Aus dem Institut für Mikrobiologie und Tierseuchen
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Functional plasmid analysis of ESBL-producing *Escherichia coli* of pandemic sequence types ST131 and ST648

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LIST OF ABBREVIATIONS

***afa*:**	Gene for afa/Dr adhesion
AmpC:	Ampicillinase C
AMR:	Antimicrobial resistance
(c)AMP:	(Cyclic) adenosine monophosphate
APEC:	Avian pathogenic *E. coli*
ATP:	Adenosine triphosphate
BHI:	Brain-heart infusion
bdar:	Brown, dry and rough morphotype
***bla*:**	Gene for beta-lactamase
bp:	Base pair
Caco-2:	Carcinoma of the colon cell line
c-di-GMP:	Bis-(3′-5′)-cyclic dimeric guanosinmonophosphate
CF29K:	Non-fimbrial adherence protein
CR:	Congo red
CsgD:	Curlin sigma S-dependent growth protein
CTX:	Cefotaximase beta-lactamase
DNA:	Desoxyribonucleic acid
***eae*:**	Gene for *E. coli* attaching and effacing
***E. coli*:**	*Escherichia coli*
e.g.:	For example
EPEC:	Enteropathogenic *E. coli*
ESBL:	Extended-spectrum beta-lactamase
et al.:	And other people
ExPEC:	Extra-intestinal pathogenic *E. coli*
F (F+) factor:	Fertility factor
***fim*:**	Gene for fimbriae
FQ:	Fluoroquinolone
H-antigens:	“Hauch”-antigens
HGT:	Horizontal gene transfer
HUS:	Hemolytic uremic syndrome
***ibeA*:**	Gene for invasion of brain endothelium protein
ICE:	Integrative and conjugative element
***iha*:**	Gene for adhesion siderophore
IMT:	Institut für Mikrobiologie und Tierseuchen
Inc:	Incompatibility
InPEC:	Intestinal pathogenic *E. coli*
***iroN*:**	Gene for catecholate-siderophore receptor
IS:	Insertion sequence
kbp:	Kilo base pairs
MGE:	Mobile genetic element
MLST:	Multi-locus sequence typing
MOB:	Mobility

pdar:	Pink, dry and rough morphotype
PAI:	Pathogenicity island
PBRT:	PCR-based replicon typing
PCR:	Polymerase chain reaction
PCV:	Plasmid "cured" variant strain
PFGE:	Pulsed-field gel electrophoresis
PPA:	Plasmid-profile analysis
PM:	Phenotype MicroArray
pMLST:	Plasmid multi-locus sequence typing
QS:	Quorum sensing
rdar:	Red, dry and rough morphotype
Rep:	Replicon
R(1)-plasmid:	Resistance (1) plasmid
(r)(m)RNA:	**(**Ribosomal) (messenger) ribonucleic acid
***sat*:**	Gene for secreted autotransporter toxin
SHV:	Sulfhydryl variable beta-lactamase
SNP:	Single-nucleotide polymorphism
T:	Transformant strain
TA:	Toxin-antitoxin
TEM:	Temoneira beta-lactamase
tra:	Transfer region of plasmid
UPEC:	Uropathogenic *E. coli*
***usp*:**	Gene for uropathogen-specific protein
UTI:	Urinary tract infection
VF:	Virulence factor
WGS:	Whole genome sequencing
WT:	Wild-type strain
16S:	Component of the ribosomal subunit 30S

1. INTRODUCTION

Successful bacterial lineages and clones attract global attention due to their individual or combined pathogenic, infectious, and zoonotic characteristics [1-3]. While these characteristics are already problematic, their often antimicrobial (multi-) resistant (AMR) phenotype [4-7] considerably limits therapeutic options, intensifying their pandemic risk in both human [7-9] and veterinary medicine [10, 11].

Extra-chromosomal elements such as AMR-conferring plasmids are among the driving forces behind the emergence of high-risk bacterial lineages and clones in terms of resistance [9]. Interestingly, successful bacteria carrying AMR plasmids are also detected in environments with low and moderate antimicrobial selection pressures [12-15]. This not only calls into question the old dogma of fitness costs for bacteria that harbor plasmids but also raises the question of how AMR plasmids contribute to the clonal emergence of pathogenic strains beyond resistance factors.

A prime example of AMR plasmids are those mediating resistance to third-generation cephalosporins through carriage of extended-spectrum beta-lactamase- (ESBL) genes [16]. ESBL-plasmid carriage seems often associated with certain bacterial phylogenetic backgrounds, as with *Escherichia (E.) coli* sequence type (ST) ST131, which has led to our hypothesis that AMR plasmids interact with the bacterial chromosome to help explain the bacterial host's success in various habitats [17].

This thesis has aimed at contributing to the understanding of the mechanisms of successful bacterial lineages and clones exemplified by AMR *E. coli* of ST131 and ST648, focusing on the influence of ESBL-plasmids on non-resistance features including fitness and virulence.

HYPOTHESIS I: The carriage of ESBL-plasmids in *E. coli* does not result in fitness-associated disadvantages for the bacterial host.

Work objectives I:

- To establish an ESBL-plasmid extraction ("curing") technique to generate ESBL-plasmid "cured" variants (PCV) of ST131 and ST648 wild-type (WT) strains as base for further analyses;
- To retransform the ESBL-plasmid into PCVs resulting in transformants (T) as verification for further analyses; and
- To comparatively analyze WT, PCV and T strains in fitness-associated assays.

HYPOTHESIS II: An interaction of ESBL-plasmids with the *E. coli* chromosome results in non-resistance factor-associated advantages for the bacterial host.

Work objectives II:

- To phenotypically compare WT, PCV and T strains in biofilm and motility assays;
- To genotypically compare one WT, PCV and T strain combination in RNA sequence analysis focusing on non-resistance factor-associated transcriptomes; and
- To characterize the ESBL-plasmids to identify candidate genes and pathways responsible for the interaction.

2. LITERATURE

2.1 Bacterial lineages & clones

Successful bacterial lineages that are often clonally related and antimicrobial resistant derive from Gram-negative and Gram-positive bacterial genera, including *Acinetobacter* [18], *Staphylococcus* [19], *Klebsiella* [20] and *Escherichia* [6, 21]. All having in common a frequent occurrence in various global settings, one might ask what enables their pandemic success. Most likely it is a favorable combination of AMR, virulence, and fitness [22].

My thesis addressed this question by exemplarily studying successful pandemic *E. coli* of ST131 and ST648. The distinction between lineage, clonal lineage, and clone seems ambiguous. According to Mathers et al. [9] and based on Dijkshoorn et al. [23] a clone refers "to the progeny of one bacterial cell through asexual reproduction, implying that the same clonal lineage consists of highly closely related isolates that have recently diverged from a common ancestor". Isolates assigned to one clonal group, however, may differ in their genotype due to recombination and rearrangement events [24]. Clones should be defined as highly similar or indistinguishable after using a specific molecular characterization technique [9]. Specifically, isolates within one ST that have similar features (e.g. serotype and AMR resistance patterns) are normally termed "lineage" while clonally related isolates within one lineage (based on macrorestriction and whole genome sequence [WGS] analysis) are termed "clone". As the literature does not use these terms consistently, they will be applied alternately throughout this thesis, dependent on the relevant reference.

