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Autoinducer 2 in *Campylobacter jejuni*

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List of Abbreviations

AB	Autoinducer bioassay medium
AHL	acyl- homoserin- lactone
AI	Autoinducer
AMC	activated methyl cycle
BBA	BB containing 0.4% agar
BB	Brucella broth
CFS	cell free supernatants
Cm	chloramphenicol
Cytolethal distending toxin	Cytolethal distending toxin
DPD	4,5-dihydroxyl-2,3-pentanedion
HC	homocysteine
HSL	N–(3-hydroxy-butanosyl)-L-homoserine lactone
Km	kanamycin
MH	Mueller-Hinton-Broth
MHA	MH containing 0.4% agar
Pfs	5´methylthioadenosine/S-adenosyl-homocysteine nucleosidase
Qrr	Quorum regulatory RNAs
QS	Quorum sensing
R-THMF	R-2-methyl-2,3,3,4-tetrahydroxytetrahydrofuran
SAH	S-adenosyl-homocysteine
SAM	S-adenosyl-methionine
sRNAs	small regulatory RNAs
S-THMF-borate	S-2-methyl-2,3,3,4-tetrahydroxytetrahydrofuran-borate
TCA	tricarboxylic acid cycle
VBNC	viable but not culturable

Abstract

Autoinducer 2 in *C. jejuni*

The ability of *Campylobacter* (*C.*) *jejuni*, the leading cause of food borne bacterial enteritis worldwide, to produce the Quorum sensing molecule autoinducer-2 (AI-2) provides new insights into the mechanisms by which *C. jejuni* regulates its behavior. The AI-2 mediated Quorum sensing system is widely conserved over both gram-negative and gram-positive bacteria and has been demonstrated to play a critical role in the environmental adaptation of other enteric pathogens such as *Escherichia coli* and *Salmonella* spp. Since the discovery of a *luxS* gene in the *C. jejuni* genome, which is capable of producing AI-2, various studies have been conducted to explore the function and role of AI-2 in *C. jejuni*.

AI-2 is a byproduct of the conversion of s-ribosylhomocystein into homocystein in the methionine cycle. Therefore, the *C. jejuni luxS* mutant phenotypes can either be a result of a changed metabolism or the absence of AI-2. Most studies lack sufficient complementation resulting in not knowing whether phenotypes of *luxS* mutants should be attributed to a disrupted metabolism or a lack of AI-2. Furthermore, the analysis of phenotypes of the existing *C. jejuni luxS* mutant could be influenced by differences in strain background, kind of mutation and culture conditions. Additionally, no AI-2 receptor has been found yet for *Campylobacter*. All this contributes to an extensive discussion about the exact role of AI-2 in *C. jejuni*. Our work addresses two critical questions regarding AI-2 mediated Quorum sensing of *C. jejuni*.

First, we provide insight as to why literature about phenotypes of *C. jejuni luxS* mutants is extremely contradictory. Further, some *luxS* mutant phenotypes could be partially complemented by AI-2, suggesting that *C. jejuni* can regulate its behavior by AI-2 dependent Quorum sensing.

Secondly, we demonstrate that AI-2 was not actively taken up by *C. jejuni*, so further search of AI-2 receptors in *C. jejuni* should focus on two-component signaling systems or chemoreceptors rather than transporter systems.

Zusammenfassung

Autoinducer 2 bei *C. jejuni*

Campylobacter (*C.*) *jejuni* ist der häufigste, durch Lebensmittel übertragene, bakterieller Auslöser einer Enteritis. Seine Fähigkeit das Quorum Sensing (QS) Molekül Autoinducer-2 (AI-2) herzustellen, eröffnet neue Ansätze für die Forschung von Anpassungsmechanismen bei *C. jejuni*. Sowohl bei gram-negativen als auch bei gram-positiven Bakterien sind AI-2 vermittelte QS-Systeme weit verbreitet. Bei anderen Spezies wie z.B. *Escherichia coli* oder *Salmonella* spp. spielen AI-2 vermittelte QS-Systeme eine entscheidende Rolle bei der Anpassung an Umwelteinflüsse. Seit im Genom von *C. jejuni* das *luxS*-Gen gefunden wurde, welches für die AI-2 Produktion verantwortlich ist, haben sich zahlreiche Studien mit der Funktion und Rolle von AI-2 in *C. jejuni* befasst. AI-2 entsteht als Beiprodukt während der Umwandlung von S- Ribosylhomocystein zu Homocystein im Methioninzyklus. Somit können die Phänotypen von *luxS*-Mutanten durch den veränderten Stoffwechsel oder den Mangel an AI-2 bedingt sein. In den meisten Studien wird nicht ausreichend komplementiert, wodurch unklar bleibt ob die beobachteten Phänotypen der *luxS*-Mutanten auf einen allgemein veränderten Stoffwechsel oder auf einen Mangel an AI-2 zurückzuführen sind. Die Phänotypen von *C. jejuni luxS*-Mutanten könnten zudem durch stammspezifische Unterschiede, die Mutationsart oder abweichende Kulturbedingungen beeinflusst werden. Außerdem wurde bisher kein AI-2-Rezeptor bei *Campylobacter* gefunden. Diese Situation führt zu einer intensiven Diskussion über die genaue Rolle von AI-2 in *C. jejuni*. Unsere Studie befasst sich mit zwei Kernfragen bzgl. AI-2 vermittelten QS Prozessen in *C. jejuni*.

Erstens untersuchten wir die Gründe warum sich vorhandene Studien bzgl. Phänotypen von *C. jejuni luxS*-Mutanten so stark widersprechen. Wir konnten weiterhin zeigen, dass einige Phänotypen von *luxS*-Mutanten synthetisches AI-2 komplementiert werden konnten, was darauf schließen lässt, dass *C. jejuni* sein Verhalten mittels AI-2 abhängigem QS regulieren kann.

Zweitens zeigten wir, dass AI-2 nicht aktiv von *C. jejuni* aufgenommen wird. Somit sollte sich die weitere Suche nach AI-2-Rezeptoren in *C. jejuni* nicht auf Transportersysteme, sondern auf Zwei-Komponenten-Signalsysteme oder Chemorezeptoren konzentrieren.

Chapter 1: Introduction and Literature Review

1.1 Quorum sensing

Historically, bacterial cells were considered to be solitary individuals that do not interact with each other. In the 1960s, this simplistic view began to change with the observation of bioluminescence in the marine bacterium *Vibrio* (*V.*) *fischeri*, which ultimately lead to the discovery of Quorum Sensing (QS). QS is a regulatory mechanism of gene expression. In recent years, it has become evident that this mechanism allows bacteria to communicate with each other, enabling the microorganisms to adjust their activity at a multicellular level and in turn letting the bacteria coordinate their collective behavior. Today, QS is known to be common in bacteria and many different behavioral adjustments, e.g. in response to changes in environmental factors or population density are observed among a vast variety of bacteria.

1.2 Quorum sensing mediated processes

Processes controlled by QS are usually unproductive when undertaken by an individual bacterium but become effective when undertaken by a group. For example, virulent bacteria refrain from secreting toxins prematurely, to avoid elimination by the hosts immune system. The bacteria sense a sufficiently high cell-density, allowing them to produce toxins simultaneously and in a concerted effort. This way, the bacteria are in a better position to overpower the immune system.

QS regulated processes are for example production and secretion of virulence factors, biofilm formation, motility and bioluminescence (Passador *et al.* 1993, Davies *et al.* 1998, Sperandio *et al.* 2001, Miller *et al.* 2002, Engebrecht *et al.* 1983, Lilley and Bassler 2000, Elvers and Park 2002).

1.3 Quorum sensing systems: different signalling molecules

The term "Quorum" is derived from Latin and originally means „the number of participations that must be cast to be valid". The question "How do they know when the Quorum is reached?" arises. An integral part of QS are the signaling molecules. Increased synthesis of the signal molecule creates a positive feedback loop, which is why QS molecules are commonly called Autoinducers (AI) (Sifri 2008). QS involves the synthesis, secretion and detection of AI (Bassler 1999). The cells continuously secrete AI into the environment. Simultaneously, cells measure the AI concentration via specific receptors. During growth, the AI concentration increases in the cell environment and thus reflects the bacterial cell-density. At a critical threshold concentration, the AI is recognized by the cells and the expression of specific genes become up- or down regulated (Fuqua *et al.* 1994). Many pathogenic bacteria species have been proven to be able to communicate via QS. So far there are three different QS systems known, which are regulated through different Autoinducers:

(1) intraspecies specific communication: AI-1 (acyl- homoserine lactone: AHL) used by gram-negative bacteria, and oligopeptides used by gram-positive bacteria

(2) intra- as well as interspecies specific communication: AI-2 used by gram-negative and gram-positive bacteria

(3) interkingdom signaling system: AI-3 signaling is used as an interkingdom chemical signaling system between microbes and their hosts (mammals or plants) (Sperandio *et al.* 2001).

1.3.1 Autoinducer 1 and oligopeptides

Gram-negative bacteria

The principle of cell density dependent gene expression was first discovered in 1970 in the luminescent bacterium *V. fischeri* (Nealson *et al*. 1970). *V. fischeri* lives either in solitary or in the light organ of the Hawaiian squid *Euprymna scolopes*. Inside this organ, *V. fischeri* cells grow to high cell density, inducing genes which encode enzymes for

bioluminescence. The squid utilizes the light provided by *V. fischeri* for illumination to mask its shadow, thus allowing better predation. The bacteria also benefit since the light organ is rich in nutrients. In the light organ *V. fischeri* can achieve a cell density of 10^{11} cells/ml. At this point the bacteria start to illuminate. It is a cell density dependent regulation mechanism, which controls bioluminescence in *V. fischeri*. This regulation is enabled through the *lux*-operon *luxCDABE*. This operon includes the genes, which are necessary for bioluminescence and two regulatory genes, *luxI* and *luxR*. LuxI is the autoinducer synthase that catalyzes synthesis of the acyl- homoserine lactone (AHL) autoinducer. LuxR is the autoinducer receptor and transcriptional activator and is only active if an autoinducer is bound. The constitutive expression of *luxI* at low cell density leads to the production and secretion of AI-1, which can freely diffuse in the environment. With an increasing cell density the AI-1 accumulates extra- and intracellular. Upon reaching a critical threshold concentration, AI-1 binds to LuxR in the cytoplasm. LuxR is activated and functions as a transcriptional regulator by binding the promotor region of the *luxICDABE* operon, which controls the expression of QS target genes (Fig. 1). In this case the bacteria produce luminescence (Bassler *et al.* 1993).

Since the discovery of the LuxI/LuxR QS system many comparable systems have been discovered in different bacteria. AI-l structures of these signal molecules show identical homoserine- lactone backbone, but differ in length and structure of acyl-groups (Fuqua and Greenberg 2002). LuxI/LuxR like systems are associated with regulation processes like virulence factor production, chemotaxis and cell division (de Kievit and Iglewski 2000, Miller and Bassler 2001, Withers *et al.* 2001).

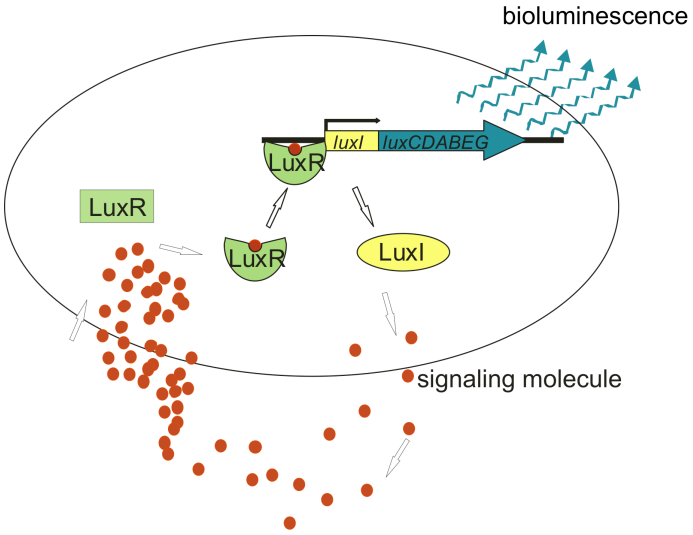

Figure 1: Mechanism of AI-1 mediated signalling in *V. fisheri* (Gölz *et al.* 2012). The AI-1 synthase LuxI produces AI-1 signaling molecules which diffuse across the cell wall. The complex of AI-1 molecules with the intracellular receptor LuxR functions as transcriptional activator of the *lux*-operon resulting in enhanced expression of *lux*I and other genes necessary for production of bioluminescence.

Gram-positive bacteria

QS in gram-positive bacteria involves a different type of signaling molecule. Typically gram-positive bacteria use small peptides as AI. The autoinducing peptide is secreted outside of the cell through an ATP-binding cassette (ABC) transporter. This peptide AI increases in concentration depending on cell density. The AI is bound by receptors present in the cell membrane. The receptor is a two-component-type membrane-bound sensor histidine kinase. If the AI binds to the histidine kinase, the receptor undergoes a conformational change that results in phosphorylation of proteins in the cytoplasm. Phosphorylation of a regulator protein activates itself, allows it to bind DNA and to transcribe the QS-controlled target genes (Fig. 2) (Kleerebezem *et al.* 1997).

QS regulates a variety of processes in gram-positive bacteria, for example competence for DNA uptake and sporulation in *Bacillus subtilis*, competence in *Streptococcus pneumonia* and virulence in *Staphylococcus* (Alloing *et al.* 1998, Hamoen *et al.* 2003, Xu *et al.* 2006).

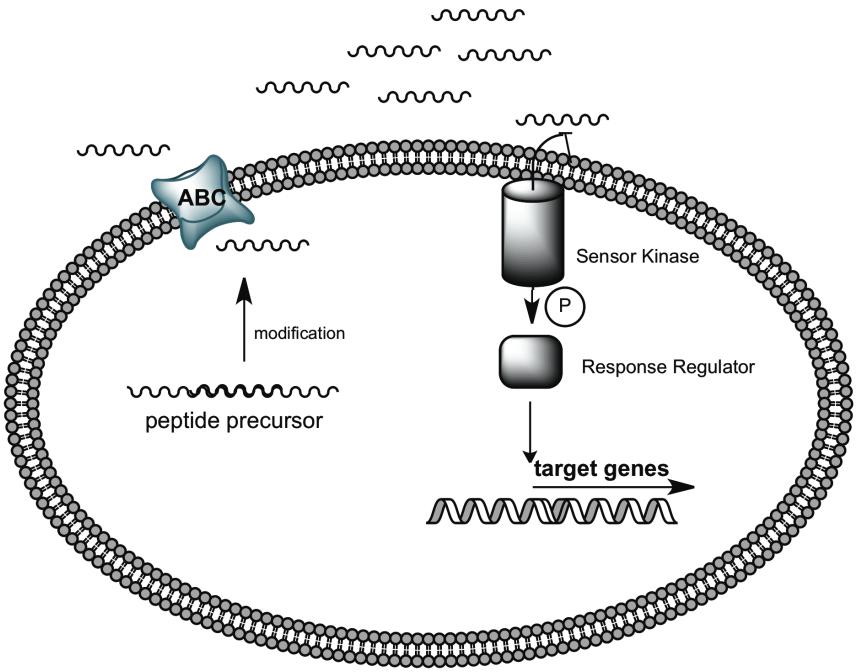

Figure 2: Peptide based quorum sensing in gram-positive bacteria: ABC- Transporters process and export peptide autoinducers. Peptides (wavy lines) are recognized by membrane bound two-component sensor kinase proteins. The sensors autophosphorylate on a conserved histidine residue, and subsequently transfer the phosphoryl group to cognate response regulators. Following phosphorylation, response regulator proteins activate or repress transcription of specific target genes.

