c	0.632	M	Cell wall/membrane/envelope biogenesis	Cell wall/membrane/capsule
Cj1130c	0.585	P	Inorganic ion transport and metabolism	Cell wall/membrane/capsule
Cj1131c	0.471	M	Cell wall/membrane/envelope biogenesis	Cell wall/membrane/capsule
Cj1134	0.495	M	Cell wall/membrane/envelope biogenesis	stress response (general)
Cj1182c	0.268	J	Translation, ribosomal structure and biogenesis	Protein synthesis, Amino Acid metabolism
Cj1188c	0.409	D	Cell cycle control, cell division, chromosome partitioning	Protein synthesis, Amino Acid metabolism
Cj1220	0.540	O	Post-translational modification, protein turnover, and chaperones	stress response (general)
Cj1221	0.405	O	Post-translational modification, protein turnover, and chaperones	stress response (general)
Cj1227c	0.335	K	Transcription	signal transduction
Cj1228c	0.561	M	Cell wall/membrane/envelope biogenesis	stress response (general)
Cj1257c	0.495	EGP	Amino acid transport and metabolism; Carbohydrate transport and metabolism; Inorganic ion transport and metabolism	membrane transport
Cj1258	0.208	T	Signal transduction mechanisms	signal transduction
Cj1267c	*0.125*	C	Energy production and conversion	stress response (oxidative)
Cj1283	0.647	P	Inorganic ion transport and metabolism	membrane transport
Cj1284	0.811	P	Inorganic ion transport and metabolism	membrane transport
Cj1364c	0.292	C	Energy production and conversion	Carbohydrate metabolism
Cj1385	0.524	C	Energy production and conversion	stress response (oxidative)
Cj1386	0.376	S	Function unknown	stress response (oxidative)
Cj1387c	0.825	S	Function unknown	cell motility
		J	Translation, ribosomal structure and biogenesis	Protein synthesis, Amino Acid metabolism
		C	Energy production and conversion	stress response (oxidative)
		I	Lipid transport and metabolism	Lipid metabolism
		G	Carbohydrate transport and metabolism	Carbohydrate metabolism
		F		Carbohydrate metabolism
		H	Coenzyme transport and metabolism	stress response (oxidative)
		M	Cell wall/membrane/envelope biogenesis	Cell wall/membrane/capsule

Cj1416c	0.248	M	Cell wall/membrane/envelope biogenesis	Cell wall/membrane/capsule
Cj1443c	*0.156*	M	Cell wall/membrane/envelope biogenesis	Cell wall/membrane/capsule
		M	Cell wall/membrane/envelope biogenesis	Cell wall/membrane/capsule
		M	Cell wall/membrane/envelope biogenesis	Cell wall/membrane/capsule
		GM	Carbohydrate transport and metabolism; Cell wall/membrane/envelope biogenesis	Cell wall/membrane/capsule
		S	Function unknown	unknown
		S	Function unknown	signal transduction
		S	Function unknown	DNA metabolism and repair
		S	Function unknown	unknown
Cj1534c	0.313	P	Inorganic ion transport and metabolism	stress response (oxidative)
Cj1536c	0.684	M	Cell wall/membrane/envelope biogenesis	Cell wall/membrane/capsule
Cj1574c	0.560	C	Energy production and conversion	unknown
		S	Function unknown	unknown
Cj1577c	0.467	C	Energy production and conversion	stress response (oxidative)
Cj1578c	0.516	C	Energy production and conversion	stress response (oxidative)
Cj1579c	0.667	C	Energy production and conversion	stress response (oxidative)
Cj1586	0.201	C	Energy production and conversion	stress response (oxidative)
Cj1588c	0.360	EGP	Amino acid transport and metabolism; Carbohydrate transport and metabolism; Inorganic ion transport and metabolism	membrane transport
Cj1638	0.444	L	Replication, recombination and repair	DNA metabolism and repair
Cj1640	*0.111*	S	Function unknown	unknown
Cj1641	0.604	M	Cell wall/membrane/envelope biogenesis	Cell wall/membrane/capsule
Cj1642	0.388	S	Function unknown	unknown
Cj1643	0.833	M	Cell wall/membrane/envelope biogenesis	unknown
Cj1644	0.636	H	Coenzyme transport and metabolism	Metabolism of cofactors and vitamins
Cj1645	0.300	G	Carbohydrate transport and metabolism	Carbohydrate metabolism
Cj1650	0.762	S	Function unknown	unknown
Cj1651c	0.860	E	Amino acid transport and metabolism	Protein synthesis, Amino Acid metabolism
Cj1652c	0.722	M	Cell wall/membrane/envelope biogenesis	Cell wall/membrane/capsule
		P	Inorganic ion transport and metabolism	other
Cj1684c	0.431	P	Inorganic ion transport and metabolism	membrane transport
Cj1687	0.689	EGP	Amino acid transport and metabolism; Carbohydrate transport and metabolism; Inorganic ion transport and metabolism	membrane transport
		J	Translation, ribosomal structure and biogenesis	Protein synthesis, Amino Acid metabolism