I will mostly follow the definition of "international high-risk multi-drug resistant clones" that are defined as following [25]: i) globally distributed, ii) associated with several AMR factors, iii) able to persist in hosts for a long time (> 6 months), iv) able to transmit across different hosts, v) equipped with enhanced virulence and fitness and vi) able to cause severe and/or recurrent infections [9]. *E. coli* is a potential species that harbors such clones; I will introduce it in the following paragraphs.

2.1.1 *Escherichia coli*

This Gram-negative, facultative, anaerobic, rod-shaped bacterium was discovered by Theodor Escherich in 1885 and is one of the best investigated and most used bacteria in research [26]. Apathogenic (commensal) are distinguished from intestinal pathogenic (InPEC) and extra-intestinal pathogenic *E. coli* (ExPEC) [27, 28], which mostly depends on production of virulence factors. Commensal *E. coli* are components of the resident microbiota in humans and warm-blooded animals and birds [26, 29, 30]. While already transmitted through the mother at birth [31] they are among the first colonizers in a newborn's gut. *E. coli* provide the host with vitamin K2 and may prevent gut colonization with pathogens [32]. InPEC and ExPEC subtypes are a ubiquitous cause of diarrhea [26], urinary tract infections (UTI), bacteremia, soft tissue, and other infections in humans [33] and animals [34, 35].

Many pathogenic *E. coli* are resistant against a broad range of different antimicrobial classes, which results in multi-resistant phenotypes and limitations in antimicrobial therapy options [36]. The respective resistance genes are often carried on mobile genetic elements (MGE) with large ESBL-plasmids as prominent representatives. ESBL-enzymes are effective against one of the most important antimicrobial classes in human and veterinary medicine, the beta-lactams, which exacerbates the global health risk in the antibiotic era.

Given their versatility, a detailed characterization is necessary to determine the *E. coli's* molecular characteristics and phylogenetic population structures. Traditional phenotypic and genotypic methods including serotyping and classical PCR-techniques are increasingly substituted and/or expanded by bioinformatics approaches based on WGS data [37, 38]. Phylogenetic grouping allows a first *E. coli* phylogenetic categorization, sophisticated by multi-locus sequence typing (MLST) based on the allelic variation of seven housekeeping genes, which assigns isolates to STs [39]. *E. coli* are then clonally analyzed with macrorestriction techniques followed by pulsed-field gel electrophoresis (PFGE) [40].

E. coli belong to four phylogenetic groups: A, B1, B2 and, D [41, 42]. ExPEC belong mainly to group B2 and, to a lesser extent, group D. Commensal types are mostly found in groups A and B1 [43]. At this time, almost 6,000 different STs have been described representing the tremendous species phylogenetic diversity. The more surprising is the dominance of certain STs and even lineages in the global health context. Also interesting is the suggested association of ESBL-plasmid carriage with a minority of the large group of *E. coli* STs, including ST131, ST648, ST224, and ST410 among others.

2.1.2 *E. coli* sequence type ST131

ST131 is an *E. coli* ST harboring international multi-drug resistant high-risk clones. As mentioned above, the assignment to STs allows a first, rough strain characterization, followed and expanded by other classification and typing methods to identify bacterial lineages and clones.

Originally identified in human clinical isolates [44], ST131 seems particularly associated with community-acquired infections [9]. A rapid pandemic dissemination was observed in different settings since 2008 including domestic animals and the environment, such as wastewater and wildlife [12, 33, 45-49]. Mathers et al. [9] describe the ST131 pandemic as a human phenomenon, thus substantially more prevalent in humans than in animals, food, or the environment, which might be due to limited study numbers. Transmission of ST131 among individuals in one family [50] and among humans and pets has been previously described [51]. ST131 belongs to phylogenetic group B2, which is known for harboring isolates causing extra-intestinal infections [21], commonly UTIs and bacteremia [21]. ST131's prevalence depends on geographic regions and host population [9]; it is estimated between 10 and 30% of all human clinical *E. coli* isolates [51]. Individual and combinative approaches explain the ST131's ubiquity: i) selective advantages including antimicrobial multi-resistance [52], ii) a broad environmental presence [53], iii) a transmission via companion [11] and wild animals [48], iv) a human to human transmission [54], v) contaminated food [55] and vi) travel [56].

Strains of ST131 may possess plasmids often associated with the production of ESBL enzymes, mostly CTX-M-15 [44, 57]. While horizontal gene transfer (HGT) constitutes the main driving force for AMR dissemination in *E. coli* [58] other data suggest that clonal expansion of ST131 is also of considerable importance [58, 59]. Although first gaining attention due to their third-generation cephalosporin-resistant phenotype in 2008, large-scale studies suggest that ST131 strains were first, in the early 2000s, negative for ESBL-production, but resistant against fluoroquinolones [49]. ESBL-resistance gene acquisition seemed to happen over time [51]. In addition to third-generation cephalosporin and fluoroquinolone resistance, ST131 strains often confer resistance towards tetracyclines, aminoglycosides, and trimethoprim-sulfamethoxazole among others [49].

The possession of ExPEC-associated virulence genes including *iha* (adhesion siderophore), *sat* (secreted autotransporter toxin) and *usp* (uropathogen-specific protein) demonstrates the ST131's high virulence potential [60]. Van der Bij et al. [61] showed that

ST131 displayed a significantly higher virulence factor (VF) level than other STs. The combination of specific VFs with AMR seems to affect ST131 isolates positively when compared to less resistant and/or less virulent *E. coli* STs [62]. However, a different study [63] did not detect a higher virulence potential of ST131 isolates when compared to other STs in a mouse-sepsis model. A Spanish research group assigned a large collection of ST131 isolates to four virotypes with VFs distinctive for each group: *afa* (afa/Dr adhesion), *iroN* (catecholate-siderophore receptor), *ibeA* (invasion of brain endothelium), and *sat*. [64].

ST131's most prevalent lineage contains the mannose-specific type1 fimbriae *fimH30* allele [21, 65]; it was termed *H*30 [58, 65, 66] (Fig. 1) and first detected in the early 2000s [9]. Within this lineage, clinically important and expanded sub-lineages are fluoroquinolone resistant *H*30R or clade C1 and CTX-M-15 (mostly)-producing *H*30Rx or clade C2 [58, 66], as recently proposed. Not all CTX-M-15-producing ST131 isolates, however, belong to *H*30Rx [9]. Interestingly, there seems to be an association of *H*30Rx with VF accumulation [67, 68]. Other clades are clade A (*fimH41*) and B (*fimH22*) [21]. *fimH30*, *fimH22* and *fimH35* have the same serotype, O25b:H4 [9] (Fig. 1). As previously mentioned, a combination of virulence, AMR, and phylogenetic background may explain the success of ST131 and especially of the sub-lineage *H*30Rx [69]. Strains of ST131 represent a prime example of international multi-drug resistant high-risk clones.