1.3.2 Autoinducer 2

A QS system that is widespread across the bacterial kingdom use a signaling molecule collectively called Autoinducer 2 (AI-2). Schauder *et al.* (2001) and Winzer *et al.* (2002) showed that the synthase LuxS produces a molecule with AI-2 activity. LuxS is an enzyme which is involved in the activated methyl cycle (AMC). Genome analysis shows that LuxS is widespread among bacteria species. This gene is widely found in Bacteroidetes, Actinobacteria, and β-, γ-, ε -Proteobacteria, Bacilli and Deinococci but not in Archaea or Eukarya (Rezzonico and Duffy 2008).

Autoinducer 2 synthesis

Signal molecules are produced by a specific synthase and recognized by a corresponding receptor. The LuxS enzyme synthesizes the precursor of AI-2. In addition, LuxS is involved in the AMC of cells (Fig. 3). The AMC is an important metabolic pathway in cells. The starting compound is S-adenosyl-methionine (SAM), which is the general

methyl donor. It donates its methyl group to diverse cellular components such as DNA, RNA and proteins. SAM is thereby converted to S-adenosyl-homocysteine (SAH), which is a toxic compound and has to be recycled. For recycling of SAH, two different pathways are known so far: a one-step and a two-step pathway. Only in the two-step pathway AI-2 is produced. In the two-step pathway Pfs (5´methylthioadenosine/S-adenosyl-homocysteine nucleosidase) hydrolyzes SAH to S-ribosylhomoscysteine (SRH) and adenine. LuxS catalyzes the cleavage of SRH to 4,5-dihydroxyl-2,3-pentanedion (DPD) and homocysteine (Winzer *et al.* 2002, Vendeville *et al.* 2005). DPD spontaneously cycles into AI-2, while homocysteine is converted by MetE or MetH to methionine. Methionine is then converted by MetK into SAM. Two different forms of AI-2 are known so far. The first structure of AI-2 is a cycle borated form called S-2-methyl-2,3,3,4-tetrahydroxytetrahydrofuran-borate (S-THMF-borate). The second is a non borated R-2-methyl-2,3,3,4-tetrahydroxytetrahydrofuran (R-THMF) AI-2 ligand (Miller *et al.* 2004). A peculiarity of AI-2 signaling is that diverse bacteria have different AI-2 receptors which recognize distinct forms of AI-2. S-THMF-borate is sensed by *Vibrio* spp. (Chen *et al.* 2002), whereas *Salmonella* Typhimurium and *Escherichia* (*E.*) *coli,* recognize the R-THMF form of AI-2 (Miller *et al.* 2004). However, each species can react to AI-2 produced by other species, because the two forms are in equilibrium and interconvert.

Crystal structure analyses shows that LuxS, for example from *Helicobacter* (*H.*) *pylori* contains two homodimeres in which each monomere contains four alpha helices and five antiparallel beta-sheets. The “active site” contains one zinc ion. It is assumed that the zinc ion plays an important role in cutting ribose during AI-2 synthesis (Hilgers and Ludwig 2001).

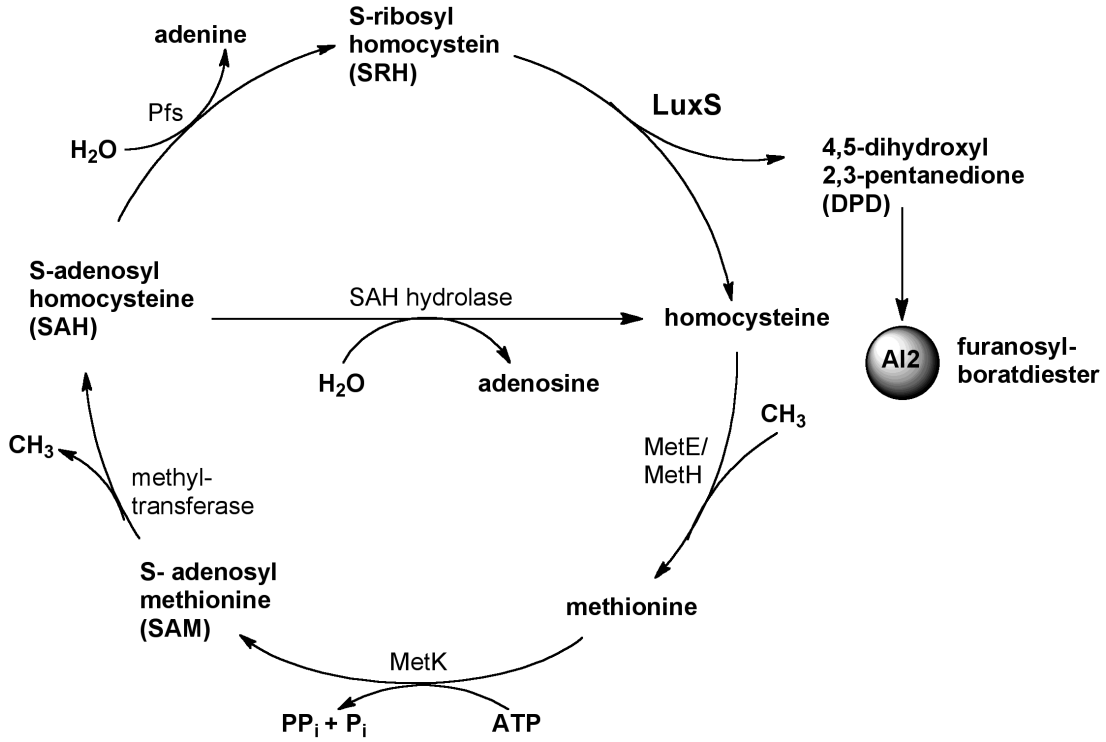

Figure 3: Metabolic function of LuxS. Methionine recycling, methylation of DNA and proteins is affected by the AMC. The SAM-synthetase MetK converts methionine to *S*-adenosylmethione (SAM). The cleavage of methyl residues from SAM results in the formation of *S*-adenosylhomocysteine (SAH), which can be converted to homocysteine either in a one- or two-step reaction. The SAH-hydrolase metabolizes SAH to adenosine and homocysteine in a one-step reaction. In the other pathway, Pfs cleaves SAH into adenine and *S*-ribosylhomocysteine (SRH), which in turn is cleaved into 4,5-dihydroxyl-2,3-pentanedione (DPD) and homocysteine by LuxS. Homocysteine can be converted to methionine by the methyltransferase (MetE or MetH).

AI-2 perception

So far distinct classes of AI-2 receptors have been discovered. The AI-2 signal could be sensed by ABC- Transporters, special two component systems or chemoreceptors (Xavier and Bassler 2005, Reading and Sperandio 2006, Rader *et al.* 2011).

ABC- Transporter

In *E. coli* AI-2 is imported into the cell via an Lsr transporter (ABC- Transporter). LsrB (LuxP homolog) is thereby exposed at the cell surface. By passing through, AI-2 is phosphorylated by the kinase, LsrK, to form phospho-AI-2. Phosphorylated AI-2 binds the transcriptional repressor LsrR so that LsrR is inactivated and the *lsr* operon can be transcribed (Fig. 4) (Xavier and Bassler 2005).

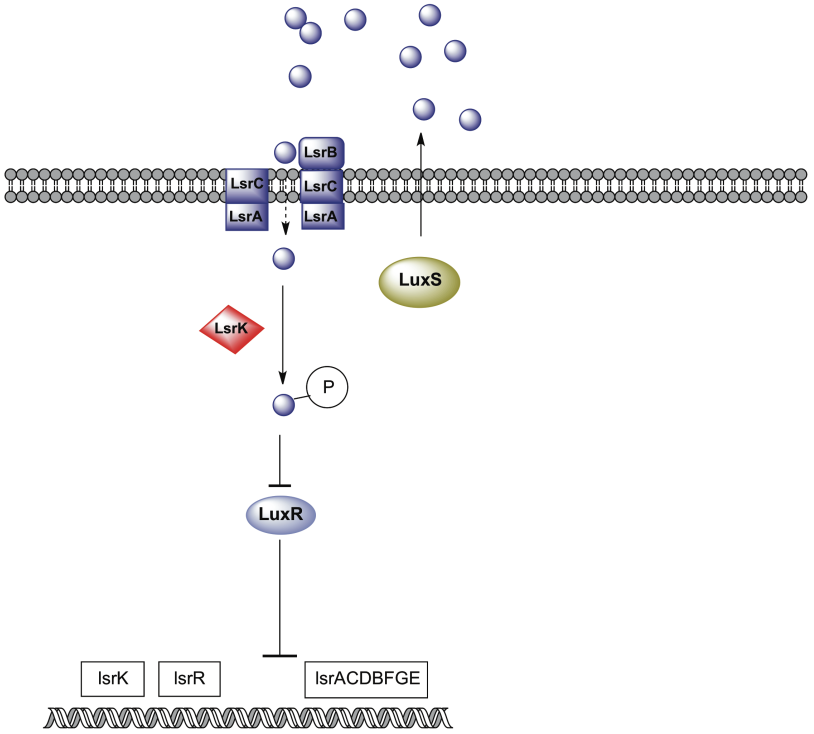

Figure 4: Quorum sensing system of *E. coli*: The import of AI-2 (circles) by the ABC-Transporter (composed of LsrA, LsrB and LsrC) in *E. coli* results in phosphorylation of the signal molecules by LsrK. Phosphorylated AI-2 inactivates LsrR (transcriptional repressor) and thereby increases the expression of the *lsr*-operon and can modulate the transcription of other target genes.

AI-2 perception: Two component signaling system

The AI-2 receptor in *Vibrio* spp., is the periplasmic two component sensor kinase LuxPQ (Reading and Sperandio 2006). Here, just the signal but not the AI-2 molecule is transduced inside the cell. In *V. harveyi*, AI-2 binds to the LuxP receptor protein, thereby inducing a phosphorylation-dependent signalling cascade of LuxQ, LuxU and LuxO. Phospho-LuxO together with a transcription factor σ^{54} activates the expression of non-coding small regulatory RNAs (sRNAs), called Qrr (Quorum regulatory RNAs). An RNA chaperone, Hfq, interacts with Qrr sRNAs and destabilizes the mRNA encoding the transcriptional activator termed LuxR (no homolog to *V. fischeri* LuxR) (Lenz *et al.* 2004). In the absence of LuxR, luminescence is not induced (Fig. 5).

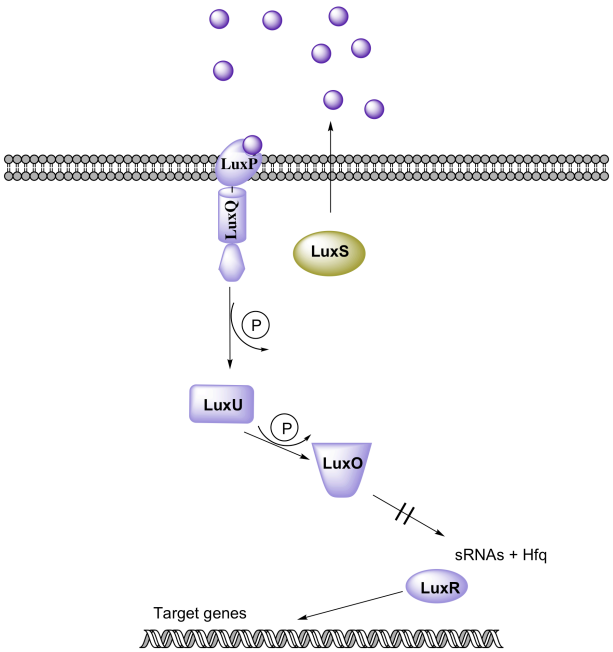

Figure 5: Two component signaling system: AI-2 (circles) binds to the LuxP receptor protein, thereby inducing a phosphorylation-dependent signalling cascade of LuxQ, LuxU and LuxO. Dephosphorylated LuxO enhances protein synthesis of the transcriptional activator LuxR, which results in increased expression of the *lux*-operon.

Chemoreceptors

Recently, chemoreceptors in *E. coli* and *H. pylori* have been described as third class of AI-2 receptors (Hegde *et al*. 2011; Rader *et al*. 2011). *E. coli* sense AI-2 as a chemoattractant via the chemoreceptor Tsr and LsrB. In contrast, AI-2 is perceived as chemorepellent in *H. pylori* by the chemoreceptor TlpB, but the signal recognition mechanism is not clear so far.

1.3.3 Quorum sensing in *V. harveyi*: parallel Quorum sensing circuits

V. harveyi is a gram-negative marine luminous bacterium, which uses different QS systems to control the *luxCDABE* operon (Fig. 6). The QS system of *V. harveyi* consists of three different AIs and their cognate receptors (Freeman and Bassler 1999, Henke and Bassler 2004). AI-1 is an acylated homoserine lactone (AHL), produced by the synthase LuxM, and binds to the membrane bound protein sensor kinase LuxN. AI-2 is a furanosylboratdiester, produced by LuxS and binds to the periplasmic binding protein. LuxP, The LuxP-AI-2 complex then interacts with the sensor kinase LuxQ in the

membrane. The third *V. harveyi* signal is called CAI-1, (S)-3-hydroxytridecan-4-one, and is produced by the CqsA enzyme (Higgins *et al.* 2007). CAI-1 interacts with a membrane bound sensor histidine kinase, CqsS.

At low cell density LuxQ, LuxN and CqsS act as kinases and transfer a phosphate group to the cytoplasmic protein LuxU, which in turn passes the phosphate group to the DNA-binding response regulator protein LuxO. Dephosphorylated LuxO enhances translation of the transcriptional activator LuxR mRNA, resulting in increased expression of the *lux*-operon (Freeman and Bassler 1999).

At high cell density, LuxQ, LuxN and CqsS act as phosphatases. Therefore, LuxU and LuxO are dephosphorylated. The dephosphorylated LuxO is inactive and does not induce the expression of sRNAs. In the absence of sRNAs, LuxR is expressed and initiates the expression of genes responsible for bioluminescence (Ng and Bassler 2009, Freeman and Bassler 1999).

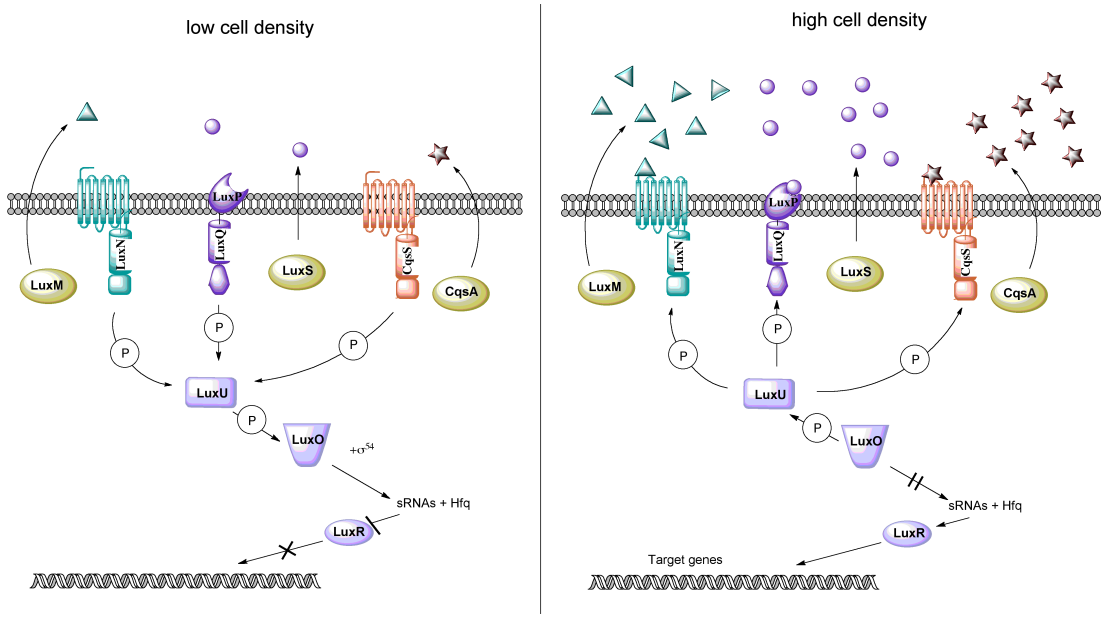

Figure 6: Hybrid quorum sensing in *V. harveyi*. At low cell density, phosphate fluxes from the membrane receptors, LuxN, LuxPQ and CqsS, to LuxU and then LuxO. Accumulation of phosphorylated LuxO together with σ^{54} activates the transcription of regulatory sRNAs, which in turn destabilize the *luxR* mRNA and inhibit expression of this regulator. At high cell density, the autoinducers CAI-1 (asterix), AI-2 (circles) and HSL (triangles) accumulate in the environment and bind to their corresponding receptors, CqsS, LuxPQ, and LuxN, respectively. Ligand binding promotes the phosphatase activity of these proteins, so that phosphate flow through the pathway is reversed. The resulting unphosphorylated LuxO does not induce the transcription of the sRNAs; LuxR is produced, which regulates the quorum sensing regulon of *V. harveyi*.