Suppl. Tab. S2: Sheet2 “Overview func. cat.”

functional category	number
DNA metabolism and repair	12
Cell wall/membrane/capsule	14
membrane transport	15
stress response (general)	5
stress response (oxidative)	14
cell motility	5
Lipid metabolism	6
Protein synthesis, Amino Acid metabolism	17
signal transduction	5
Metabolism of cofactors and vitamins	6
Carbohydrate metabolism	5
other	2
unknown	20
sum	126



8.2.4 Publication 5: "Take it or leave it"-Factors regulating competence development and DNA uptake in *Campylobacter jejuni*.

Table S1: Generation time and final pH of BfR-CA-14430 grown under different conditions as shown in Figures 4, 5 and S3 Mean generation time for CO_2 conditions was calculated for each experiment for pH range of 6.5-8; nd, not determined.

condition	experiment	generation time	final pH	mean generation time per condition	SD generation time per condition
7% CO_2	day 1	1.125	6.28	1.136	0.10183388
7% CO_2	day 1	1.119	6.52		
7% CO_2	day 1	1.110	6.62		
7% CO_2	day 1	1.140	7.01		
7% CO_2	day 1	1.129	7.08		
7% CO_2	day 2	1.244	6.81		
7% CO_2	day 2	1.214	6.88		
7% CO_2	day 2	1.246	7.12		
7% CO_2	day 2	1.205	7.24		
7% CO_2	day 2	1.298	7.36		
7% CO_2	day 2	1.263	7.42		
7% CO_2	day 2	1.299	7.51		
7% CO_2	day 2	1.291	7.60		
7% CO_2	day 2	1.347	7.71		
7% CO_2	day 3	1.057	7.27		
7% CO_2	day 3	1.064	7.43		
7% CO_2	day 3	1.065	7.55		
7% CO_2	day 3	1.086	7.30		
7% CO_2	day 3	1.046	7.45		
7% CO_2	day 3	1.063	7.57		
7% CO_2	day 3	1.032	6.64		
7% CO_2	day 3	1.026	7.10		
7% CO_2	day 3	1.013	7.29		
7% CO_2	day 3	1.037	7.56		
7% CO_2	day 4	1.203	6.67		
7% CO_2	day 4	1.219	7.30		
7% CO_2	day 4	1.240	7.55		
7% CO_2	day 4	nd	7.68		
7% CO_2	day 5	1.088	6.63		
7% CO_2	day 5	1.039	7.01		
7% CO_2	day 5	1.036	7.21		
7% CO_2	day 5	1.061	7.48		
7% CO_2	day 6	0.971	7.45		
7% CO_2	day 7	1.100	7.52		
0% CO_2	day 4	3.411	7.11	3.352	0.68912011
0% CO_2	day 4	3.144	7.30		
0% CO_2	day 6	2.523	7.70		
0% CO_2	day 7	4.667	6.67		
0% CO_2	day 7	3.671	7.25		
0% CO_2	day 7	3.207	7.56		
0% CO_2	day 7	2.840	7.81		
0% CO_2	day 7	5.597	8.23		
1% CO_2	day 2	1.025	7.12	1.122	0.11010026
1% CO_2	day 2	1.052	6.91		
1% CO_2	day 2	1.082	6.56		
1% CO_2	day 2	1.045	7.21		
1% CO_2	day 2	1.034	7.41		