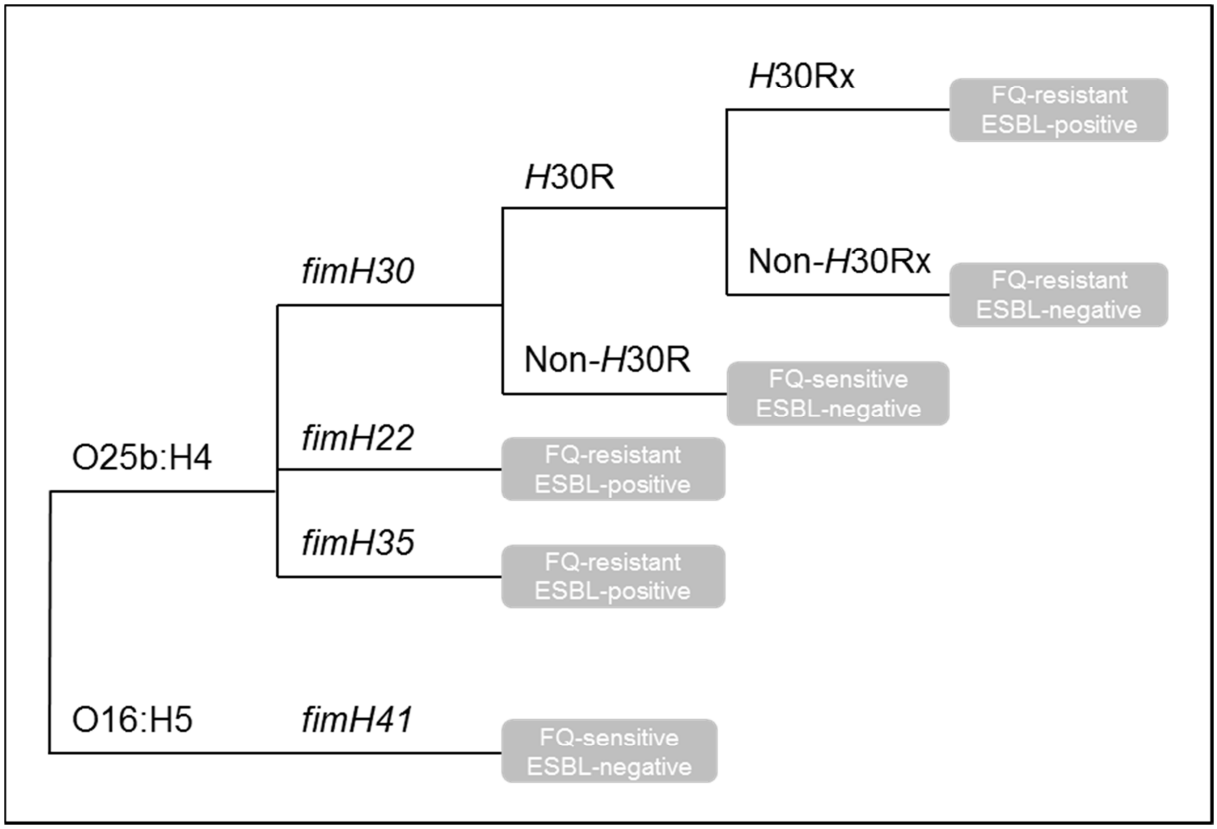

Figure 1: *Structure of E. coli sequence type ST131 of two serotypes (O25b:H4 and O16:H5) with lineages and sub-lineages producing or not producing extended-spectrum beta-lactamase-(ESBL) enzymes and being resistant or sensitive against fluoroquinolones (FQ). Adapted from Mathers et al., 2015 [9].*

2.1.3 *E. coli* sequence type ST648

Another *E. coli* ST seemingly associated with CTX-M-production is ST648. It belongs to phylogenetic group D and was first described by Nicolas-Chanoine et al., who detected it in a human isolate from Spain [57]. Since then ST648 has been isolated from human clinical samples in China [70, 71], The Netherlands [72], Canada [73] and Brazil [74], from poultry farms in Spain [75], from wild birds in Germany and Mongolia [76, 77], from clinical samples of companion animals and horses in Europe [78, 79] and from surface waters in Switzerland [80]. The authors of this last study also found ST648 isolates in healthy humans, suggesting an anthropogenic origin of ST648. Results from China demonstrate that a multi-drug resistant ST648 clone, which produces CTX-M-enzymes, might have emerged in the Chinese community [81].

In a recent study from Nepal [82], ST648 was the second most common ST (15 of 105 clinical ESBL-producing *E. coli* isolates), only surpassed by ST131. These authors performed further analyses on non-ESBL resistance factors and virulence profiles of ST131 and ST648, comparing it to non-ST131/648. ST648 showed a proportionally higher resistance to non-beta-lactam antimicrobials than ST131 and non-ST131/648 but had a lower overall virulence score as compared to ST131. The lower virulence potential aligns with other studies [83], and is not surprising as ST648 belongs to phylogenetic group D with generally lower numbers of highly virulent isolates compared to phylogenetic group B2. While the exact role of ExPEC-associated virulence genes in ST648 is still unknown, the combination of those with AMR and the phylogenetic background might be the reason for its success, comparable to ST131 [72].

Further investigation is still necessary to determine whether strains of ST648 meet the above mentioned criteria for an international multi-drug resistant high-risk clone.

2.2 The role of plasmids in the emergence of bacterial lineages & clones

MGEs are important contributors to the distribution of AMR, virulence [9] and the emergence of pandemic multi-resistant high-risk clones. Received from other organisms through HGT [84], MGEs are a driving force behind bacterial evolution and ecological diversity, and contribute to the bacterial host's adaption to changing environments and conditions [84, 85]. Based on different characteristics MGEs are differentiated into plasmids, bacteriophages, insertion sequences (IS), transposons, integrative and conjugative elements (ICE), and pathogenicity islands (PAI) [86-88].

2.2.1 General plasmid characteristics

Plasmids are extra-chromosomal, mostly circular and double-stranded genetic elements that replicate independently of the bacterial chromosome [89, 90]. Sizes range from 800 base pairs (bp) up to 300 kbp. Plasmids exist in a varying quantity of copies per cell, called copy number. Plasmidic genes and interactions between host cell and plasmids influence this number. Smaller plasmids have copy numbers of up to 1000 per cell.

Plasmids often contain genes essential for their transfer, known as conjugation fertility (F) factors. F factors induce the formation of protein cylinders (sex-pili) to enable the

connection of donor and recipient cells for a subsequent exchange of genetic elements [29]. Naturally occurring bacteria often contain small, cryptic plasmids that only carry replication genes and sequences of unknown function and are transferred to other hosts via larger conjugative plasmids, referred to as mobilization [86, 91].