1.3.4 Autoinducer 3

Bacterial cells respond to the human stress hormones epinephrine (adrenaline) and norepinephrine (noradrenaline). These hormones are sensed by a receptor named QseC, which is a sensor kinase in the membrane. QseC also responds to a bacterial hormone-like molecule named autoinducer-3 (AI-3). This third group of autoinducers, was initially described in enterohemorrhagic *E. coli* (EHEC) by Sperandio *et al.* (2001). This group of Autoinducers mimics eukaryotic hormones and mediates inter-kingdom signaling events among bacteria and mammals or plants and vice versa.

1.4 *Campylobacter* spp.

Campylobacter (*C.*) spp. belong to the class of epsilonproteobacteria. The most important *Campylobacter* species are *C. jejuni* and *C. coli*. *Campylobacter* spp. are microaerophilic, gram-negative, spiral curved bacteria, with an either unipolar or bipolar flagellum. Growth takes place between 30°C and 42°C under microaerobic conditions (5% O_2, 10% CO_2).
Reports showed that *C. jejuni* and *C. coli* are the most common bacterial cause of gastroenteritis worldwide (WHO, 2011). *Campylobacter* spp. are widely distributed and can be found with high prevalence in food animals such as poultry, cattle and pigs. The routes of transmission are fecal-oral through contaminated food or water. Campylobacteriosis is an infection caused mostly by *C. jejuni* and *C. coli*. The incubation time ranges from two to five days. Most common clinical symptoms of *Campylobacter* infections include diarrhea, frequently with blood, abdominal pain, fever, headache, nausea, and/or vomiting (Skirrow *et al.* 1997, Butzler 2004). Sequelae like meningitis, miscarriage, reactive arthritis or Guillain-Barré syndrome have been described (Dedie and Bockemühl 1993).
It appears that pathogenic factors like chemotactic motility, adhesion and invasion ability, the toxin production and the variability of surface structures play an important role (van Vliet and Ketley 2001). In *C. jejuni,* motility is achieved by a single flagellum at one or both ends of the bacteria. The flagellum has an important role in virulence because it is required for the bacteria to reach the attachment sites and penetrate into the intestinal cells (Nachamkin *et al.* 1993, Wassenaar *et al.* 1993). The flagella of *C. jejuni* are composed of proteins, mainly encoded by two genes *flaA* and *flaB* (Nuijten *et al.* 1990). *C. jejuni*

mutants without a flagellum are unable to colonize in an animal model organism (Nachamkin *et al.* 1993, Wassenaar *et al.* 1993). Adhesion to the intestine epithelia and invasion into the cells is vital for the pathogenicity of *Campylobacter* spp. Adhesion factors of *C. jejuni* have already been identified which include the fibronectin-binding protein CadF (Konkel *et al.* 1997). *C. jejuni* causes watery diarrhea that progresses into a bloody diarrhea. These findings are consistent with the idea that toxins play a role in this disease. Cytolethal distending toxin (CDT) is the only verified *Campylobacter* spp. toxin identified to date (Elmi *et al.* 2012, Johnson and Lior 1988).

1.4.1 Metabolism in *Campylobacter* spp.

C. jejuni lacks a glucokinase and 6-phosphofructokinase gene and consequently lacks the ability to catabolize many common carbohydrates as carbon sources (Parkhill *et al.* 2000). Although *C. jejuni* is generally considered to be unable to utilize most sugars, certain strains were shown to utilize L- fucose as an energy source (Muraoka and Zhang 2011, Stahl *et al.* 2012). *C. jejuni* relies on the uptake and utilization of amino acids like aspartate, glutamate, serine and proline or tricarboxylic acid intermediates as their primary source of energy (Leon-Kempis Mdel *et al.* 2006, Guccione *et al.* 2008) (Velayudhan and Kelly 2002). The genome of *C. jejuni* encodes all enzymes which are required for a complete oxidative tricarboxylic acid cycle (TCA) in which amino acids are incorporated (Parkhill *et al.* 2000). Given that *C. jejuni* does not utilize the glycolytic pathway, this organism must perform anaplerotic reactions to replenish key TCA intermediates.

1.4.2 Quorum sensing in *Campylobacter* spp.

AI-1

To date no homology to AI-1 synthase, *luxI* and *ainS*, have been found (Moorhead and Griffiths 2011). But recently Moorhead and Griffiths (2011) discovered that *C. jejuni* produces N–(3-hydroxy-butanosyl)-L-homoserine lactone (HSL). This HSL is able to increase the transition rate to a viable but not culturable (VBNC) state. Furthermore the *C. jejuni* HSL, termed CjA, inhibits biofilm formation, significantly affects virulence gene expression and increases the production of IL-8.

AI-2

The existence of LuxS as well as the dependent AI-2 production in *C. jejuni* was first described in *C. jejuni* NCTC 11168 by Elvers and Park (2002). Additionally, LuxS and AI-2 were found in *C. coli*, *C. fetus* and *C. upsaliensis* and others (Gölz *et al.* 2012). LuxS could not be found in *C. lari* (Tazumi *et al.* 2011). In *C. jejuni* maximal AI-2 production was induced in the late exponential growth phase (Cloak *et al.* 2002, Quinones *et al.* 2009). Cloak *et al.* (2002) were able to demonstrate AI-2 production from *C. jejuni* in milk and chicken broth. So far no receptor homologues like LuxP, LsrB or chemoreceptors like TlpB and Tsr had been found in *C. jejuni* (Rezzonico and Duffy 2008).

AI-2: a true Quorum sensing signal?

Due to the fact that in many organisms no AI-2 Receptor has been found yet, the exact role of AI-2 in these species as a QS signal remains unclear. For example receptors like *luxP* and *lsrB* are missing in *C. jejuni* (Rezzonico and Duffy 2008). The disruption of *luxS* itself could lead to changing phenotypes due to the absence of AI-2 or a disturbed methionine cycle, so that experimental analysis with *luxS* knockout mutants needs to be complemented with pure AI-2.

The role of AI-2 as a byproduct of the AMC leads to the question, if AI-2 is indeed a true QS signal molecule. Some researchers argue that inactivating *luxS* leads to changes in phenotypes only due to the defect in metabolism (Winzer *et al.* 2003).

The degradation of the toxic byproduct SAH can occur via a one- or two-step pathway. AI-2 is only produced in the two-step pathway. So why should an organism use the assumingly more complex way, involving two enzymatic steps? It could be argued that the utilization of the more difficult pathway resulted in an evolutionary advantage. The AI-2 production could represent this advantage (Diggle *et al.* 2007).

Growth

Different studies have demonstrated equal growth rates between wild type and the *luxS* mutant of *C. jejuni* 81116, M129, and NCTC 11168 (Elvers and Park 2002, Jeon *et al.* 2003, Reeser *et al.* 2007, Holmes *et al.* 2009). Interestingly, He *et al.* (2008) showed that in *C. jejuni* 81–176 the *luxS* mutant had a statistically significant longer doubling time compared to the wild type strain incubated at 37°C, but not at 42°C. Also Quinones *et al.*

(2009) demonstrated that the *luxS* mutant of the 81–176 strain had a decreased growth rate during the exponential phase but reached stationary phase at the same time as the wild type at 42°C.

Motility

Motility in *C. jejuni* could be AI-2 regulated. In literature it is controversially discussed. Thereby temperature plays an important role. For example, *C. jejuni* 81-176 *luxS* mutants showed the same motility at 42°C, but at 37°C a reduction in motility was observed compared to the wild type (He *et al*. 2008). Many other studies have also demonstrated that motility is decreased in the *luxS* mutant (Jeon *et al.* 2003, Holmes *et al.* 2009, Quinones *et al.* 2009, Plummer *et al.* 2012). In contrast, one study of a *C. jejuni* 81-176 *luxS* mutant strain showed no reduction in motility (Guerry *et al.* 2006). Furthermore it was shown that *flaA* transcription, the major flagellin gene, which is important for motility, is reduced in *luxS* nullmutants of *C. jejuni* 81116 at 42°C, whereas *flaB* as well as the protein levels of FlaA and FlaB are not affected (Jeon *et al.* 2003). In microarray studies with *C. jejuni* NCTC 11168, He *et al.* (2008) demonstrated that *luxS* mutants of this strain grown at 42°C showed an altered expression profile for some flagellar structures (*flgD*, *flgE*, *fliD*, *fliS*, *flgR*, *flgI*, *flgK*, *flaA*, *flgG2*), even though the motility was not affected. Another microarray study by Holmes *et al.* (2009) with *C. jejuni* 81-176 showed a down regulated gene expression profile of 15 flagellar genes including 12 genes which were shown to be up-regulated in the study by He *et al*. (2008). During the study from He *et al*. (2008) they did not compare microarray analysis of *C. jejuni luxS* mutants in the presence or absence of pure AI-2. Without adequate complementation it is difficult to discern if the change was associated with metabolic disruption of *luxS* or *luxS* as AI-2 synthase. Thus, experimental analysis with *luxS* mutants needs to be complemented with AI-2 and/or a metabolic replacement substance like homocysteine (HC) and genetic complementation to exclude polar effects. In contrast, Holmes *et al.* (2009) complemented the *luxS* null mutant with AI-2. They were unable to find any genes that were differentially expressed in the presence of AI-2 and concluded that the different transcriptional changes observed in *luxS* mutants of *C. jejuni* were the result of metabolic dysfunction of *luxS* in AMC.

Chemotaxis

Campylobacter spp. regulate their motility by chemotactic signaling systems, which allow the bacteria to follow chemical gradients in their host environment. Quinones *et al*. (2008) described an enhanced chemoattraction towards amino acids and reduced chemoattraction towards organic acids for the *C. jejuni* 81-176 *lux*S mutant as compared to the wild type strain. Neither the expression of the core signal proteins *cheA* and *cheW* nor the expression of accessory proteins *cheB*, *cheR* and *cheV* were differentially regulated in the *C. jejuni* 81-176 *lux*S mutant grown at 42°C (He *et al*. 2008). However, from these results it was concluded that the described swarming motility regression of the *C. jejuni* 81-176 *lux*S mutant are likely due to defects in flagellar regulation and not in chemotaxis (He *et al*. 2008). Contradictory to He *et al*. (2008), a down-regulation of *cheA* in the *luxS* mutant of *C. jejuni* NCTC 11168 was described by Holmes *et al*. (2009). Since little is known about AI-2 mediated chemotaxis in *C. jejuni* no conclusion towards possible functions can be drawn yet.

Biofilm formation

Biofilms are an assemblage of microbial cells that are irreversibly attached to a surface and are enclosed in a matrix of primarily polysaccharide materials (Donlan 2002). *C. jejuni* is able to form biofilms (Joshua *et al.* 2006). Reeser *et al.* (2007) demonstrated that *luxS* and flagellar structure are important for biofilm formation in *C. jejuni*. *luxS* mutants showed decreased ability of biofilm formation. The formation of biofilms is influenced by AI-2, since the presence of cell free supernatants (CFS) from wild type *C. jejuni* increases biofilm formation of the mutant. The exact role of AI-2 in terms of biofilm formation is unknown so far.

Surface structures

Jeon *et al.* (2003) were able to demonstrate decreased agglutination ability of the *luxS* mutant *C. jejuni* strain. They speculated that QS is involved in the formation of surface structures. Similarly, Guerry *et al.* (2006) demonstrated that a *luxS* mutant of *C. jejuni* strain 81–176 had a decreased autoagglutination compared to the wild type strain (Guerry *et al*. 2006). Autoagglutination has been associated with the presence of flagellar

assemblies (Misawa and Blaser 2000, Golden and Acheson 2002) but it is unclear whether the observed change in autoagglutination is mediated by changes in flagellar assembly.

Stress response

Compared to the wild type strain, the *luxS* mutant of *C. jejuni* 81-176 was more sensitive to the toxic effects of hydrogen peroxide and hydroperoxide after incubation at 42°C (He *et al.* 2008). In contrast, a *C. jejuni* NCTC 11168 *luxS* mutant incubated at 37°C was not altered in oxidative stress response compared to the wild type (Elvers and Park 2002). He *et al*. (2008) demonstrated that the transcriptional expression of the *aphC, tpx* and *groES* genes, three stress response genes, appeared to be down regulated in the *luxS* mutant strain (He *et al*. 2008). The oxidative stress regulator CosR negatively regulates the transcription of LuxS as well as the stress response associated proteins SodB, Dps and Rrc, while it positively regulates the transcription of the stress response protein AhpC in *C. jejuni* NCTC 11168 (Hwang *et al.* 2011). Deletion of *cosR* rendered the strain more resistant to oxidative stress. Based on this data it is suggested that LuxS is somehow involved in the oxidative stress response of *C. jejuni*.

Virulence factors and pathogenicity

Putative virulence factors of *C. jejuni* include genes for motility and chemotaxis, binding and adhesion, as well as invasion and toxins (Dasti *et al.* 2010). One biological function of CDT is that cells are arrest in G2/M cell cycle, leading to enlarged or distended cells. Jeon *et al.* (2003) demonstrated that *cdt* transcription is decreased in *luxS* mutated *C. jejuni* 81116. Furthermore they show that CFS of mutants induced diminished cell cycle arrest, compared to CFS from the wild type. However, Holmes *et al.* (2009) did not find any down regulation of the three CDT encoding genes *cdtA*, *cdtB* and *cdtC*.

Elvers and Park (2002) were unable to demonstrate any differences in *in vitro* adherence and invasion assays between *C. jejuni* NCTC 11168 *luxS* mutant and the wild type. Inactivation of *luxS* in *C. jejuni* 81-176 reduced chicken colonisation and adherence to LMH chicken hepatoma cells compared to the *C. jejuni* wild type (Quinones *et al.* 2009). Plummer *et al.* (2012) recently showed that the highly virulent sheep abortion strain IA3902 completely lost its ability to colonize the intestinal tract of guinea pigs, when *luxS* is knocked out, while this *luxS* mutant strain was still virulent after intraperitoneal

inoculation of the guinea pigs. Genetic complementation of *luxS* restored the virulent phenotype. Furthermore, these authors showed that a *luxS* mutant of W7 (a motile clone of *C. jejuni* NCTC 11168) showed comparable colonization capabilities as the corresponding wild type strain in the chicken model but after co-infection of the *luxS* mutant with the wild type strain W7, the mutant was outcompeted by the wild type strain after several days (Plummer *et al.* 2012). The phenotypes of genetically complemented *luxS* in the mutant strains of W7 and IA3902 were comparable to the wild type phenotypes.

The formation of biofilms in *C. jejuni luxS* mutants was only investigated once. Reeser *et al.* (2007) showed that biofilm formation is AI-2 dependent. It is not yet clear how AI-2 is involved in this process. All other phenotypes of *C. jejuni luxS* mutants were controversially described in the literature. The lack of proof by genomic complementation of wild type *luxS* or the addition of exogenous AI-2 hampers evaluating whether the obtained phenotypes are a consequence of missing AI-2 molecules or disrupted metabolic LuxS function. In addition, phenotypes like motility in *luxS* mutants seem to be dependent on experiment conditions. Therefore and because of missing data on putative AI-2 receptors, no clear conclusions can currently be drawn in regard to processes that are regulated by AI-2 QS mechanisms.