1% CO_2	day 2	1.022	7.65		
1% CO_2	day 2	1.037	7.84		
1% CO_2	day 2	1.077	7.95		
1% CO_2	day 2	1.179	8.11		
1% CO_2	day 4	1.242	7.11		
1% CO_2	day 4	1.251	7.27		
1% CO_2	day 4	1.252	7.60		
1% CO_2	day 4	1.370	7.87		
1% CO_2	day 5	1.032	7.08		
1% CO_2	day 5	1.063	7.28		
1% CO_2	day 5	1.138	7.57		
1% CO_2	day 5	1.230	7.88		
15% CO_2	day 5	1.180	6.39	1.141	0.027423
15% CO_2	day 5	1.138	6.90		
15% CO_2	day 5	1.116	7.16		
15% CO_2	day 5	1.130	7.42		
35% CO_2	day 4	1.567	6.14	1.514	0.11842482
35% CO_2	day 4	1.379	6.73		
35% CO_2	day 4	1.598	6.93		
50% CO_2	day 4	1.629	6.04	1.558	0.327892
50% CO_2	day 4	1.427	6.72		
50% CO_2	day 4	2.143	6.93		
50% CO_2	day 5	1.278	6.02		
50% CO_2	day 5	1.255	6.78		
50% CO_2	day 5	1.613	6.98		
50% CO_2	day 5	nd	7.14		
pH 5.7	day 21	1.482	5.75	1.929	0.4960276
pH 5.7	day 22	1.842	5.68		
pH 5.7	day 23	2.463	5.75		
pH 6.3	day 9	1.090	6.36	1.189	0.11983312
pH 6.3	day 10	1.166	6.36		
pH 6.3	day 11	1.108	6.28		
pH 6.3	day 12	1.156	6.30		
pH 6.3	day 15	1.261	6.27		
pH 6.3	day 17	1.237	6.21		
pH 6.3	day 18	1.392	6.34		
pH 6.3	day 19	1.292	6.41		
pH 6.3	day 20	1.361	6.24		
pH 6.3	day 24	1.099	6.38		
pH 6.3	day 26	1.108	6.38		
pH 6.3	day 27	0.998	6.42		
pH 7.5/37°C	day 8	1.198	7.53	1.176	0.10480859
pH 7.5/37°C	day 9	0.971	7.45		
pH 7.5/37°C	day 10	1.121	7.44		
pH 7.5/37°C	day 11	1.346	7.46		
pH 7.5/37°C	day 12	1.234	7.48		
pH 7.5/37°C	day 13	1.100	7.52		
pH 7.5/37°C	day 14	1.208	7.51		
pH 7.5/37°C	day 15	0.929	7.51		
pH 7.5/37°C	day 16	1.102	7.57		
pH 7.5/37°C	day 17	1.214	7.51		
pH 7.5/37°C	day 18	1.130	7.56		
pH 7.5/37°C	day 19	1.346	7.54		
pH 7.5/37°C	day 20	1.186	7.46		
pH 7.5/37°C	day 22	1.160	7.51		

pH 7.5/37°C	day 23	1.209	7.48		
pH 7.5/37°C	day 24	1.267	7.58		
pH 7.5/37°C	day 25	1.235	7.59		
pH 7.5/37°C	day 26	1.185	7.59		
pH 7.5/37°C	day 27	1.209	7.60		
42°C	day 12	0.891	7.51	0.916	0.14744584
42°C	day 13	0.782	7.45		
42°C	day 14	1.074	7.50		
32°C	day 9	3.038	7.49	3.141	0.31153584
32°C	day 16	3.491	7.53		
32°C	day 17	2.893	7.52		

Supplementary Figures

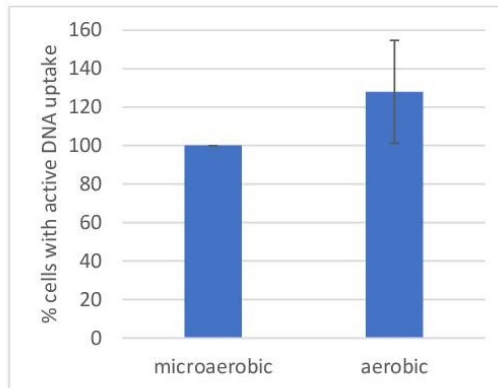


Figure S1. DNA uptake is favored under aerobic compared to microaerobic conditions. Two aliquots of competent *C. jejuni* BfR-CA-14430 were incubated with fluorescent DNA under microaerobic (set to 100%) or aerobic conditions. n = 20, error bars indicate standard deviation.