Characterization of plasmids is based on their relatedness, which is important for representing their distribution, phylogenetic origins, and their relationship to the host cell [16, 91]. In 1971, a characterization scheme was developed due to the plasmids' ability to maintain during conjugation- the so-called incompatibility (Inc) [92]. Plasmids of one Inc group are unable to stably coexist in one bacterial cell, which is dependent on their replicon (Rep) machinery [93]. Inc typing does not necessarily reflect true evolutionary divergence [94]. Besides, a single bp change in the element conferring Inc might render two similar plasmids compatible [91]. Thus, a new scheme for plasmid comparison and typing has since been widely accepted, where DNA sequences essential for replication, the so-called conserved replicons, are used. These replicons are targeted by PCR-based replicon typing (PBRT), which is originally based on Southern blot hybridization, providing the typing of both conjugative and non-conjugative plasmids [16, 95, 96]. PBRT targets different Rep types of the major plasmid families in Enterobacteriaceae including HI2, HI1, I1-γ, X, L/M, N, FIA, FIB, FIC, W, Y, P, A/C, T, K, and B/O [95, 97-99]. Rep elements, however, are often unrelated as bacterial plasmids often contain several and even recombinant regions [91]. Generally, since Rep typing determines the Inc group, these two terms are exchangeable [93]. Plasmids of different Inc/Rep types exhibit different behaviors, e.g. regarding their host specificity: narrow-host-range plasmids often belong to Inc group F (IncF), with replicons FIA and FII and others, and those with a broad-host-range belong to IncA/C and IncN [9]. Broad-range plasmids are easily transmittable between different bacterial species [16].

Other methods to determine plasmidic backbones include plasmid MLST (pMLST) [100] and PCR-based detection of genes beyond plasmid replication and maintenance [101, 102]. The one common feature of all transmissible plasmids is the relaxase protein, which is the crucial factor for plasmid transfer initiation [91]. The evolutionary relatedness among relaxases was investigated resulting in a total of six relaxase (MOB from mobility) families [103]. As noted by the authors [91] "plasmid mobilization is an almost universal procedure for gene spread among bacteria" and "a classification of plasmids according to their mobilization properties could be universal and will be of paramount importance, since it could provide a more suitable alternative to classification by Rep regions".

Many plasmids have co-evolved with bacteria, they carry genes affecting their bacterial host's phenotype considerably; besides resistance (e.g. antimicrobial and heavy metal resistance) and virulence (e.g. enterotoxins and hemolysins) also physiological determinants (e.g. lactose metabolism) [26, 89, 90]. Plasmids help their bacterial host adapt to and develop in unsuitable environments [9].

2.2.2 AMR plasmids

AMR-carrying plasmids [94] attract exceptional attention all over the world. They are central to the global prevalence and rapid emergence of AMR bacteria [9]. Often encoding resistance mechanisms against multiple antimicrobial classes [104], they confer antimicrobial multi-resistance phenotypes. Moreover, they frequently carry non-resistance factors including those important for virulence [105, 106] and regulation [107].

As large non-AMR parts of plasmid sequences, however, remain rather unexplored one is left only to speculate about their function [108]. AMR plasmids give their host an edge in terms of antimicrobial selection advantages, yet little is known about their stability and persistence in environments without high antimicrobial selection pressures [9]. A well-known AMR plasmid group is the one encoding ESBL-enzymes, with the main beta-lactamase gene families $bla_{\text{CTX-M}}$, bla_{TEM}, and bla_{SHV}. The carriage of ESBL-genes seems to be associated with particular Inc/Rep types; CTX-M-15, for instance is mainly encoded by plasmids of the IncF group [16], which is important in studying high-risk clonal lineages. These clones, such as ST131, are predominated by narrow-host-range plasmids (e.g. IncF) [9] and seem to provide a stable environment for the AMR plasmid genes.

2.2.3 Horizontal gene transfer

The main mechanism for plasmid transfer and thus selection-favoring features from one bacterial host to another is conjugation. Conjugation is a major contributing force to bacterial genomic evolution [109] and has evolved to transport the plasmid from donor to recipient cell, in contrast to transformation and transduction [86]. The concept of HGT was introduced in the early 1990s to explain the incongruences of conflicting phylogenies, which based upon the grouping of microorganisms by using original molecular markers, such as the 16S sequencing [110, 111]. While transfer frequency seems higher among closely related organisms, different studies have revealed that HGT is not limited to bacteria but is also

occurring among archaea and eukarya [111-114]. Successful HGT is both dependent on the transfer (e.g. conjugation, transduction and transformation) of the genetic material but also on its maintenance (e.g. influenced by toxin-antitoxin [TA]-systems and positive selection) during several bacterial generations [111]. To capture the HGT's exact role to precisely reconstruct bacterial phylogenies remains difficult. Some authors question whether reconstruction is possible at all [115] and some state that different core genes are in fact never transferred, thus providing a reliable base upon which to build microbial phylogenetic trees [116]. Regardless, a study analyzing the pan-genome (the set of genes present in a group of sequenced bacterial genomes) of 573 bacterial whole genomes revealed that only about 8% of the genes in a bacterial genome are assigned to the core genome [117], demonstrating the HGT's high impact on bacterial evolution.

Back to conjugation and plasmids: the location of the transfer genes on the plasmid determines its conjugative ability [118] (e.g. the F-plasmid from *E. coli*), the required genes are located in the transfer (tra) region of the plasmid. Not all plasmids are conjugative. The donor cell (termed F^+ cell) forms a sex-pilus (for F-plasmids it is called F-pilus), which contacts the recipient cell (termed F^- cell) and subsequently retracts [119]. Genetic material is then transferred through a conjugation bridge [89, 90]. Following breakage of the double-stranded plasmid DNA, it is transferred to and replicated in the recipient cell. Concurrently, complementary DNA-strand formation takes place in the donor cell.

2.2.4 Plasmid "curing"

One unconventional approach to study the AMR plasmids' exact role in the dissemination of clonal bacterial lineages, especially in terms of rather unexplored non-resistance genes, is the manual plasmid elimination- the so-called "curing" and the comparison of the "cured" isolates to plasmid-carrying WT strains. While performing "curing", plasmid replication is inhibited without repressing the chromosomal replication, which leads to plasmid attenuation during cell division. This either happens spontaneously or by applying chemicals [120], such as fluorescent dyes like acridine orange [121] and phenothiazines [122] including promethazine. As plasmid replication and subsequent elimination are dependent on bacterial growth, the use of sub-inhibitory concentrations is required for most chemical components. Activity of chemicals relies on their binding to the plasmid DNA: super-helical conformations bind more components than a linear DNA plasmid formation. In general, chemical compounds do not act mutagenic on bacterial hosts, which

provides interesting opportunities for biotechnological studies and the development of novel drugs [120].

Elevated temperatures (normally 5-8°C above the optimal bacterial growth temperature) to "cure" plasmids can also be employed (Fig. 2). The strains under investigation are incubated until they reach the late log phase and are again re-incubated at the elevated temperature [123], which might take numerous generations and several days or weeks of sub-cultivations. Dependent on the temperature-sensitivity of the plasmids, the "curing" procedure is either crowned with success- or not [124].

"Curing" poses difficulties as many plasmids acquired properties to ensure their maintenance in the bacterial host cell, such as TA systems (below) [125]. From our own experience, some bacterial strains prefer to die than lose their plasmid, which makes studies with "cured" bacteria exceptionally difficult.