1.5 References for Introduction and Literature Review

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Chapter 2: Phenotypes of *C. jejuni luxS* mutants are depending on strain background, kind of mutation and experimental conditions

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Phenotypes of *Campylobacter jejuni luxS* mutants are depending on strain background, kind of mutation and experimental conditions

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2.1 Abstract

Since the discovery that *Campylobacter* (*C.*) *jejuni* produces Autoinducer 2 (AI-2), various studies have been conducted to explore the function and role of AI-2 in *C. jejuni.* However, the interpretation of these analyses has been complicated by differences in strain backgrounds, kind of mutation and culture conditions used. Furthermore, all research on AI-2 dependent phenotypes has been conducted with AI-2 synthase (*luxS*) mutants. This mutation also leads to a disruption of the activated-methyl-cycle. Most studies lack sufficient complementation resulting in not knowing whether phenotypes of *luxS* mutants depend on disrupted metabolism or lack of AI-2. Additionally, no AI-2 receptor has been found yet. All this contributes to an intensive discussion about the exact role of AI-2 in *C. jejuni*. Therefore, we examined the impact of different experiment settings on three different *C. jejuni luxS* mutants on growth and motility (37°C and 42°C). Our study showed that differing phenotypes of *C. jejuni luxS* mutants depend on strain background, mutation strategy and culture conditions. Furthermore, we complemented experiments with synthetic AI-2 or homocysteine as well as the combination of both. Complementation with AI-2 and AI-2+homocysteine significantly increased the cell number of *C. jejuni* NCTC 11168Δ*luxS* in stationary phase compared to the non-complemented *C. jejuni* NCTC 11168Δ*luxS* mutant. Genetic complementation of both *C. jejuni* 81-176 *luxS* mutants resulted in wild type comparable growth curves. Also swarming ability could be partially complemented. While genetic complementation restored swarming abilities of *C. jejuni* 81-176Δ*luxS*, it did not fully restore the phenotype of *C. jejuni* 81-176::*luxS*, which indicates that compensatory mutations in other parts of the chromosome and/or potential

polar effects may appear in this mutant strain. Also with neither synthetic complementation, the phenotype of the wild type-strains was achieved, suggesting yet another reason for differing phenotypes other than communication and methionine metabolism for *C. jejuni luxS* mutants.

2.2 Introduction

Numerous bacteria communicate via the small interspecies-specific signalling molecule autoinducer-2 (AI-2) generated via LuxS [1]. This process is commonly known as Quorum sensing (QS). QS is a regulatory mechanism of gene expression, which enables bacteria to change their behaviour when the population reaches a particular cell-density. QS allows bacteria to communicate with each other and therefore coordinate their activities at a multicellular level. QS regulated processes are for example secretion of virulence factors, biofilm formation, motility and bioluminescence [2-4].
AI-2 is generated as a by-product via LuxS during the activated methyl cycle (AMC) [5,6]. The AMC is an important metabolic pathway in cells. The starting compound is S-adenosyl-methionine (SAM), which is the general methyl donor. It donates its methyl group to diverse cellular components such as DNA, RNA and proteins. SAM is thereby converted to S-adenosyl-homocysteine (SAH), which is a toxic compound and has to be recycled. For recycling of SAH, two different pathways are known so far: a one-step and a two-step pathway. Only in the two-step pathway AI-2 is produced. In the two-step pathway Pfs (5´methylthioadenosine/S-adenosyl-homocysteine nucleosidase) hydrolyzes SAH to S-ribosylhomoscysteine (SRH) and adenine. LuxS catalyzes the cleavage of SRH to 4,5-dihydroxyl-2,3-pentanedion (DPD) and homocysteine [7,8]. DPD is spontaneously cyclized into AI-2, while homocysteine is converted by MetE or MetH to methionine. Methionine is then converted by MetK into SAM [9].

In *V. harveyi,* AI-2 binds to the periplasmic binding protein LuxP. In many other bacteria e.g. *Salmonella* and *Escherichia coli*, AI-2 binds to LsrB, the ligand binding protein of an ABC transporter. So far, no homologues of the known AI-2 receptors like LuxP or LsrB were identified in *Campylobacter* spp. [10,11].
Recently, Rader et al. [12] described that the chemoreceptor TlpB functiones as AI-2 receptor in *Helicobacter pylori*. Despite of the existence of chemoreceptors in *C. jejuni*, which would suggest the existence of a corresponding receptor, no TlpB receptor homolog has been found yet.

The existence of LuxS, as well as the LuxS-dependent AI-2 production in *C. jejuni* NCTC 11168, was first described by Elvers and Park [13]. The fact that AI-2 is a by-product of the AMC and that a receptor is yet to be found, leads to the question, if AI-2 in *C. jejuni* is

indeed a true QS signal molecule. The disruption of *luxS* could lead to changing phenotypes due to the absence of AI-2 or disrupted methionine cycle. Thus, experimental analysis with *luxS* mutants needs to be complemented with AI-2 and/or a metabolic replacement substance like homocysteine (HC). Several studies of *C. jejuni luxS* mutants showed various results with diverse phenotypes in *luxS* mutants. For instance, motility and growth seems to be influenced through *luxS* disruption but in slightly different ways depending on the study design and conditions [13-15].

These sometimes opposing phenotypes might be due to different culture conditions. Furthermore, the authors conducted their studies with *luxS* mutants of different *C. jejuni* strains and used different mutation strategies. Additionally, most studies lack proof of complementing the *luxS* mutant strains with AI-2 and/or a metabolic substance to confirm whether resulting phenotypes are due to metabolic function of LuxS or a consequence of disrupting cell communication.

Therefore, we examined the impact of strain background, mutation strategy and culture condition on three different *C. jejuni luxS* mutants on growth and motility. Furthermore complementation experiments with synthetic AI-2 and/or homocysteine (HC) were conducted.

2.3 Material and Methods

Bacterial strains and growth conditions

Campylobacter (*C.*) strains described in Tab. 1 were cultured at 37°C or 42°C in Brucella broth (BB) (BD, Heidelberg, Germany), cation adjusted Mueller-Hinton-Broth (MH) (BD) or on Mueller-Hinton blood agar plates (MHB) (Oxoid, Wesel, Germany) under microaerobic conditions (5% O_2, 10% CO_2) generated by an Anoxomat (Omni Life Science, Bremen, Germany). *V. harveyi* was cultured in Autoinducer bioassay medium (AB). AB medium contained 0.3M NaCl, 0.05M MgSO4, and 0.2% vitamin-free casamino-acids (Difco, BD, Heidelberg, Germany). After adjusting the pH to 7.5 with KOH the medium was sterilized by autoclaving and then allowed to cool to room temperature. Finally, 1ml of sterile 1M potassium phosphate (pH 7.0), 1ml of 0.1M L-arginine (free-base) and 2ml of 50% glycerol were added per 100ml of AB medium.

The mutation of *luxS* was genetically confirmed. Therefore all *luxS* mutants were verified by PCR, followed by DNA sequencing of the amplified products. Additionally, the absence of AI-2 activity of all *luxS* mutants, as well as the presence of AI-2 in genetically complemented mutants was routinely tested in *V. harveyi* bioluminescence assay [16].

V. harveyi bioluminescence assay

Overnight cultures of *C. jejuni and V. harveyi* BB152 (positive control) were diluted in BB or AB to a cell density of $1x10^8$ CFU/ml. Culture supernatants were collected and centrifuged at 8000x g for 10 min. The supernatants were sterilized by passing through a 22 µm filter (VWR, Darmstadt, Germany) and stored at -20°C until used. In parallel the absorbance was measured at the same time point to determine cell growth.

The *V. harveyi* autoinducer assay was performed as described previously [16]. The reporter strain (BB170) was grown over night in AB medium and diluted (1:5000) into fresh AB medium. CFS and uninoculated AB respectively BB medium were then added to the diluted *V. harveyi* culture at 10% (v/v) final concentration. As another positive control AI-2 (10µM) alone was tested. The reporter strain with CFS, AI-2 or uninoculated media were incubated at 30°C with aeration (750 rpm). After 4 hours of incubation, luminescence of

100 µl aliquots in microtiter plates were measured (10s per well) using Luminometer (CentroPro, Berthold, Bad Wildbach). For each of three experiments, triplicates of relative light units (RLU) were measured. n- fold luminescence induction values were calculated from RLU obtained with conditioned CFS vs. RLU obtained with sterile medium.

Chemical complementation

AI-2 activity was quantified with the bioluminescence assay and compared to wild-type *C. jejuni* grown to an OD600 nm of 1.0, at which maximal AI-2 activity was obtained. To test for complementation of growth and motility, AI-2 (OMM Scientific, Dallas, USA) at a physiological concentration of 10µM and non-limiting concentration of 100µM was used. Homocysteine (HC; Sigma Aldrich, St. Louis, USA) was tested at 1µM, 10µM and 100µM.

To exclude non-specific effects of AI-2 and homocysteine, the same concentrations were added to wt strains.

Genetic complementation

To exclude potential polar effects in the mutant strains, genetic complementation was performed exemplarily in the insertion and deletion mutant of strain *C. jejuni* 81-176. The complemented *C. jejuni* 81-176::*luxS* strain was kindly provided by Quiñones et al. [15].

Quiñones et al. [15] complemented the *luxS* mutation in strain *C. jejuni* 81-176::*luxS* with pBQ117 (plasmid pWM1015 containing a 1.3-kb fragment with the promoter-proximal region and intact *luxS* gene from strain 81-176).

In this study both plasmids, pWM1015 and pBQ117, were introduced into *C. jejuni* 81-176Δ*luxS* by electroporation [17].

Growth assay

For growth assays, cultures of wild type (wt) and *luxS* mutants of *C. jejuni* NCTC 11168 and *C. jejuni* 81-176 strains were grown overnight in BB or MH at 37°C. Precultures were inoculated in BB or MH to approx. $2x10^5$ CFU/ml and incubated under microaerobic conditions at 37°C and 42°C. For chemical complementation assays 10µM AI-2, HC or AI-2+HC were added to the cultures.

Numbers of viable bacteria were determined over 48h by plating serial dilutions of the bacterial suspensions. Results reported are the average of at least three independent assays.

Swarming assay

For swarming either BB containing 0.4% agar (BBA) or MH containing 0.4% agar (MHA) were used. The swarming ability of *C. jejuni luxS* mutants was investigated at 37°C and 42°C on swarming plates. For chemical complementation 10µM each of AI-2, HC or AI-2+HC were added to the molten agar. Overnight cultures of *C. jejuni* strains were adjusted to 10^8 CFU/ml and 1µl dropped on BBA or MHA. After 24h incubation at 37°C or 42°C the diameters of the swarming halos were measured. Halos of *luxS* mutants were normalized to the wild type halos (100%). Results reported are the median of six independent assays.

Statistical Analysis

For statistical analyses, all experiments were repeated at least three times in three independent experiments.

Statistical analyses were performed using GraphPad Prism v6.0 (GraphPad Prism, San Diego, USA). To calculate significant differences a two- tailed Mann-Whitney test was used. For all statistical analyses, a confidence level of 95% was defined.

2.4 Results

Growth assays

Strain background

Growth of both *C. jejuni* wild type strains did not differ at 37°C and 42°C, whereas growth profiles of the three *luxS* mutants were not equal (Fig. 1A-F). Compared to the wild type the Δ*luxS* mutant of *C. jejuni* NCTC 11168 showed significantly reduced cell numbers within mid-exponential (8h) and mid-stationary phase (32h) while cell numbers converged during late stationary phase (48h) at both temperatures (Fig. 1A-B). *C. jejuni* 81-176Δ*luxS* and ::*luxS* mutants showed comparable cell numbers to the wild type at 37°C with a slight decrease at late stationary phase (Fig. 1C/E). At 42°C *C. jejuni* 81-176Δ*luxS* showed significantly decreased cell numbers in exponential and stationary phase, but *C. jejuni* NCTC 11168Δ*luxS* showed much lower levels (Fig. 1D). However, the cell number of *C. jejuni* 81-176::*luxS* only decreased significantly at mid-stationary phase at 42°C (Fig. 1F). These results demonstrate that strain background has an impact on growth profiles of *C. jejuni luxS* mutants.

Culture conditions

When comparing growth of *C. jejuni* NCTC 11168 wt and mutant in MH versus BB it becomes obvious that the growth profiles were quite similar between these media at both temperatures (Fig. 1A-B and 2A-B). In contrast, growth of *C. jejuni* 81-176 wt was slightly reduced in late stationary phase in MH. Both mutants of strain *C. jejuni* 81-176 showed growth defects in MH at 37°C that were not exhibited in BB (Fig. 2C-F). In addition, growth profiles of *C. jejuni* 81-176Δ*luxS* mutants differed between MH and BB, thus the cell numbers of *C. jejuni* 81-176Δ*luxS* in MH were substantially reduced in the stationary phase while cell numbers in BB showed only a partially slight reduction. This indicates that composition of media could influences growth of *luxS* mutants. Temperature influences growth profiles of the *C. jejuni* 81-176::*luxS* mutant strain in MH. However, temperature did not have an impact on growth of the other *C. jejuni luxS* mutants.

Impact of mutation strategy on growth of luxS mutants

To investigate the influence of mutation strategy, three different *C. jejuni luxS* mutants were used. Considering growth profiles of *C. jejuni* in MH the influences of mutation strategy became apparent. The Δ*luxS* mutants of both *C. jejuni* NCTC 11168 and *C. jejuni* 81-176 showed growth defects which were not exhibited in the insertional mutant *C. jejuni* 81-176::*luxS* at 42°C (Fig. 2D/F). These results demonstrated the impact of mutation strategy.

Genetic complementation

Based on the varying growth profiles between *C. jejuni* 81-176 wt and its *luxS* mutant strains in MH, genetic complementation of the *C. jejuni* 81-176 mutant strains were conducted. Genetic complementation of both *C. jejuni* 81-176 *luxS* mutants in MH resulted in wild type comparable growth curves (Fig. 2). Introduction of the isogenic plasmid pWM1015 alone did not alter growth of *C. jejuni* 81-176 mutants. This indicates that no polar effects caused varying growth profiles of *C. jejuni* 81-176 *luxS* mutants in MH.

Chemical complementation

Because of the varying growth profiles between *C. jejuni* NCTC 11168 wt and its Δ*luxS* mutant we examined if the addition of exogenous AI-2 and HC influences growth of *C. jejuni* NCTC 11168Δ*luxS* in BB. Complementation with AI-2 (10µM) and AI-2+HC (both 10µM) significantly increased the cell number of *C. jejuni* NCTC 11168Δ*luxS* in stationary phase at 37°C and 42°C compared to the non-complemented Δ*luxS* mutant (Fig. 3A/B). In contrast, HC alone did not show any significant effect on cell numbers at any of the investigated temperatures (data not shown). Full restoration of wild type cell numbers was not achieved by the chemical complementation strategy used in *luxS* mutants indicating that alternative mechanisms for these different phenotypes exist. The same effects have been observed with 100µM AI-2 and 100µM HC (data not shown). Non-specific effects through AI-2 and homocysteine could be excluded since no alteration in phenotypes of the wild type was observed (data not shown).

With the addition of AI-2 and AI-2+HC to strain *C. jejuni* 81-176Δ*luxS* in MH media slightly increased cell numbers during stationary phase were observed at both temperatures, but these were not statistically significant (Fig. 3C/D). The addition of HC alone did not lead to increased cell numbers (data not shown).

Swarming ability

Strain background

Swarming ability *of C. jejuni* wild types did not differ between the different strains (data not shown). Swarming ability of *luxS* mutants were normalized to the wild type (100%). *C. jejuni* NCTC 11168Δ*luxS* showed reduced swarming ability (Fig. 4A/D, Fig. 5A) (approx. 42% of wt swarming in BBA). In contrast, *C. jejuni* 81-176Δ*luxS* showed no significant reduction in swarming ability in BBA (Fig. 4B/E). Our data clearly indicate the influences of strain background on swarming ability of *C. jejuni* Δ*luxS* mutants under these conditions (Fig. 4A-F).