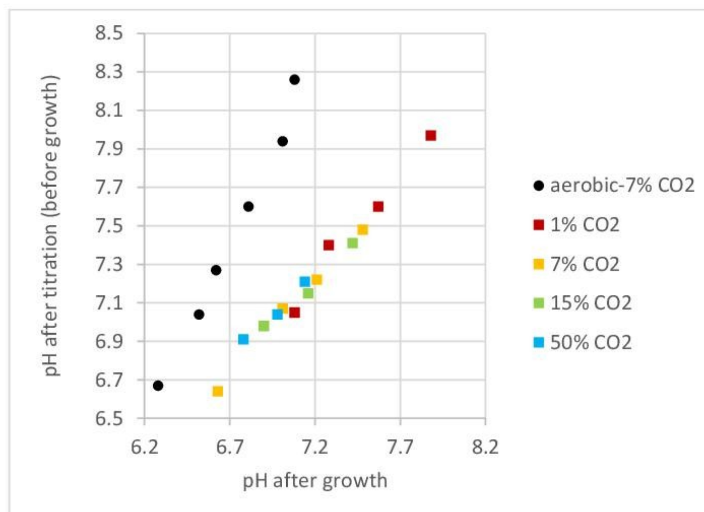


Figure S2. pH of pH-adjusted growth medium before and after growth of *C. jejuni* to exponential phase. Due to low number of bacterial cells in exponential phase, the pH of the growth medium remained constant but dependent on CO_2 concentration. Bolton broth was titrated with HCl or NaOH under aerobic conditions and incubated for at least 30 min under the indicated microaerobic conditions before pH was measured (time point "before growth", y-axis). In order to show the influence of microaerobic incubation on pH reduction, one medium was measured before growth under aerobic conditions (black dots). *C. jejuni* BfR-CA-14430 was grown until exponential growth phase and pH was measured "after growth" (x-axis). Atmospheres contained 3.5% H_2, 6% O_2 and the indicated CO_2 concentration (red, 1% CO_2, yellow, 7% CO_2, green, 15% CO_2, blue, 50% CO_2) and corresponding N_2 levels. pH after growth was measured within 5 min after opening the jar (see Fig. S5).

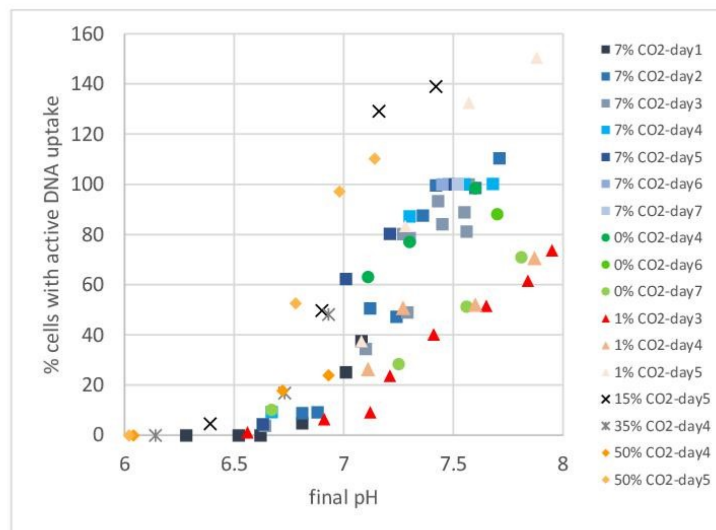


Figure S3. pH-dependent competence development in *C. jejuni* BfR-CA-14430 is independent of CO_2 concentration. Fraction of competent cells grown for 18 ± 4 h in Bolton to indicated final pH values into exponential phase were normalized per experimental day to the pH 7.5 condition at 7% CO_2 (set to 100%). DNA uptake was performed under microaerobic conditions. All tested atmospheres contained 3.5% H_2 and 6% O_2 and varying levels of CO_2 (and corresponding N_2). circles/green colors, 0% CO_2; triangles/red colors, 1% CO_2; squares/blue colors, 7% CO_2; cross/dark grey, 15% CO_2; crosses/light grey with orthogonal bar, 35% CO_2; rhombus/orange colors, 50% CO_2. Generation times for all conditions were 1.3 h ± 0.3 h, except for the atmosphere containing 0% CO_2 and 50 % CO_2., under which generation times were enhanced up to 4.7 h or 2.1 h, respectively (see also Tab. S1).