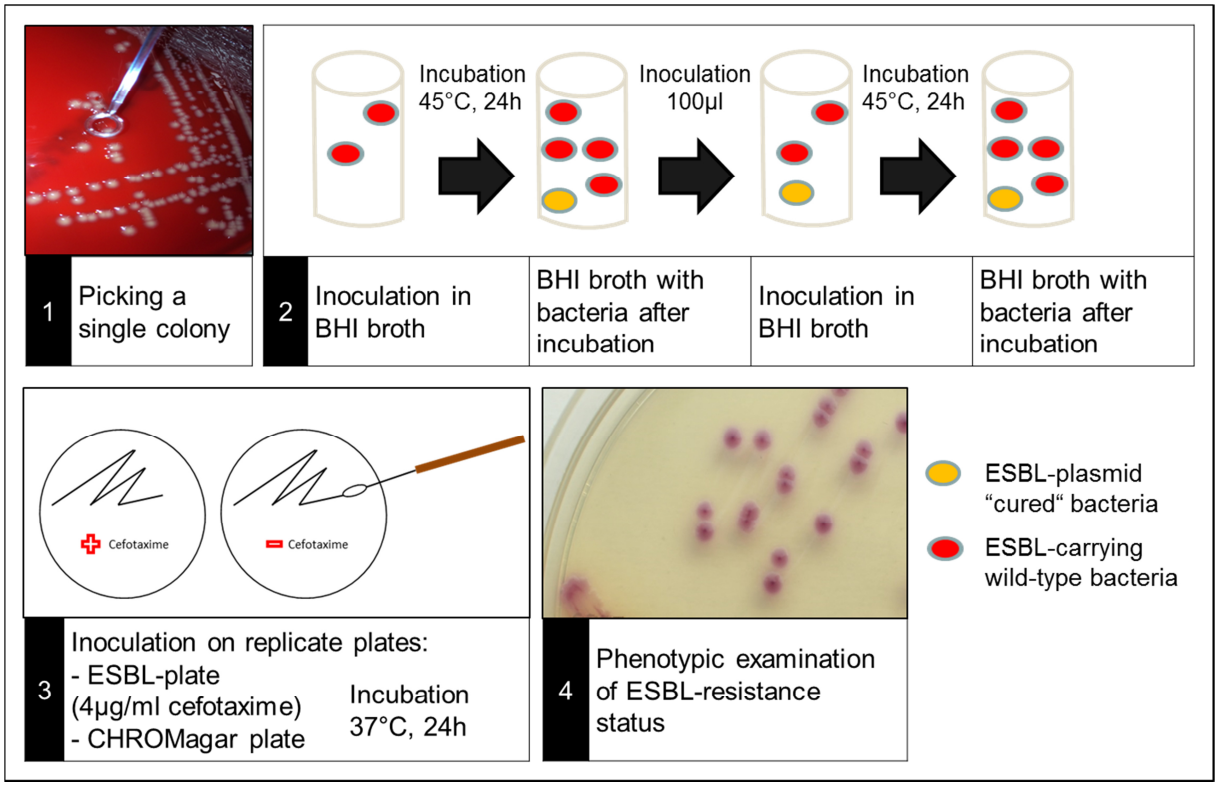

Figure 2: *Plasmid "curing" by applying heat stress exemplary for extended-spectrum beta-lactamase-(ESBL) plasmid-carrying bacteria.*

Single colonies (1) of ESBL-plasmid-carrying wild-type strains (red) are incubated for 24 hours at elevated temperature (45°C) in brain-heart infusion (BHI) broth to force the loss of the ESBL-plasmid. One hundred microliters of the over-night culture are re-incubated in fresh BHI broth daily. Cultivation takes up to several weeks (2). Success of ESBL-plasmid "curing" is evaluated on cefotaxime-containing and -lacking CHROMagar plates (3). Successfully ESBL-plasmid "cured" strains (yellow) do not show colony growth on cefotaxime-containing agar plates. Strains that show growth on cefotaxime-containing agar plates still contain the ESBL-plasmid (4).

2.2.5 Toxin-antitoxin systems

Another factor for the dissemination of pandemic successful bacterial lineages that should be considered is TA systems as these provide stable host conditions for selection-favoring plasmids. Those systems are chromosomally-encoded or they are part of the mobilome [126]. Several TA systems have been described, always consisting of a stable toxin, which causes bacterial cell death or bacteriostasis, and an unstable antitoxin. The systems differ in functionality and type of the antitoxins. In type I systems the antitoxin is a small RNA molecule, which interacts with the toxin-encoding mRNA, while in type II systems the toxin protein is inhibited by an antitoxin protein [127]. In type III systems, the

pseudoknot-containing RNA antitoxin binds the toxin protein [128] and in type IV systems the antitoxin protein interacts with the toxins targets, such as cytoskeletal proteins [129]. Type V antitoxin proteins split toxin mRNA molecules [130].

Plasmid-encoded TA systems are involved in "post-segregational killing", therefore ensuring the maintenance of the plasmid in bacterial cells. This mechanism bases upon the variable stability of the toxin and antitoxin. While the antitoxin is degraded rapidly in plasmid-free daughter cells during replication, the stable toxin is released off the antitoxin-toxin complex and might cause death or growth stasis of the bacterial cell [126]. Also, competing plasmids of the same Inc group are degraded by TA-systems, if they do not carry the systems on their own [131]. A well-studied TA system in Gram-negative bacteria is type I *hok/sok.* It was first described in 1986 on *E. coli* plasmid R1 [132]. In the case of *hok/sok,* the translation of the toxin is dependent on a third gene, called *mok,* therefore the antitoxin does not bind directly to the toxin but to *mok* [133]. Other type I systems are *tisB/istR1, ldr/rdl* and *flmA/B* [134-136]. In large part, system I toxins induce cell membrane porins, which leads to impaired ATP-synthesis and subsequent death of the bacterial cell due to the inhibition of replication, transcription and translation [127]. Examples for type II TA systems are *ccdA/B* [137], *mazE/F [138]* and *vapB/C* [139]. Toxins of this group often work as nucleases [140]. Type III systems include *ceptI/N* [128], type IV systems *yeeU/V* [129] and type V systems *ghoS/T* [130], where toxins have different functions, such as ghost cell formation of the bacterial host [130]. TA systems are applied to biotechnological approaches, including to maintain plasmids in large bacterial cell cultures [141] or as future targets for antibiotics [142].

Evolutionary, TA-systems might be considered "selfish" or "addictive" DNA due to the fact that they do not always contribute to bacterial fitness [143] and, as mentioned before, compete with other plasmids. However, they might as well be anti-addictive, given their involvement in the protection against phages [144, 145] and that they confer fitness advantages under post-segregational conditions [146]. As TA-systems ensure the plasmid's maintenance in the bacterial cell, they confer selective advantages for the bacterial host when these plasmids co-encode for selective advantages including virulence and AMR. This may be among the many factors involved in the successful emergence of international high-risk multi-drug resistant clones.