Impact of mutation strategy on swarming ability of luxS mutants

Insertion and deletion of *luxS* in strain *C. jejuni* 81-176 resulted in different swarming abilities. In our experimental setting, only the *C. jejuni* 81-176::*luxS* mutant exhibited smaller swarming halos (Fig. 4C/F) compared to the wt but not the deletion mutant of this strain (Fig. 4B/E). Our data indicate that mutation strategy influences the ability to swarm in *C. jejuni* 81-176 *luxS* mutants.

Culture conditions

At 42°C diameters of swarming halos of all strains and their mutants increased compared to halos at 37°C (data not shown). Neither temperature nor media influenced the reduced swarming abilities of *C. jejuni* NCTC 11168Δ*luxS* (Fig. 4A/D) and *C. jejuni* 81-176::*luxS* (Fig. 4C/F) compared to their wt. In contrast, swarming ability of *C. jejuni* 81-176Δ*luxS* (Fig. 4B/E) was slightly increased compared to the wt in BBA at both temperatures and

decreased in MHA. The difference in swarming halos of *C. jejuni* 81-176Δ*luxS* was statistically significant between both media. This result indicates that culture conditions could have an impact on swarming ability of *C. jejuni luxS* mutants.

Chemical complementation in BB

Hence, we examined if the addition of exogenous AI-2 and HC influences the reduced swarming of *C. jejuni luxS* mutants on BBA at 37°C and 42°C (Fig. 6). In BBA complementation with AI-2 and AI-2+HC contributed to an increased swarming ability compared to the non-complemented mutant of *C. jejuni* NCTC 11168Δ*luxS* at 37°C (Fig. 6) but not at 42°C (Fig. 6A/D). However, the addition of HC alone did not alter the swarming ability at both temperatures. Only the addition of both AI-2+HC to *C. jejuni* 81-176::*luxS* mutant increased the swarming motility at 37°C (Fig. 6C), while the swarming ability of *C. jejuni* 81-176Δ*luxS* was not significantly altered by any condition investigated (Fig. 6B/F). Our data implicate that partial complementation of *luxS* mutants is possible in BBA depending on temperature and strain background. Complementation only occurs if AI-2 is admitted. With neither chemical complementation of *luxS* mutants, the phenotype of the wild type-strains was achieved.

Chemically complementation in MH

The swarming ability of *C. jejuni* NCTC 11168Δ*luxS* was increased through the addition of AI-2, HC and AI-2+HC at 42°C compared to the non-complemented mutant strain on MHA (Fig. 7). At 37°C only a slightly increased swarming ability could be observed through the addition of AI-2 and AI-2+HC. However, the addition of AI-2 to *C. jejuni* 81-176::*luxS* yielded increased swarming motility at both temperatures. Furthermore, with the addition of AI-2+HC swarming motility could also be increased at 37°C in this mutant. In contrast, the swarming ability of *C. jejuni* 81-176Δ*luxS* was not significantly changed by any complementation investigated. With neither chemical complementation, the phenotype of the wild type-strains was achieved. Complementation in MHA is likely to occur but depends on strain background and mutation strategy.

Genetic complementation

Introduction of the isogenic plasmid pWM1015 alone did not alter swarming ability of *C. jejuni* 81-176 mutants. Genetic complementation (+pBQ117) restored swarming abilities of *C. jejuni* 81-176Δ*luxS* (Fig. 7B/D) in MH, whereas in strain *C jejuni* 81-176::*luxS* genetic complementation did not fully restore the phenotype at 37°C and 42°C in both media (Fig. 6A/D and Fig. 7A/D). The incomplete restoration hints at appearing polar effects in this mutant strain.

2.5 Discussion

Since the discovery that *C. jejuni* produces AI-2, various studies have been conducted to explore the function and role of AI-2 in *C. jejuni* [18,19]. However, the interpretation of these analyses has been complicated by differences in strain background, kind of mutation and culture conditions. Furthermore most studies lack sufficient complementation resulting in not knowing whether phenotypes of *luxS* mutants depend on disrupted metabolism or lack of AI-2. Additionally, no AI-2 receptor has been found yet. All this contributes to an intensive discussion about the exact role of AI-2 in *C. jejuni*.

In the literature, various motility and growth phenotypes have been described for *C. jejuni luxS* mutants [14,15,20]. Therefore, we investigated if the strain background, kind of mutation and different culture conditions impact the occurring phenotypes in *C. jejuni luxS* mutants.

To verify that our *luxS*-mutant strain truly deficient in AI-2 production a *V. harveyi* bioluminescence reporter assay was conducted. *V. harveyi* only responds to the borate diester derived from (2S,4S)-THMF [9]. The wild type strain of *C. jejuni* as well as the synthetic AI-2 exhibit positive signals in this reporter assay, indicating that the absence of a positive signal (in the reporter assay) of the mutant strain is equatable to the absence of AI-2 production. Further the *V. harveyi* reporter assay indicates that the synthetic AI-2 contains a similar equilibrium of AI-2 as the one produced by *C. jejuni* NCTC 11168.

Culture conditions

During growth of *C. jejuni* wild types there were no significant differences observed in BB and MH. Also Ng et al. [21] described that there was no significant difference between cell numbers of *C. jejuni* among these basal media. Growth and swarming abilities of *C. jejuni* NCTC 11168Δ*luxS* were quite similar in BB and MH at both temperatures (37°C and 42°C), which leads to the assumption that these culture conditions do not have an impact on the *luxS* mutant phenotype of this strain.

Further, He et al. [14] described that the cell numbers of *C. jejuni* 81-176 differed between wild type and Δ*luxS* mutant at 37°C and 42°C in the mid-exponential phase, while cell numbers converged in late stationary-phase in MH medium. In our study, we also observed reduced cell numbers of *C. jejuni* 81-176Δ*luxS* in exponential as well as in late stationary phase in MH at both temperatures. In contrast, growth of *C. jejuni* 81-176Δ*luxS* in BB only showed significant differences to growth of wild type in late stationary phase at 37°C. These results demonstrate that the choice of the culture medium has a large impact on the resulting phenotypes of *C. jejuni* 81-176 *luxS* mutants. Growth of the *C. jejuni* 81-176::*luxS* is also influenced by temperature in MH. However, growth curves at 37°C and 42°C are quite equal in *C. jejuni* 11168Δ*luxS* and *C. jejuni* 81-176Δ*luxS*, which indicate that growth differences are independent of temperature but dependent on culture media for deletion mutant strains. Motility observation confirmed this assumption as well. Like He et al. [14] we observed reduced swarming abilities of *C. jejuni* 81-176Δ*luxS* at 37°C on MHA media in contrast to the wild type. However, this phenotype was only observed on MHA media but not on BBA. The components of these two different basal mediums differ. For example only BB contains dextrose. Wang et al. [22] showed that glucose affected the gene expression of *luxS* in *E. coli.* Furthermore, they observed that the expression of *pfs* was reduced by the presence of glucose. Our findings clearly illustrate that resulting phenotypes of *C. jejuni luxS* mutants can be influenced by the choice of culture medium and components in media could also influence resulting phenotypes of *C. jejuni luxS* mutants.

Strain background

C. jejuni is a highly diverse species [23]. To investigate the influence of strain background on phenotypes of *luxS* mutants we used *C. jejuni* NCTC 11168 and *C. jejuni* 81-176Δ*luxS* mutants. Comparing these phenotypes we observed differences between *luxS* mutants of *C. jejuni* NCTC 11168 and *C. jejuni* 81-176 especially in BB (Fig. 1A-D). Also, the comparison of *luxS* mutant strains of *C. jejuni* 81-176 used by He et al. [14] and *C. jejuni* NCTC 11168 used by Elvers and Park [13] revealed different outcomes in terms of growth. Comparing wild types of *C. jejuni* in our study, cell numbers of strain *C. jejuni* NCTC 11168 decline under cell numbers of strain *C. jejuni* 81-176 in late stationary phase. Cell numbers in all other time points did not differ between these two wild type strains, whereas cell numbers of *C. jejuni* NCTC 11168Δ*luxS* in early stationary phase in BB are lower than cell numbers of *C. jejuni* 81-176Δ*luxS*. Furthermore, the ability to swarm differed between the *luxS* mutants of both strains. Here, we observed that *C. jejuni* NCTC 11168Δ*luxS* exhibits the greatest reduction of swarming ability, whereas *C. jejuni* 81-176Δ*luxS* did not exhibit reduced swarming abilities at all in BB. The different observed phenotypes in *C. jejuni* NCTC 11168 and *C. jejuni* 81-176 may be a consequence of genetic diversity between these strains [24]. For instance previous analysis of the complete flagellin glycosylation locus of *C. jejuni* strain 81–176 revealed a less complex genomic organization than the corresponding region in the genome of strain *C. jejuni* NCTC 11168 [25]. In addition, Dugar et al. [17] identified strain-specific transcriptome organization and sRNAs that could contribute to differential gene regulation among these strains.

Mutation strategy

An explanation for the differences in growth profiles between *C. jejuni* NCTC 11168 and *C. jejuni* 81-176 *luxS* mutants might be the genetic differences between these two strains. However, it seems equally probable that the phenotypic differences observed were due to different mutation strategies applied. Previously Haigh *et al.* [26] showed that mutation design and strain background influenced phenotype *of E. coli luxS* mutants. The authors concluded that one explanation could be the different orientation of antibiotic resistance cassette in the mutants. The kanamycin resistance cassette of *C. jejuni* NCTC 11168Δ*luxS*

is orientated in the same direction as the *luxS* gene, whereas the *C. jejuni* 81-176Δ*luxS* mutant has the chloramphenicol resistance cassette in the opposite direction of the deleted *luxS* gene. Both mutants had reduced cell numbers when cultured in MH but only the *luxS* mutant of *C. jejuni* NCTC 11168 showed lesser swarming abilities compared to the corresponding wild type. Recently, it has been demonstrated that the regulatory small RNA MicA is located closely upstream of the *E. coli luxS* gene [27,28] and could be an obvious target for polar effects of a *luxS* mutation. In *C. jejuni*, Dugar et al. [17] showed that a small RNA is located downstream of the *luxS* gene, but no function of this small RNA has been described so far. However, the expression of this small RNA could be influenced in a mutation strategy dependent manner. Since this molecule can have regulatory effects, its dysregulation can affect other pathways. Another reason for the observed different phenotypes could be the size of the deleted region. In contrast to our results, the *C. jejuni* NCTC 11168Δ*luxS*-mutant constructed by Elvers and Park [13] showed similar growth compared to the wt at 37°C. The Δ*luxS* mutant of Elvers and Park [13] has the same strain background and orientation of antibiotic resistance cassette as the Δ*luxS* mutant used in this study. However, the mutant used in our study has a larger deletion region, including those with functional domains from *luxS* (Fig. 8 illustrated the differences in mutation strategies of *C. jejuni luxS* mutants), whereas the mutant from Elvers and Park [13] still retains the functional domain regions. Even though the functionality relating to AI-2 production is disabled, it cannot be ruled out that other regions within this sequence exert an influence on other processes. Also the *C. jejuni* NCTC 11168::*luxS* mutant described by Plummer et al. [20] (an insertion mutant up-stream of the functional domains) showed similar growth like the wt. However, the mutant of Plummer et al. [20] and Elvers and Park [13] exhibited decreased motility. Examining the mutant in the current study, decreased motility haloes in semisolid media have been observed, which indicates that disruption of *luxS* causes a reduction of swarming ability in *C. jejuni* NCTC 11168 whether or not the functional domain regions are deleted.

To investigate the influence of mutation strategy on phenotypes of *C. jejuni luxS* mutants we additionally conducted our study with an insertion mutant of *C. jejuni* 81-176. The resistance cassette of this mutant strain is also (like in *C. jejuni* 81-176Δ*luxS*) orientated in the opposite direction of the *luxS* gene. By examining growth in MH at 37°C and 42°C cell numbers of the deletion mutant are smaller than cell numbers of insertion mutant in stationary phase. Again, one reason could be the lack of the region among the functional

domains within *luxS* in the deletion mutant, whereas this region is still present in the insertion mutant of *C. jejuni* 81-176. Additionally, Quinones et al. [15] replaced the *luxS* gene of strain 81-176 by a mutated *luxS* gene of the *C. jejuni* strain RM1221, which is another possible explanation for differing phenotypes of *luxS* mutants. Furthermore, Quiñones et al. [15] used larger up- and downstream regions of *luxS* for their mutation construct. Even though there is a high DNA sequence identity (96.8%) between the *luxS* genes of strains RM1221 and 81-176, there are some differences which might influence phenotypes of *luxS* mutants.

The observation of swarming ability additionally indicates the importance of mutation strategy. Like Quiñones et al. [15] we observed a significantly reduced swarming ability of *C. jejuni* 81-176::*luxS* mutant compared to the wild type on BBA, whereas the swarming ability of *C. jejuni* 81-176Δ*luxS* is not reduced on BBA in contrast to the wt.

While genetic complementation restored swarming abilities of *C. jejuni* 81-176Δ*luxS*, genetic complementation restored swarming abilities of *C. jejuni* 81-176::*luxS* only partial. The phenotype of wt strain *C. jejuni* 81-176 was not fully achieved. The incomplete restoration was probably caused by polar effects in this insertion mutant strain. Also Haigh et al. [26] argued that insertion may always result in adverse polar effects.

Combined, these findings suggest that growth deficits of *C. jejuni luxS* mutants might be associated with deletion of a larger region of *luxS*, while motility might be influenced by polar or compensatory mutation effects of the *luxS* mutation.

Complementation

It remains unclear whether phenotypes of *luxS* mutants are due to the lack of AI-2 or result from the metabolic deficits caused by disruption of this enzyme in the activated methyl cycle. Additionally, occurring phenotypes may be appearing through polar effects depending on the kind of mutation. Genetic complementation of both *C. jejuni* 81-176 *luxS* mutants resulted in wild type comparable growth curves, which indicates that no polar effects influence growth of *C. jejuni* 81-176 *luxS* mutants.

Therefore, we investigated the phenotypes of *C. jejuni luxS* mutants chemically complemented with AI-2, HC and AI-2+HC (Fig. 3). Growth of *luxS* mutants could be

partially complemented by AI-2 but not to wt level, implicating that altered phenotypes of *luxS* mutants not solely occur as a consequence of lacking AI-2. The addition of AI-2 and AI-2+HC to mutant strain *C. jejuni* 81-176Δ*luxS* leads to slightly increased cell numbers, too. Addition of HC to the mutant strains did not alter the *luxS* mutant phenotype indicating that disruption of the activated methyl cycle downstream of LuxS might not be responsible for the observed phenotypes (data not shown). The accumulation of components upstream of LuxS within the AMC as well as other unknown functions of LuxS could also have an impact on the observed phenotypes.
Furthermore, in solution, DPD exists in an equilibrium that contains diastereomeric mixtures of dihydroxytetrahydrofurans (DHMF) and tetrahydroxytetrahydrofurans (THMF) through cyclization and hydration [29].

A peculiarity of AI-2 signaling is that diverse bacteria have different AI-2 receptors which recognize distinct forms of AI-2. For example, *V. harveyi* responds to the borate diester derived from (2S,4S)-THMF [30,31], whereas *Salmonella* Typhimurium [9], *Sinorhizobium meliloti* [32] and *Yersinia pestis* [33] respond to (2R,4S)-THMF. Thereby it is possible that the DPD used in this study might not have harboured adequate amounts of the relevant DPD variant for *C. jejuni*. However, as the synthetic DPD also induced luminescence in the *V. harveyi* assay, it can be concluded that AI-2 produced by *C. jejuni* and synthetic DPD contained a similar variation of AI-2. The response observed following chemical complementation would suggest the existence of the adequate structure of AI-2 for receptor recognition. Nevertheless it remains unclear if the lack of complete complementation could be caused by an inappropriate chemically equilibrium of the AI-2 structures.