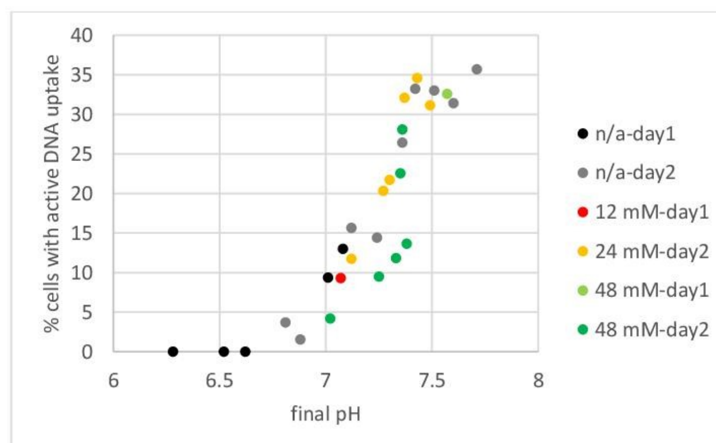


Figure S4. Competence development depends on pH but not on the concentration of sodium hydrogen carbonate ($NaHCO_3$). *C. jejuni* BfR-CA-14430 was grown for 18 ± 4 h in Bolton broth supplemented with $NaHCO_3$. n/a, no addition. Data are shown from two independent experiments (day1 and day2).

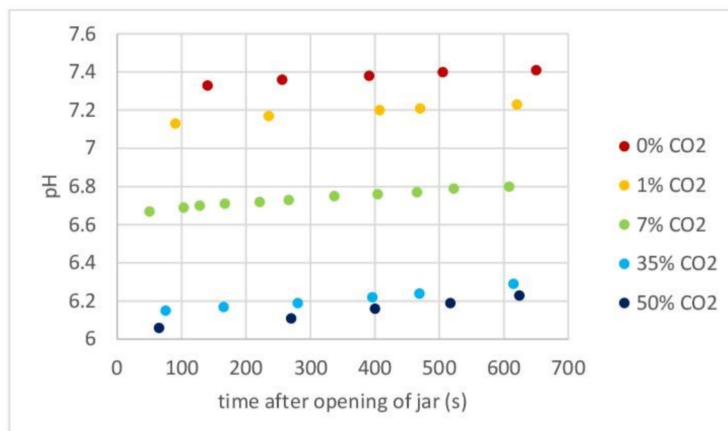


Figure S5. pH change of Bolton broth as a function of time after opening the jar. pH of Bolton was measured for around 10 min after opening the jar containing 3.5% H_2, 6% O_2 with the indicated CO_2 concentration and corresponding N_2 levels under constant agitation.

8.2.5 **Danksagung**

Zu allererst gilt mein Dank meiner Erstgutachterin Frau Dr. Kerstin Stingl für die Möglichkeit dieses interessante Thema im Rahmen einer Promotion im Nationalen Referenzlabor für *Campylobacter* untersuchen zu dürfen. Ich danke ihr für die fortwährende Unterstützung und Betreuung meiner Arbeit und zugleich dem mir entgegengebrachten Vertrauen und der Freiheit verschiedenen Fragestellungen und Seitenprojekten nachgegangen sein zu dürfen.

Mein Dank gilt Herrn Prof. Dr. Reinhard Kunze für die Übernahme des Zweitgutachten meiner Doktorarbeit.

Ferner danke ich allen aktuellen und ehemaligen Mitgliedern des Nationalen Referenzlabors für *Campylobacter* für die sehr angenehme Arbeitsatmosphäre und die große Hilfsbereitschaft untereinander. Danke vor allem an Christiane, Maja und Marie, die mich die gesamte Zeit meiner Promotion begleitet haben.

Ich danke Herrn Dr. Matthias Fischer und Sarah für das Lesen meiner Arbeit und die wertvollen Hinweise.

Mein Dank gilt ferner den Kolleginnen und Kollegen aus der Fachgruppe 41 und der Nachwuchsgruppe 4NG für die schöne Zeit in den Pausen.

Darüber hinaus danke ich allen Mitgliedern des PAC-CAMPY Konsortiums für die gute Zusammenarbeit und dem Bundesministerium für Bildung und Forschung für die Finanzierung.

Mein besonderer Dank gilt meiner Familie vor allem meinen Eltern, die mich immer unterstützen und mir diesen Weg erst ermöglicht haben. Ich danke außerdem all meinen Freunden, die mich teilweise schon über so viele Jahre begleiten.