2.3 Interplay of bacterial lineages & clones with AMR plasmids

Interplay among AMR plasmids and bacterial host mostly includes: i) the occurrence of AMR plasmid-encoded non-resistance factors such as fitness and virulence, which favors the bacterial host through co-selection and ii) the cost or gain of AMR plasmid carriage for the bacterial host, which results in metabolic costs and decreased or enhanced non-resistance factors associated with fitness and virulence.

2.3.1 Fitness

Most AMR mechanisms, either following HGT or chromosomal mutation, are associated with fitness costs in bacteria, typically noticed by a reduced growth rate of the bacterial host. Dependent on this cost's dimension is the AMR development rate, the AMR stability and the AMR decrease rate in case of the absence or attenuation of antimicrobial substances [147, 148] suggesting that if antibiotic pressure decreases, susceptible bacteria might benefit and outcompete resistant rivals [149, 150]. This is not necessarily the case; owed to processes like compensatory evolution, which compensates fitness costs through plasmid or chromosomal mutations, and AMR plasmid-encoded co-selection, which confers benefits beyond resistance [147, 151, 152].

Bacterial fitness is commonly tested in classic growth curve models using different media. The OmniLog Phenotype MicroArray (PM) technology represents a more holistic, high-throughput approach to investigate fitness/metabolic activity and bacterial resistance phenotypes concurrently (www.biolog.com). 96-well microtiter plates spotted with substrates ranging from carbons over heavy metals and nitrogen sources, to antibiotics [153] represent ideal test preconditions to simultaneously evaluate the investigated bacteria regarding their metabolic potential and too their potential to emerge successfully as metabolism also plays a crucial role in colonization and virulence [154, 155]. Following bacterial incubation, which can last up to several days, each well displays a respiration curve, in cases of positive reactions as sigmoid graphs similar to growth curves. Analyzed data [156] estimate statistically relevant differences among different STs, plasmid-carrying strains, plasmid "cured" strains, or knock-out mutants [157].

A study from 2014 comparing ST131 and non-ST131 strains in fitness assays including the OmniLog system suggested only slight metabolic differences on the ST level [158]. Interestingly, however, a different study, again comparing ST131 and non-ST131

ExPEC strains in 47 biochemical tests revealed a higher metabolic potential of those belonging to ST131, with ESBL-producing ST131 showing the highest values [159].

2.3.2 Virulence

Besides fitness costs, bacteria carrying AMR plasmids show decreased virulence [147]. This does not always seem to apply, however; AMR and virulence per se are often connected: for example in microbial biofilms [160].

Several studies report on co-selection among beta-lactamase-production and virulence in Enterobacteriaceae, particularly in terms of plasmid-encoded adhesion factors [161, 162]. A non-fimbrial protein termed CF29K, which adheres to microvilli of the human Caco-2 cell line detected in 1991 [163] exemplifies these features. The genes encoding for this protein are located on a conjugative R-plasmid, which confers resistances against beta-lactamases and other antibiotics. In the study, this plasmid was transformed into K-12 *E. coli* mutants lacking the plasmid. While they first did not show any adhesion, they adhered to approximately 92% of the Caco-2 cells after transconjugation. CF29K shows similarities to plasmid-encoded, non-fimbrial adhesion factors produced by *E. coli* [163, 164]. Beyond non-fimbrial adhesion factors, serum resistance [165] and avoidance of the opsonin-mediated phagocytosis through granulocytes [166] are important in ESBL-producing bacteria. Sahly et al. [167] showed that ESBL-producing *Klebsiella pneumoniae* showed a higher expression of fimbrial adhesins, leading to enhanced cell invasion rates, contrary to non-ESBL-producers. This might be explained by co-expression of fimbrial proteins important for invasion, the mannose-sensitive type 1, and the mannose-resistant type 3 proteins in ESBL-producers. Interestingly, ESBL-plasmid elimination did not influence cell adhesion or invasion capacity, which speaks against plasmid-encoded fimbrial factors. The ESBL-plasmid might have increased the translation frequency of the chromosomally-encoded type 3 fimbriae gene, subsequently influencing the cell invasion rate. Indeed, in the study, most strains possessed genes for both fimbrial protein types but only few expressed both phenotypes. This might indicate an influence of the ESBL-plasmid on chromosomally-encoded VFs. In 1995, researchers detected that non-AMR plasmids activated chromosomally-encoded *eae* expression, a gene necessary for the attachment of EPEC to epithelial cells [168]. Other studies have not suggested ESBL-plasmid influences on bacterial virulence. Dubois et al. [169] investigated the influence of a CTX-M-1-encoding plasmid of K1 *E. coli* strains on the meningitis incidence using a mouse model. The enzyme production did not affect the bacteria's virulence but the clinical outcome.

Similar results were shown by a different study [170] for a metallo-beta-lactamase-producing *Pseudomonas aeruginosa* isolate, where the enzyme did not significantly influence VFs. In the event of AmpC-producing *Salmonella enterica* isolates, the expression of this enzyme was connected with decreased bacterial growth- and invasion rates [171].

A connection among *E. coli* conjugative plasmids and biofilms has also been reported [172-174]. May and Okabe [175] investigated the influence of a natural IncF F-plasmid, a plasmid that does not produce F-pili continuously, on biofilm formation and maturation. Both conjugative and also non-conjugative plasmid genes seemed to play a role. After initial F-pilus formation, natural conjugative F-plasmids stimulated the induction of extracellular matrix components. Moreover this study detected significant differences in the biofilm-related gene expression among F-plasmid-containing and F-plasmid-free strains.

Further investigations are essential to estimate the extent to which beta-lactamases and ESBL-plasmids affect (chromosomally-encoded) bacterial virulence and fitness [176]. The role of the bacterial host's phylogenetic background should also be considered as interactions demand two associates.

2.4 Phenotypic assays to study bacterial lineages & clones

Interactions of AMR plasmids and bacterial lineages and clones can be studied in phenotypic assays. Only those contexts most relevant for the thesis are introduced in further detail.

2.4.1 Biofilm formation

The ability to form biofilms might be one crucial VF to explain the pandemic success of bacterial multi-resistant clonal lineages, be it through AMR plasmids, the bacterial host's phylogenetic background or their interaction. Infection pathogenesis such as the one of catheter-associated urinary tracts [177] and wounds [178] is often related to biofilms. Biofilms are multicellular communities of bacteria embedded in a self-produced extracellular matrix of exopolysaccharides, proteins (e.g. amyloid fibers), adhesins, and DNA of one or more bacterial species [179, 180]. Biofilms are attached to surfaces, they differ from single planktonic cells both morphologically and biochemically [181]. Mostly, bacteria form three-dimensional, fungoid biofilms at different developmental stages: first an initial, reversible

attachment of planktonic cells to a surface, then the formation of irreversibly attached microcolonies and the production of adhesion factors, and then finally the microcolony maturation and production of extracellular matrix components resulting in the mature biofilm. Subsequently, bacterial surface cells dissolve from the biofilm and return into a planktonic way of life and might form other biofilms in new locations [182]. Biofilm formation depends on environmental conditions, such as the availability of nutrition. In contrast to biofilms, characterized by the production of extracellular matrix components, the planktonic lifestyle is usually dependent on flagellar synthesis and motility. Flagella, however, seem also involved in the bacterial attachment [183] and have been recently suggested to play an architectural role in biofilms [184].