We observed significantly increased swarming ability at 37°C by chemical complementation with AI-2 and AI-2+HC in *C. jejuni* NCTC 11168Δ*luxS* on BBA and in *C. jejuni* 81-176::*luxS* on MHA. While genetic complementation restored swarming abilities of *C. jejuni* 81-176Δ*luxS*, it did not completely restore phenotype of *C. jejuni* 81-176::*luxS*. By the addition of AI-2, the same swarming abilitiy as shown for the genetic complemented mutant was achieved. These data indicate that reduced swarming abilities are partially due to polar mutation effects in *C. jejuni* 81-176::*luxS*, but could be partially complemented by exogenous AI-2.

Conclusion

Our study provides a clue why literature about phenotypes of *C. jejuni luxS* mutants is extremely contradictory. Our analyses demonstrated that occurring phenotypes of *C. jejuni luxS* mutants are depending on strain background, kind of mutations and experimental conditions (Tab. 2).

Further, some *luxS* mutant phenotypes could be partially complemented by AI-2, even though not to wild type levels, suggesting that *C. jejuni* can regulate its behaviour by AI-2 dependent Quorum sensing. Further studies should clarify which kind of AI-2 structure is recognized by *C. jejuni*.

Future studies could also clarify how the mutation strategy influences gene expression. Variability in the different mutants examined in this study reflects the likely presence of compensatory mutations in other parts of the chromosome. Also polar effects on down- and upstream genes are possible. Further, the influence of mutation strategy on small RNAs cannot be excluded.

One option for further research could be to investigate the expression of the small RNA located downstream of *luxS* in the settings used in this study.

2.6 Acknowledgement

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2.7 References

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2.8 Figures and Table

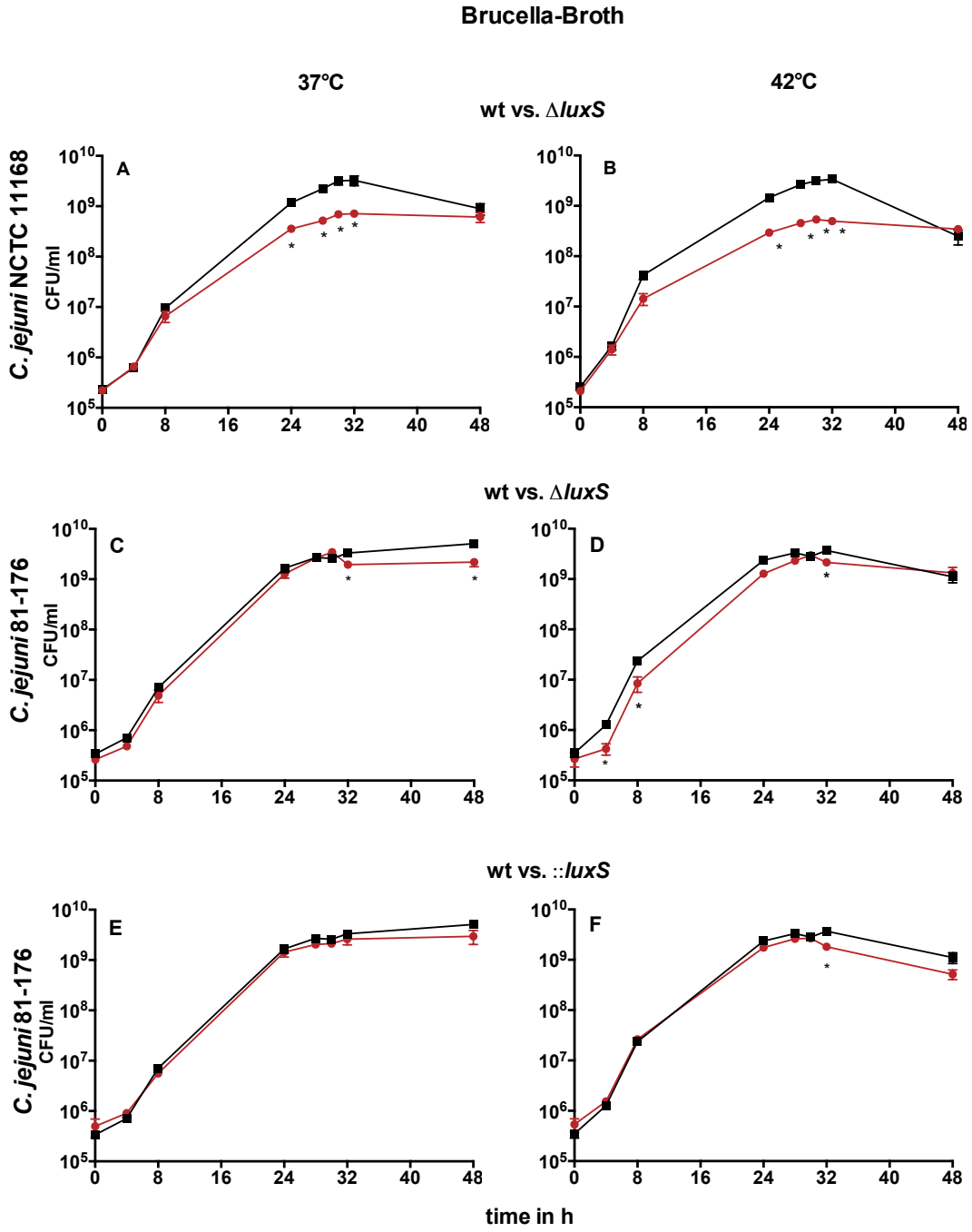

Figure 1: Growth of *C. jejuni* NCTC 11168 and *C. jejuni* 81-176 wt and *luxS* mutants at 37°C and 42°C in BB: A-B *C. jejuni* NCTC 11168 wt/Δ*luxS*, C-D *C. jejuni* 81-176 wt/Δ*luxS*, E-F *C. jejuni* 81-176 wt/::*luxS*; black- wild type, red- *luxS* mutant; shown are the means ± SD (n=3), * -$p<0.05$ (Mann-Whitney-U test)

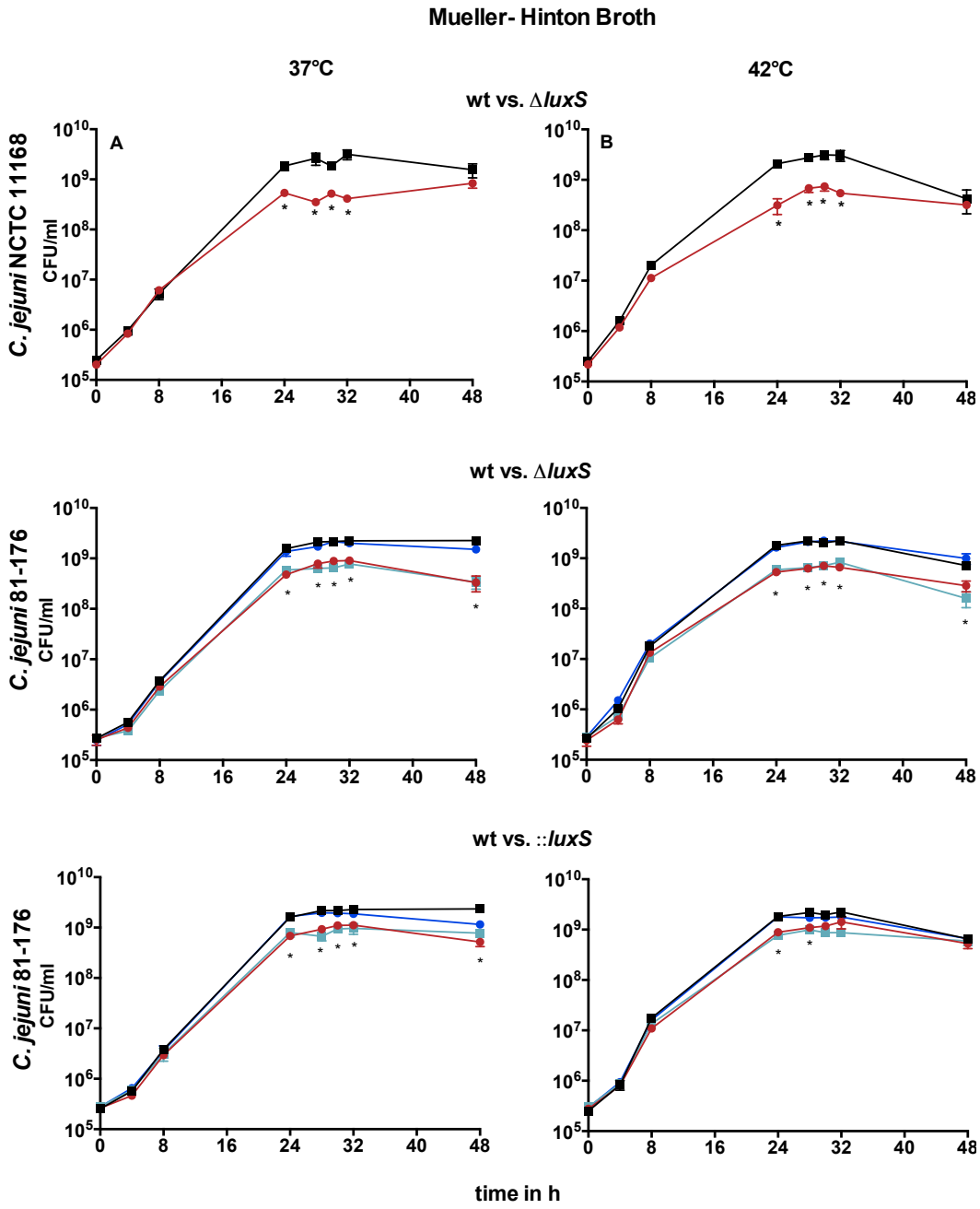

Figure 2: Growth of *C. jejuni* NCTC 11168 and *C. jejuni* 81-176 wt, *luxS* mutants and genetic complemented *C. jejuni* 81-176 mutants at 37°C and 42°C in MH: A-B *C. jejuni* NCTC 11168 wt/Δ*luxS*, C-D *C. jejuni* 81-176 wt/Δ*luxS*, E-F *C. jejuni* 81176 wt/::*luxS*; black- wild type, red- *luxS* mutant, blue- *luxS*+ pBQ117, turquoise- *luxS*+ pBQ1015; shown are the means ± SD (n=3), * -$p<0.05$ (Mann-Whitney-U test)

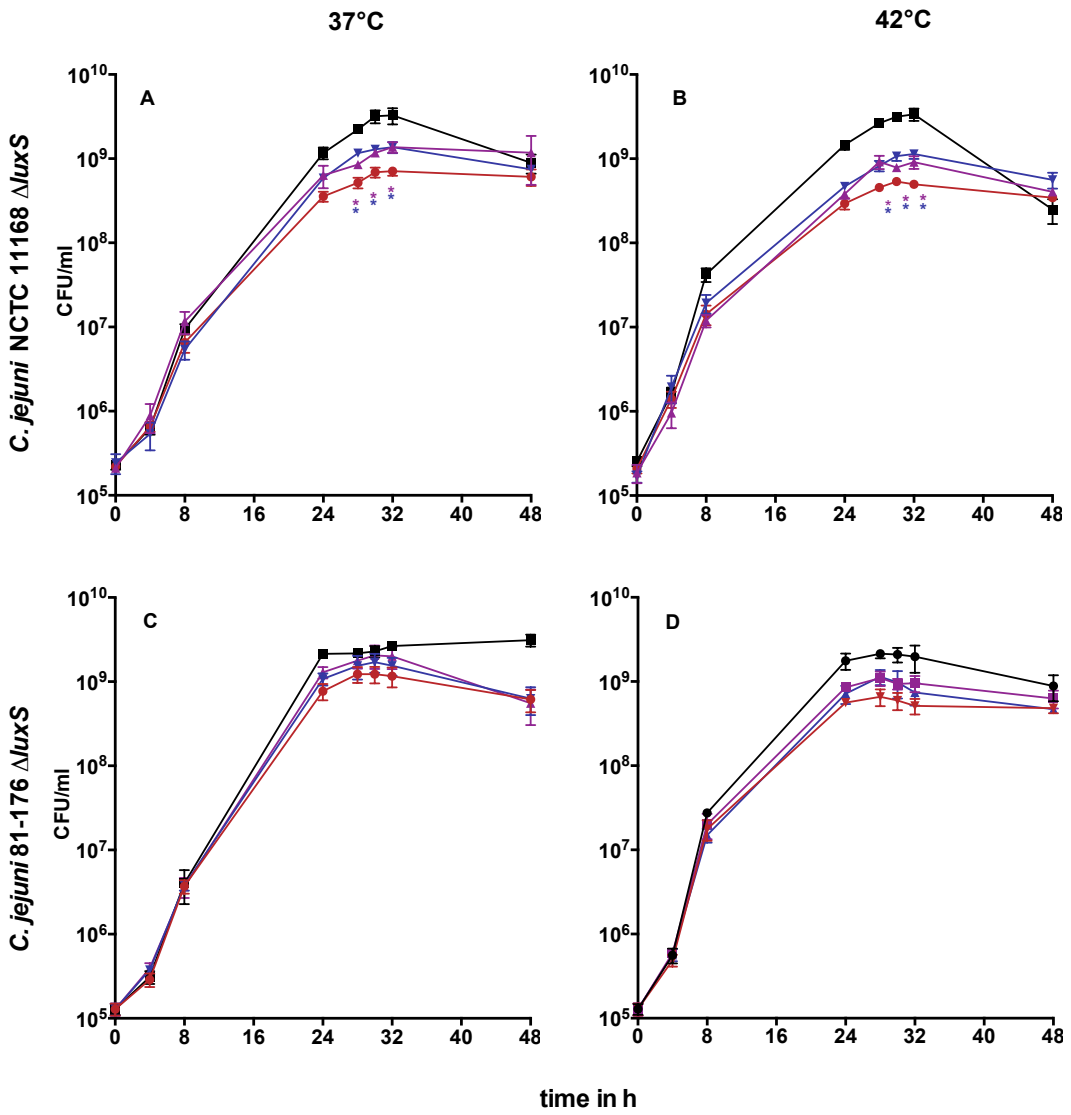

Figure 3: Growth curves of chemically complemented *C. jejuni* in BB (*C. jejuni* NCTC 11168Δ*luxS*) or MH (*C. jejuni* 81-176Δ*luxS*): A/C 37°C, B/D- 42°C; black- wild type, red- Δ*luxS*, purple- Δ*luxS*+AI-2, blue- Δ*luxS*+AI-2+HC; shown are mean ± SD (n=5), ∗- $p<0.05$ compared to Δ*luxS* (Mann-Whitney-U test)

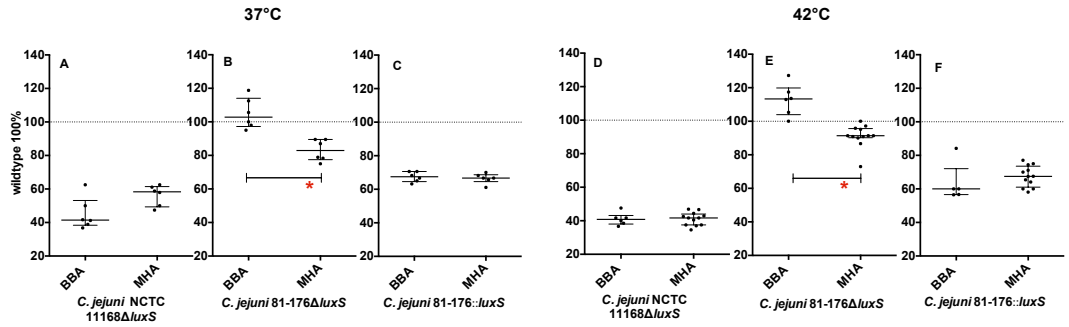

Figure 4: Swarming ability of *C. jejuni luxS* mutants on different media: A-C 37°C, D-F 42°C; shown are the normalized medians with interquartile range (n=6), ∗ -p< 0.05, (Mann-Whitney-U test); calculation of significance: BBA vs. MHA

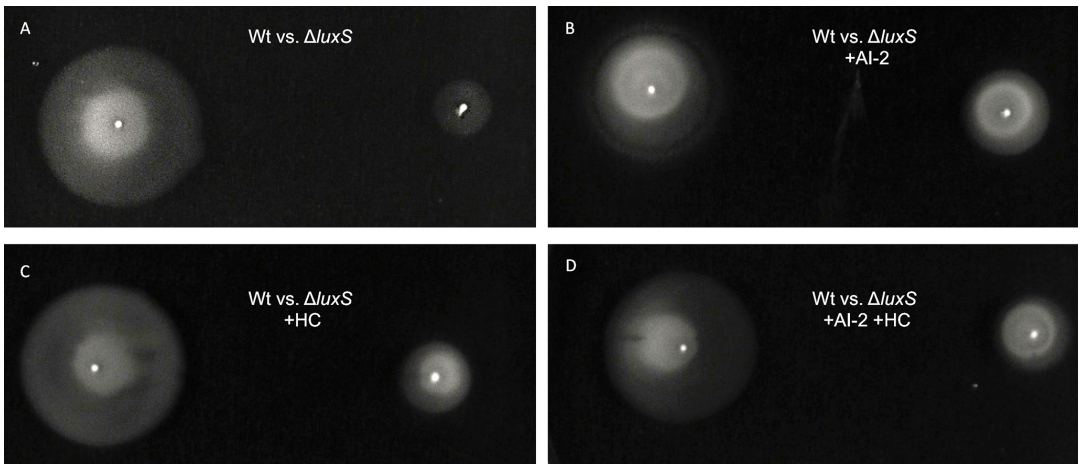

Figure 5: Swarming halos of *C. jejuni* 11168 wt and Δ*luxS* mutant in BBA (37°C): A- wt vs. Δ*luxS*, B- wt vs. Δ*luxS*+ AI-2, C- wt vs. Δ*luxS*+ HC and D- wt vs. Δ*luxS*+ AI-2+ HC

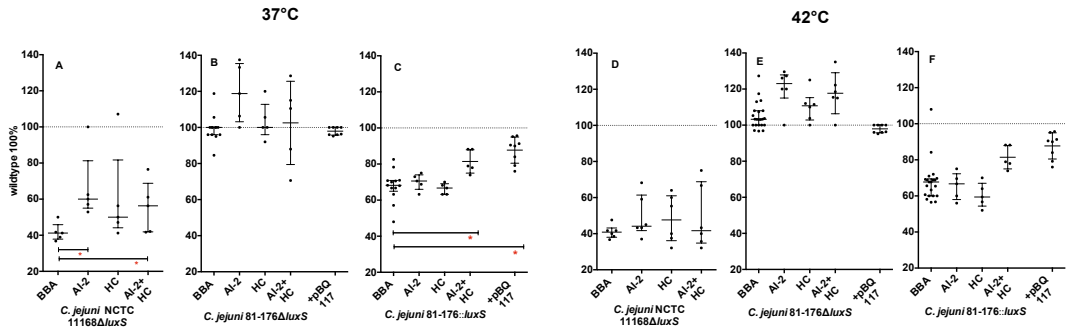

Figure 6: Swarming ability of complemented *C. jejuni luxS* mutants on BBA: A-C 37°C, D-F 42°C, complementation with: AI-2, HC, AI-2+HC and pBQ117; shown are the normalized median and interquartile range (n= 6), ∗ -p<0.05 (Mann-Whitney-U test)

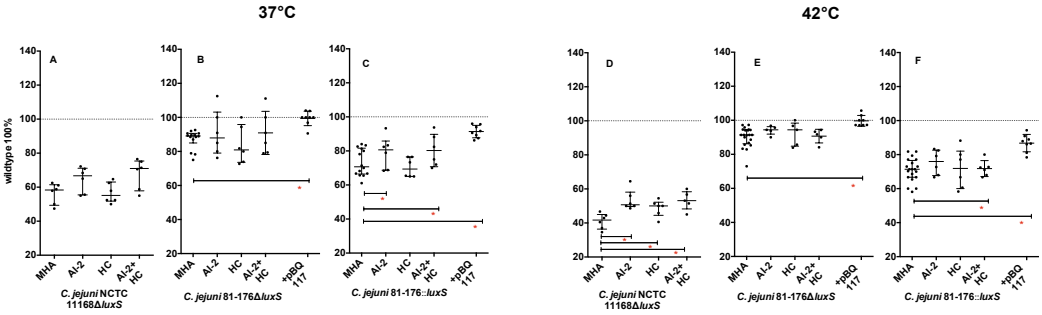

Figure 7: Swarming ability of complemented *C. jejuni luxS* mutants on MHA: A-C 37°C, C-F 42°C, complementation with: AI-2, HC, AI-2+HC and pBQ117 shown are the normalized medians and interquartile range (n= 6), ∗ -p<0.05 (Mann-Whitney-U test)

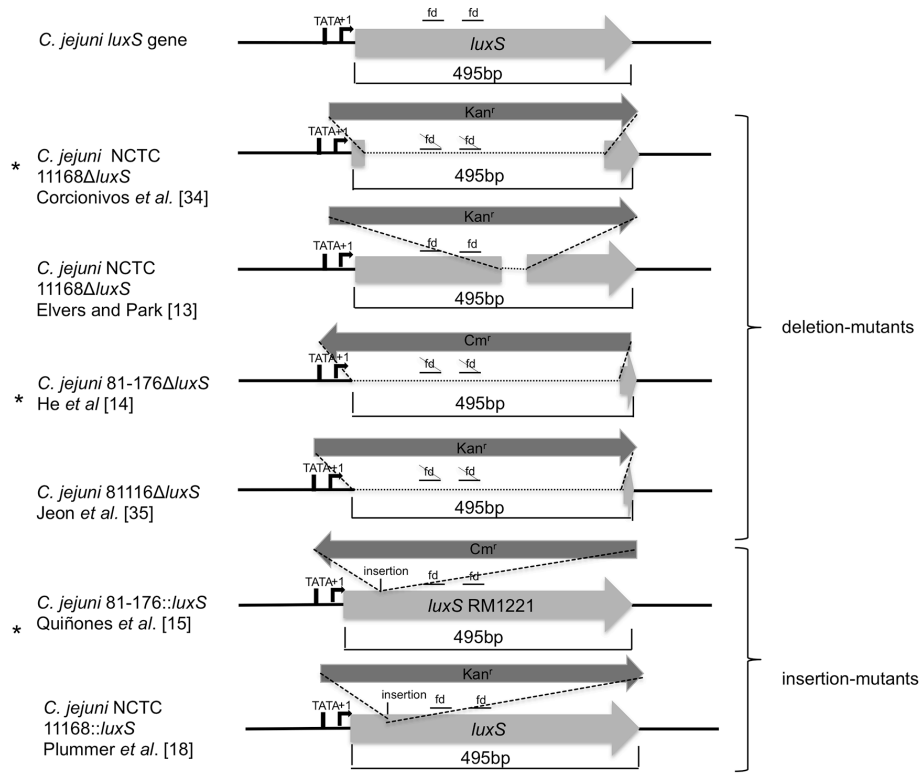

Figure 8: Comparison of mutation strategy of *C. jejuni luxS* mutants

Tab. 1: Bacterial strains used in this study

Strains	*luxS*	Description	Source or reference
C. jejuni 81-176	+	Wild type, virulent clinical isolate from a gastroenteritis outbreak	ATCC
C. jejuni NCTC 11168	+	Wild type, isolated from clinical sample in the UK in 1977	NCTC
C. jejuni 81-176Δ*luxS*	-	*luxS*- deletion mutant, Cm^r	He et al. [14]
C. jejuni 81-176Δ*luxS* +pBQ117	+	*luxS*- deletion mutant complemented with pBQ117, Cm^r, $Km^{r,}$	This study
C. jejuni 81-176*luxS* +pBQ1015	-	*luxS*- deletion mutant with *Campylobacter* vector pBQ1015, Cm^r, Km^r	This study
C. jejuni 81-176::*luxS*	-	*luxS*- insertion mutant, Cm^r	Quinones et al. [15]
C. jejuni 81-176::*luxS* +pBQ117	+	*luxS*- insertion mutant complemented with pBQ117, Cm^r, $Km^{r,}$	Quinones et al. [15]
C. jejuni 81-176::*luxS* +pBQ1015	-	*luxS*- insertion mutant with *Campylobacter* vector pBQ1015, Cm^r, Km^r	Quinones et al. [15]
C. jejuni NCTC 11168Δ*luxS*	-	*luxS*- deletion mutant, Km^r	Corcionivoschi et al. [34]
V. harveyi BB152		AI-2 positive control in luminescence Bioassay	Bassler et al. [16]
V. harveyi BB170		AI-2 reporter in luminescence Bioassay	Bassler et al. [16]

Cm, chloramphenicol; Km, kanamycin;

Tab. 2: Phenotypes (growth and swarming ability) of *luxS* mutants and its chemical complementation

***C. jejuni* strains**	**Kind of mutation**	**Medium**	**Temp.**	**Motility of *luxS* mutant vs. wt**	**Complemented with**			**Growth of *luxS* mutant vs. wt**	**Complemented with**		
					AI-2	**HC**	**AI-2+HC**		**AI-2**	**HC**	**AI-2+HC**
NCTC 11168	Δ*luxS*	BB	37°C	↓	↑	=	↑	(↓)	↑	=	↑
			42°C	↓	=	=	=	(↓)	↑	=	↑
		MH	37°C	↓	=	=	=	↓	n.a	n.a	n.a
			42°C	↓	↑	↑	↑	↓	n.a	n.a	n.a
81-176	Δ*luxS*	BB	37°C	=	n.a	n.a	n.a	↓	n.a	n.a	n.a
			42°C	=	n.a	n.a	n.a	↓	n.a	n.a	n.a
		MH	37°C	↓	=	=	=	↓	↑	=	↑
			42°C	↓	=	=	=	↓	↑	=	↑
81-176	::*luxS*	BB	37°C	↓	=	=	↑	=	n.a	n.a	n.a
			42°C	↓	=	=	=	(↓)	n.a	n.a	n.a
		MH	37°C	↓	↑	=	↑	(↓)	n.a	n.a	n.a
			42°C	↓	↑	=	=	=	n.a	n.a	n.a

↑: increased, ↓:reduced, (↓): slightly reduced, =: similar, n.a: not analysed, MH: Mueller-Hinton, BB: Brucella Broth, Temp.: temperature

Chapter 3: The signalling molecule Autoinducer-2 is not internalised in *Campylobacter jejuni*

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54-69

Chapter 4: General Discussion

There has been extensive research carried out to investigate the role of the *luxS* gene in many bacterial species; however, there still remains an intensive discussion whether its main role lies in metabolism or the production of the AI-2 signal. The presence of a cognate receptor for AI-2 is generally accepted; however, the classical LuxPQ receptor only has been clearly demonstrated in *Vibrio* spp. (Rezzonico and Duffy 2008, Pereira *et al.* 2012, Rezzonico *et al.* 2012). A second class of AI-2 receptor, the ABC transporter Lsr family, has been identified in some enterobacteriaceae and some gram-positive bacteria (Rezzonico and Duffy 2008; Rezzonico *et al*. 2012). Recently Rader *et al.* (2011) demonstrated a third group of AI-2 receptors. The chemorepellant receptor TlpB is required in *H. pylori* for AI-2 perception as chemorepellant. Alternative AI-2 receptors are still not identified for many bacterial species (Rezzonico and Duffy 2008; Rezzonico *et al*. 2012), it therefore remains unclear whether the phenotypes associated with a *luxS* mutation in such bacteria are due to the lack of AI-2 or may result solely from the metabolic imbalances caused by disruption of a key enzyme in the AMC, or are perhaps a combination of both deficits. Previous studies with *luxS* mutations in *C. jejuni* have used different strain backgrounds, culture conditions and mutation strategies to produce the *luxS* mutation, resulting in *luxS* mutants with dissimilar phenotypes. Furthermore most studies lack sufficient complementation resulting in not knowing whether phenotypes of *luxS* mutants depend on disrupted metabolism or lack of AI-2. Additionally, no AI-2 receptor in *C. jejuni* has been found yet. This situation contributes to an extensive discussion about the exact role of AI-2 in *C. jejuni*. Research from the thesis at hand provides new insights into the role of *luxS* and AI-2 in *C. jejuni*.

First, we described why literature about phenotypes of *C. jejuni luxS* mutants is extremely contradictory. Our study showed that the *C. jejuni luxS* phenotype was markedly dependent on strain background, culture conditions and mutation strategy. Further, some *luxS* mutant phenotypes could be partially complemented by AI-2, suggesting that *C. jejuni* can regulate its behavior by AI-2 dependent Quorum sensing.

Secondly, we demonstrated that AI-2 was not actively taken up by *C. jejuni* and suggest that further research on AI-2 receptors in *C. jejuni* should focus on two component signaling systems and not on transporter systems.

It is clear from multiple studies that the mutagenesis of the *luxS* gene in *C. jejuni* results in different phenotypes, e.g. motility and growth. Therefore, we investigated the impact of the strain background, kind of mutation and different culture conditions on the occurring phenotypes in *C. jejuni luxS* mutants. The corresponding study (Chapter 2) substantiates each of these parameters importance in developing the *C. jejuni luxS* mutant phenotype.
C. jejuni is a highly diverse species regarding strain-to-strain variability on the phenotypic and genomic level (Dingle *et al.* 2001). To investigate the influence of strain background on phenotypes of *luxS* mutants we used *C. jejuni* NCTC 11168 and *C. jejuni* 81-176 *luxS* mutants. Our investigation has clearly demonstrated that the genetic background of the strain is a major factor in determining the final phenotype of *C. jejuni luxS* mutant. While a *C. jejuni* NCTC 11168 *luxS* mutant showed altered phenotypes at the majority of conditions tested, the *C. jejuni* 81-176 *luxS* mutants varied in their phenotypes especially in a medium and mutation strategy dependent manner. Comparing these phenotypes we observed differences between *luxS* mutants of *C. jejuni* NCTC 11168 and *C. jejuni* 81-176 especially in BB (Chapter 2). Cell numbers of *C. jejuni* NCTC 11168Δ*luxS* in early stationary phase in BB are lower than cell numbers of *C. jejuni* 81-176Δ*luxS*. Also, the comparison of *luxS* mutant strains of *C. jejuni* 81-176 used by He *et al.* (2008) and *C. jejuni* NCTC 11168 used by Elvers and Park (2002) revealed different outcomes in terms of growth. Furthermore, the ability to swarm differed between the *luxS* mutants of both strains. Here, we observed that *C. jejuni* NCTC 11168Δ*luxS* exhibits the greatest reduction of swarming ability, whereas *C. jejuni* 81-176Δ*luxS* did not exhibit reduced swarming abilities at all in BB. The difference in observed phenotypes in *C. jejuni* NCTC 11168 and *C. jejuni* 81-176 may be a consequence of genetic diversity between these strains (Hofreuter *et al.* 2006). For instance, previous analysis of the complete flagellin glycosylation locus of *C. jejuni* strain 81–176 revealed a less complex genomic organization than the corresponding region in the genome of strain *C. jejuni* NCTC 11168 (Guerry *et al.* 2006). In addition, Dugar *et al.* (2013) identified strain-specific transcriptome organization and sRNAs that could contribute to differential gene regulation among these strains.