Mature biofilms show the maximum level of resistance towards antimicrobial substances [185], owed to multiple factors including slow bacterial growth particularly in the center [186], which results in the ineffectiveness of drugs targeting dividing cells. Increased HGT [187] with a subsequent rapid spread of AMR- and virulence-carrying plasmids, expression of biofilm-specific AMR genes, and increased mutations (e.g. in antibiotic targets) and quorum-sensing mechanisms (QS) [188] are also important. QS in biofilms contributes to enhanced virulence as it enables pathogens to express energetically costly processes as a collective; members of biofilms likely benefit from these stable communications [189]. An association of the potential to form biofilms and the co-expression of virulence genes has been described [190, 191]. Biofilm investigations have mainly used two models to study bacterial communities [184]: i) microtiter plates in which biofilms are grown in specific environments dependent on the surrounding medium [192], such as rich or minimal medium, and ii) “pellicles” on liquid surfaces or “macrocolonies” on agar [184, 193] (Fig. 3), due to the extended growth time referred to as “long-term colonies” with the development of striking morphological patterns [184].

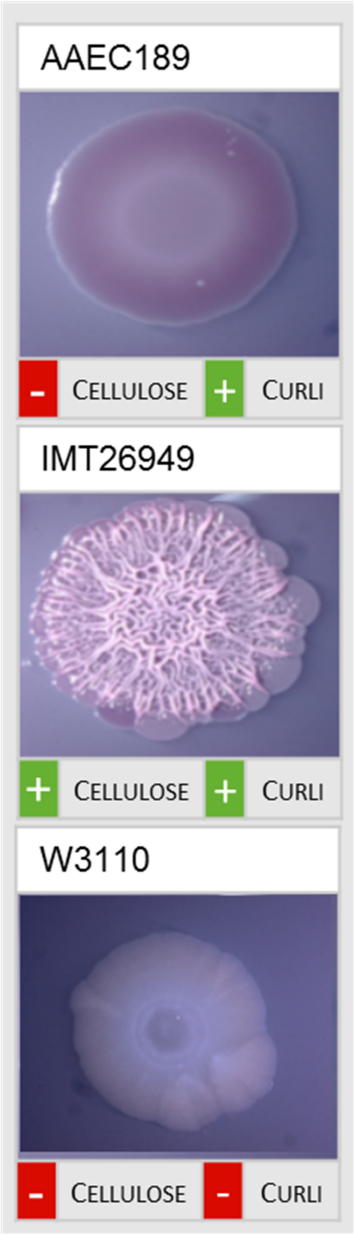

Figure 3: *Macrocolonies of three E. coli reference strains.*

Incubation for five days at 28°C, on span agar plates with sodium chloride. Purple coloration of the colony indicates curli fimbriae production (AAEC189 [194]), purple and rough, wrinkled surface indicates simultaneous curli and cellulose production (IMT [Institut für Mikrobiologie und Tierseuchen] 26949). White coloration of the colony indicates no production of cellulose nor curli fimbriae (W3110 [195]).

The manifestation of these patterns crucially depends on the bacterial production of auto-aggregative curli fibres (also: curli fimbriae), which are amyloids and bind to Congo red (CR)- thus made visible on the agar plate. In general, for the switch between a planktonic, motile lifestyle and living as part of a biofilm, bacteria undergo complex processes controlled by the flagellar master regulator FlhDC and the master regulator of the stationary phase and general stress response σ^S (RpoS) subunit of RNA polymerase (RNAP), which in turn are regulated by the second messengers cyclic AMP (cAMP) [196] and guanosine penta-/tetraphosphate [(p)ppGpp] [197] (Fig. 4). FliZ [198] and the second messenger bis-(3′-5′)-cyclic dimeric guanosinmonophosphate (c-di-GMP) influence the following transcriptional cascades, subsequently regulating, together with others (e.g. the transcription factor MlrA), CsgD, the main activator of the curli operon and other biofilm-related genes. Despite promoting biofilm formation, CsgD was shown to repress at least two flagellar operons [199], thus playing a key role in the bacterial switch of motility and biofilm formation (Fig. 4). CsgD is generally regulated by a variety of environmental and cellular signals [200]. In many *E. coli* strains, CsgD is temperature-sensitive and only expressed below 30°C. Interestingly, for the Shiga toxin-producing 2011 German outbreak *E. coli* strain, researches detected a CsgD expression at 37°C, possibly contributing to the pathogenesis of HUS (hemolytic-uremic syndrome) [201]. Besides curli fibres in biofilms, production of the exopolysaccharide cellulose determines macrocolony morphology. Bacteria produce cellulose for mainly protective reasons, following chemical and mechanical influences. For cellulose biosynthesis, four proteins are essential: BcsA, a cellulose synthase, BcsB, a c-di-GMP binding protein, BcsC

and BcsD; all encoded on the *bcsABZC* operon [202]. Cellulose is only produced upon *yaiC* expression, a gene for a transmembrane protein. In turn, YaiC is regulated by CsgD, which is why this protein is not only involved in curli- but also cellulose biosynthesis (Fig. 4) [203]. Binding of curli or cellulose alone or in combination to the CR dye in agar plates leads to various elaborate macrocolony structures that have been termed "pdar/bdar/rdar" morphotypes, "wrinkled" or "rugose" (Fig. 3) [193, 204, 205].