In addition to the aforementioned strain specificity, many environmental stimuli have been shown to affect AI-2 QS systems (Wang *et al.* 2005). Growth and swarming abilities of *C. jejuni* NCTC 11168Δ*luxS* were quite similar in BB and MH at both temperatures (37°C and 42°C), which leads to the assumption that these culture conditions do not have an

impact on the *luxS* mutant phenotype of this strain. He *et al.* (2008) described that the cell numbers of *C. jejuni* 81-176 differed between wild type and Δ*luxS* mutant at 37°C and 42°C in MH medium. In our study (Chapter 2), we also observed reduced cell numbers of *C. jejuni* 81-176Δ*luxS* in exponential as well as in late stationary phase in MH at both temperatures. In contrast, growth of *C. jejuni* 81-176Δ*luxS* in BB only showed significant differences to growth of wild type in late stationary phase at 37°C. These results demonstrate that the choice of the culture medium has a large impact on the resulting phenotypes of *C. jejuni* 81-176 *luxS* mutants. However, growth curves at 37°C and 42°C are quite equal in *C. jejuni* 11168Δ*luxS* and *C. jejuni* 81-176Δ*luxS*, which indicate that growth differences are independent of temperature but dependent on culture media for deletion mutant strains. In contrast growth of the *C. jejuni* 81-176::*luxS* was also influenced by temperature in MH. Motility observation confirmed this assumption as well. Like He *et al*. (2008) we observed reduced swarming abilities of *C. jejuni* 81-176Δ*luxS* at 37°C on MHA media in contrast to the wild type. However, this phenotype was only observed on MHA media but not on BBA. The components of these two different basal mediums were not defined and might therefore differ in their composition. However, Wang *et al*. (2005) showed that glucose affected the gene expression of *luxS* in *E. coli.* Furthermore, they observed that the expression of *pfs* was reduced by the presence of glucose. Even though *C. jejuni* lacks a glucokinase and 6-phosphofructokinase gene and consequently lacks the ability to catabolize many common carbohydrates like glucose as carbon sources, other influencing components could not be excluded to have an impact on resulting phenotypes of *C. jejuni luxS* mutants (Parkhill *et al.* 2000). Our findings clearly illustrate that resulting phenotypes of *C. jejuni luxS* mutants can be influenced by the choice of culture medium and components in media could also influence resulting phenotypes of *C. jejuni luxS* mutants.

One explanation for the differences in growth profiles between the different strains of *C. jejuni luxS* mutants might be the genetic differences between these two strains. However, it seems equally probable that the phenotypic differences observed were due to different mutation strategies applied. Previously Haigh *et al.* (2013) showed that mutation strategy and strain background influenced phenotypes of *E. coli luxS* mutants. The authors concluded that one explanation could be a difference in the mutants antibiotic resistance cassette orientation. The kanamycin resistance cassette of *C. jejuni* NCTC 11168Δ*luxS* is oriented in the same direction as the *luxS* gene, whereas the *C. jejuni* 81-176Δ*luxS* mutant

has the chloramphenicol resistance cassette in the opposite direction of the deleted *luxS* gene. Both mutants had reduced cell numbers when cultured in MH but only the *luxS* mutant of *C. jejuni* NCTC 11168 showed lesser swarming abilities compared to the corresponding wild type. Recently, it has been demonstrated that the regulatory small RNA MicA is located closely upstream of the *E. coli luxS* gene and could be an obvious target for polar effects of a *luxS* mutation (Kint *et al.* 2010, Udekwu 2010). In *C. jejuni*, Dugar *et al.* (2013) showed that a small RNA is located downstream of the *luxS* gene, but no function of this small RNA has been described so far. However, the expression of this small RNA could be influenced in a mutation strategy dependent manner. Since this molecule can have regulatory effects, its dysregulation can affect other pathways. Another reason for the observed different phenotypes could be the size of the deleted region. In contrast to our results, the *C. jejuni* NCTC 11168Δ*luxS* mutant constructed by Elvers and Park (2002) showed similar growth compared to the wild type at 37°C. The Δ*luxS* mutant of Elvers and Park (2002) has the same strain background and orientation of antibiotic resistance cassette as the Δ*luxS* mutant used in our study. However, the mutant used in our study has a larger deletion region, including those with functional domains from *luxS*, whereas the mutant from Elvers and Park (2002) still retains the functional domain regions. Even though the functionality relating to AI-2 production is disabled, it cannot be ruled out that other regions within this sequence exert an influence on other processes. Also the *C. jejuni* NCTC 11168::*luxS* mutant described by Plummer *et al.* (2012) (an insertion mutant up-stream of the functional domains) showed similar growth like the wild type. However, the mutant of Plummer *et al*. (2012) and Elvers and Park (2002) exhibited decreased motility. Examining the mutant in the current study, decreased motility haloes in semisolid media have been observed, which indicates that disruption of *luxS* causes a reduction of swarming ability in *C. jejuni* NCTC 11168 irrespective of whether the functional domain regions are deleted or retained. To investigate the influence of mutation strategy on phenotypes of *C. jejuni luxS* mutants we included an insertion mutant of *C. jejuni* 81-176 in our study. The resistance cassette of this mutant strain is also (like in *C. jejuni* 81-176Δ*luxS*) orientated in the opposite direction of the *luxS* gene. Examining growth in MH at 37°C and 42°C, the deletion mutant showed reduced cell numbers in stationary phase compared to the insertion mutant. Again, one reason could be the lack of the region among the functional domains within *luxS* in the deletion mutant, whereas this region is still present in the insertion mutant of *C. jejuni* 81-176. Additionally, Quiñones *et*

al. (2009) replaced the *luxS* gene of strain 81-176 by a mutated *luxS* gene of the *C. jejuni* strain RM1221, which is another possible explanation for differing phenotypes of *luxS* mutants. Furthermore, Quiñones *et al*. (2009) used larger up- and downstream regions of *luxS* for their mutation construct. Even though there is a high DNA sequence identity (96.8%) between the *luxS* genes of strains RM1221 and 81-176, there are some differences which might influence phenotypes of *luxS* mutants. The difference in observed swarming ability additionally indicates the importance of mutation strategy. Like Quiñones *et al*. (2009) we observed a significantly reduced swarming ability of *C. jejuni* 81-176::*luxS* mutant compared to the wild type on BBA, whereas the swarming ability of *C. jejuni* 81-176Δ*luxS* is not reduced on BBA in contrast to the wild type. While genetic complementation restored swarming abilities of *C. jejuni* 81-176Δ*luxS*, genetic complementation only partially restored swarming abilities of *C. jejuni* 81-176::*luxS*. The phenotype of wild type strain *C. jejuni* 81-176 was not fully achieved by the complementation. The incomplete restoration was probably caused by polar effects in this insertion mutant strain. Also Haigh *et al.* (2013) argued that insertion may always result in adverse polar effects. Combined, these findings suggest that growth deficits of *C. jejuni luxS* mutants might be associated with deletion of a larger region of *luxS*, while motility might be influenced by polar or compensatory mutation effects of the *luxS* mutation.

By comparing different *C. jejuni luxS* mutants we demonstrate that effects on motility and growth rates strongly depend on the strain background and culture conditions as well as the mutation strategy investigated. Only few studies complemented the *C. jejuni luxS* mutants genetically or chemically making interpretation of AI-2 dependent phenotypes more difficult. Based on the different outcomes of LuxS as both a key metabolic enzyme in the SAM recycling pathway and the synthase protein for the precursor of AI-2, it is more difficult to determine which of these mechanisms are responsible for the phenotypic changes. We investigated the phenotypes of *C. jejuni luxS* mutants chemically complemented with AI-2, HC and AI-2+HC as well as with genetical complementation. We observed that some *luxS* mutant phenotypes could be partially complemented by AI-2, suggesting that *C. jejuni* can regulate its behavior by AI-2 dependent Quorum sensing. Addition of HC to the mutant strains did not alter the *luxS* mutant phenotype indicating that disruption of the AMC downstream of LuxS might not be responsible for the observed phenotypes (data not shown). The accumulation of components upstream of LuxS within the AMC as well as other unknown functions of LuxS could also have an impact on the

observed phenotypes. Furthermore, in solution, DPD exists as equilibrium of different isomers through cyclization and hydration of DPD (Tsuchikama *et al.* 2011). A peculiarity of AI-2 signaling is that diverse bacteria have different AI-2 receptors which recognize distinct forms of AI-2. For example, *V. harveyi* responds to the borate diester derived from (2S,4S)-THMF, whereas *Salmonella* Typhimurium, *Sinorhizobium meliloti* and *Yersinia pestis* respond to (2R,4S)-THMF (Chen *et al.* 2002, Miller *et al.* 2004, Neiditch *et al.* 2006, Pereira *et al.* 2008, Kavanaugh *et al.* 2011). Thereby it is possible that the DPD used in this study might not have harboured adequate amounts of the relevant DPD variant for *C. jejuni*. However, as the synthetic DPD also induced luminescence in the *V. harveyi* assay, it can be concluded that AI-2 produced by *C. jejuni* and synthetic DPD contained a similar variation of AI-2. The response observed following chemical complementation would suggest that the structure of AI-2 was adequate for receptor recognition. Nevertheless it remains unclear if the lack of complete complementation could be caused by an inappropriate chemical equilibrium of the AI-2 structures. The fact that addition of exogenous AI-2 did not completely restore wild type levels of growth and motility in *C. jejuni luxS* mutants suggests that loss of AI-2 signaling was not the sole reason for the phenotypes observed. It remains unclear whether occurring phenotypes of *luxS* mutants, which could not be complemented by exogenous AI-2 results from the metabolic deficits caused by disruption of the AMC or by polar effects due to mutation strategies. Genetic complementation of both *C. jejuni* 81-176 *luxS* mutants resulted in wild type comparable growth curves, indicating that not polar effects of mutation but disrupted AMC are responsible for the uncomplementable growth defects of *C. jejuni* 81-176 *luxS* mutants. While genetic complementation restored swarming abilities of *C. jejuni* 81-176Δ*luxS*, it did not completely restore the phenotype of *C. jejuni* 81-176::*luxS*. By the addition of AI-2, the same swarming ability as shown for the genetically complemented mutant was achieved. These findings indicate that reduced swarming abilities are partially due to polar mutation effects in *C. jejuni* 81-176::*luxS*. The reduced swarming ability could be partially complemented by exogenous AI-2 but not with HC, indicating that disrupted AMC is not the reason for this phenotype.

Altered motility has also been described for some other *C. jejuni luxS* mutants (Elvers and Park 2002, Quinones *et al*. 2009). Further, changed expression of several flagellar associated genes has been observed in *C. jejuni luxS* mutants (Jeon *et al.* 2003, He *et al.* 2008, Holmes *et al.* 2009). No conclusive concept on the regulation mechanism for

phenotypic alteration of motility has been introduced yet. As motility is not lost completely, maybe motor proteins or their activation state are regulated by AI-2. As *C. jejuni luxS* mutants showed reduced motility in a media and mutation type dependence it seems possible that AI-2 is modulating motility in a chemotactic manner. The exact mechanisms of reduced motility in the *luxS* mutants need to be clarified.

Since the *luxS* gene was found to be widespread among the most diverse bacterial taxa, it was hypothesized that AI-2 may constitute the basis of a universal interspecies-specific microbial language. Many of the studies published in this field have drawn a direct correlation between the occurrence of the *luxS* gene and the presence and functionality of an AI-2 QS System. However, existing studies have rarely examined the existence of potential AI-2 receptors. Not knowing the appropriate AI-2 receptor makes the investigations of AI-2 dependent phenotypes much more complicated as both, the lack of AI-2 as well as the disrupted AMC, might be responsible for observed changes in *luxS* mutants. This could also been shown as aforementioned *luxS* mutant phenotypes could only be partially complemented by synthetic AI-2 (Chapter 2). To assess how AI-2 induces the altering phenotypes during complementation assays, we performed an AI-2 uptake assay. We analyzed whether the AI-2 uptake by *C. jejuni* resembles the uptake by *E. coli* and *Salmonella* spp. or if it is sensed by a two-component system as described for *Vibrio* spp. and *H. pylori*. In *E. coli*, extracellular AI-2 accumulates in exponential phase, but the amount decreases drastically upon entry into stationary phase. The rapid disappearance of AI-2 is a consequence of its import by an ABC- Transporter (Lsr). By conducting an AI-2 uptake assay we could demonstrate that AI-2 diminished in the supernatants of *E. coli* cultures but not in the supernatants of *C. jejuni* and *V. harveyi* cultures, suggesting that AI-2 is also sensed by a two-component signalling system in *C. jejuni* (Chapter 3).

As mentioned before, one characteristic of AI-2 signaling is that the different AI-2 receptors recognize distinct forms of AI-2. It seems probable that the synthetic AI-2 used in this study also has an inappropriate structure for recognition in *C. jejuni*. Again, in solution, DPD exists as equilibrium of different isomers through cyclization and hydration of DPD (Tsuchikama *et al.* 2011). Hence, the synthetic AI-2 was able to induce bioluminescence in *V. harveyi* and was taken up by *E. coli*. This suggests, that the DPD used in this study (Chapter 3) harboured adequate amounts of both AI-2 variants, and neither of these AI-2 forms is taken up by *C. jejuni*. Our data show, that AI-2 is not

internalised by *C. jejuni*. The data suggest, that yet unknown AI-2 receptors are present on the cell surface of *C. jejuni*. In any case the possibility that some bacteria which are lacking an AI-2 receptor either produce AI-2 for interference with signaling of other bacteria or that inadvertently produced AI- 2 is detected by other bacteria cannot be discarded and may have important ecological implications. Furthermore, *Campylobacter* belongs to the class of *Epsilonproteobacteria*, being evolutionary different and representing an ecologically diverse group of microorganisms that are rather evolutionarily distinct from the *Gammaproteobacteria,* such as *E. coli*, *Salmonella* spp. and *Vibrio* spp. (Gilbreath *et al.* 2011). This might also explain different kinds of AI-2 perception. The evolutionary differences as well as the different types of AI-2 recognition systems already described, let us speculate that other kinds of AI-2 receptors exist in *C. jejuni*. Furthermore, the recently described perception of AI-2 by chemoreceptors in *E. coli* and *H. pylori* could also be an opportunity for AI-2 perception in *C. jejuni* (Hegde et al. 2011; Rader et al. 2011). *E. coli* sense AI-2 as a chemoattractant via the chemoreceptor Tsr. In contrast, AI-2 is perceived as chemorepellent in *H. pylori* by the chemoreceptor TlpB, but the signal recognition mechanism is not clear so far. Therefore further search of AI-2 receptors in *C. jejuni* should focus on two component signaling systems or chemoreceptors and not on transporter systems.

Conclusion

Our findings clearly show that the culture conditions, mutation strategy used to create the mutants and the background of the strain used to analyse the impact of the *luxS* mutation are major influences on the resulting phenotype. We believe that it is important that QS researchers become aware of the difficulty of using different mutants of *luxS* in order to avoid further confusion over the exact role of AI-2 in *C. jejuni* phenotypes. In conclusion, we hope that this study may provide a convincing explanation for the wide disparity in the phenotypes which have been reported for *C. jejuni luxS* mutants. This study also provides insight into the challenges that polar mutations can cause, especially when these mutations are constructed in genes proposed to be involved in complex regulatory circuits, such as QS/AMC. Moreover it will be necessary to do further research in case of finding AI-2 receptors in *C. jejuni* and this should focus on two component signaling systems and not on transporter systems.

Future perspectives

C. jejuni encounters constantly changing environments and niches during transmission, colonization and growth. *C. jejuni* has the ability to adapt and survive in highly divergent environmental conditions. A better understanding of the molecular and cellular mechanisms responsible for adaptation to diverse environments and hosts will provide new insights into both the mechanisms of disease and potential targets for intervention. QS has been demonstrated to be an important component of environmental adaptation of several bacterial organisms and this needs to be further examined in *C. jejuni*.

The finding of another AI molecule in *C. jejuni* offers new possibilities in QS research. The identification of a putative HSL type compound (cjA) produced by *C. jejuni* demonstrates that *C. jejuni* appears to produce, as well as detect, exogenous signaling molecules and respond accordingly to aid in the survival and virulence capabilities (Moorhead and Griffiths 2011). Future work in these areas will provide critical information regarding the role of cjA in *C. jejuni.* Additional work is necessary to define the role of HSL signaling in *C. jejuni* and to determine the molecular mechanisms responsible for the synthesis and sensing of these compounds.

4.2 References for General Discussion

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Eidestattliche Erklärung

Ich erkläre hiermit, dass ich diese Dissertation selbstständig ohne Hilfe Dritter und ohne Benutzung anderer als der angegebenen Quellen und Hilfsmittel verfasst habe. Alle den benutzten Quellen wörtlich oder sinngemäß entnommenen Stellen sind als solche einzeln kenntlich gemacht.

Berlin, den

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