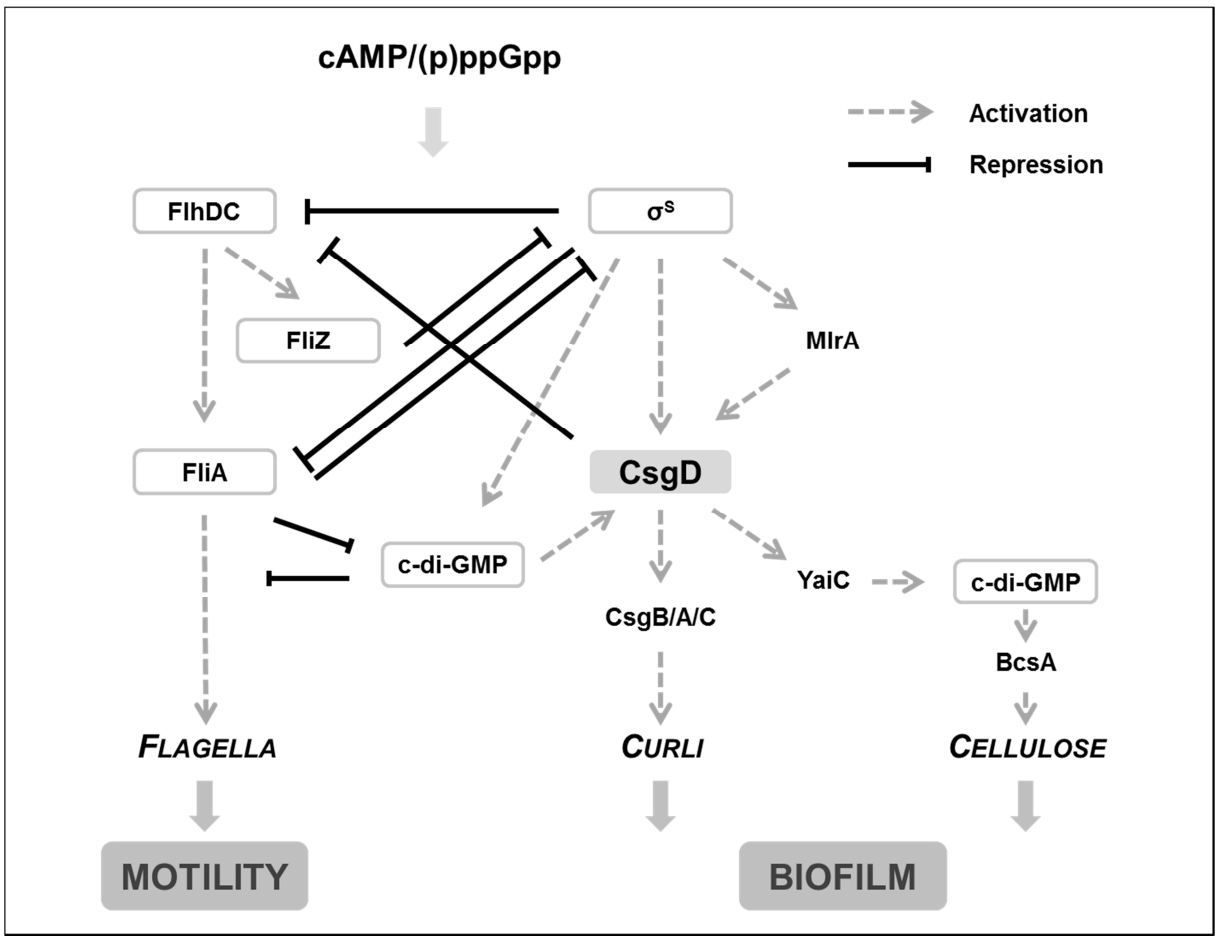

Figure 4: *Simplified regulatory network controlling bacterial motility and biofilm formation.*

The model summarizes the network of inverse regulatory cascades starting with FlhDC and σ^S. Motility is mainly regulated through FlhDC, FliA and downstream factors. Biofilm formation is regulated through σ^S, and downstream factors. Noteworthy is the central role of CsgD and c-di-GMP levels, both playing a role in suppression and activation of motility and biofilm formation. Additional factors are omitted for clarity. Adapted from Pesavento et al., 2008 [206] and Boehm et al., 2012 [200].

Curli fibers are considered virulent, as they contribute to the internalization of epithelial cells [207] and to the persistence of pathogens in the avian caecum [208]; they have even been found to take part in Alzheimer's disease [184, 209]. Cellulose is not directly connected to virulence, however, it might be important regarding the survival on abiotic surfaces [210].

Given that the ability to form biofilms represents an important virulence feature, one should note that a subgroup of the international multi-resistant ST131 *E. coli* clonal group seems to be equipped with this gift [211]. However, the 32 ST131 isolates analyzed in a different study did not form *in vivo* biofilms, highlighting the importance of further investigating the role of dissemination-favoring factors [59].

2.4.2 Motility

The supposed ancient phenomenon of motility is often linked to bacterial virulence [212]. Numerous bacterial species are motile; they often possess rotating organelles- flagella- and then show swimming and swarming [213, 214]. A twitching and often gliding locomotion arises from type IV pili [215] and sliding is caused by a passive translocation of the bacterial cells [216]. Due to chemotactic external signals, bacteria are able to regulate their locomotion to the effect that they reach environmental conditions ideal for their growth and survival [217] and to outcompete other microorganisms [218]. The subtle switch of motile bacteria between saving energy and using cost-intensive flagellar motility to seek better conditions seems dependent on environmental conditions. They carry out tactical responses to challenging environments observed as an increase of flagellar operation (motility/chemotaxis) and decrease of costly flagellar synthesis; this goes as far as saving energy outweighs the "expensive" search, which is the case in harsh environmental conditions [218].

Swimming, contrary to swarming, which is performed by a group of bacteria in semisolid agar and on surfaces, is an individual motion type of a single bacterium in liquid or very soft, semisolid media [219]. In many pathogens, flagella-dependent motility is important for rapid colonization and subsequent biofilm formation, and thus takes particularly part in the early phases of infection; the surface contact seems to induce a down-regulation of flagellar biosynthesis and an enhanced production of extracellular matrices. Whether motility or the flagellum is essential for adherence in early infection and virulence is controversially discussed [212].

Other studies report on the immunomodulatory effects of flagellar proteins, highlighting its virulence potential and, the flagella secretion system is closely related to the injectisome of Gram-negative pathogens to transfer virulence-associated components into effector cells (type III secretion system) [212, 220].

Especially during bacterial post-exponential growth, when resource availability decreases, *E. coli* synthesize flagella and become highly motile [221, 222]. Approaching zero resources, *E. coli* then enter stationary phase; they have stopped growth and flagella production. Instead, they synthesize auto-aggregative curli fibres, which leads to cellular aggregation and later on, biofilm formation [184]. After initial attachment, bacteria use type IV pili to move within the biofilm network. The flagella-hook basal-body-complex is synthesized by proteins, which are encoded over various transcription factors by the *flhDC* operon. This operon also encodes the main transcription factor FliA (Fig. 4), which again regulates other genes responsible for the production of flagellar filaments, motor and chemotactic components. Expression of the operon depends on external factors including glucose availability, heat and acetyl phosphate [219].

Motility in the internationally successful AMR resistant ST131 clone seems common [223, 224], however, this definition is based principally on serotyping of H-antigens, not on actual motility test models.

3. PUBLICATIONS

3.1 Publication 1

"ESBL-plasmids carrying toxin-antitoxin systems can be "cured" of wild-type *Escherichia coli* using a heat technique"

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3.2 Publication 2

"Carriage of extended-spectrum beta-lactamase-plasmids does not reduce fitness but enhances virulence in some strains of pandemic *E. coli* lineages"

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http://www.gutpathogens.com/content/5/1/34

Gut Pathogens

SHORT REPORT Open Access

ESBL-plasmids carrying toxin-antitoxin systems can be "cured" of wild-type *Escherichia coli* using a heat technique

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Abstract

Background: Plasmid-encoded extended-spectrum beta-lactamase (ESBL)-enzymes are frequently produced by *Escherichia coli*. Several ESBL-plasmids contain genes for toxin-antitoxin (TA) systems, which assure the maintenance of plasmids in bacteria and prevent the cells from "post-segregational killing". These systems limit options to "cure" plasmids of ESBL-wild-type strains due to the death of the bacterial cells. A helpful tool to understand the role of ESBL-plasmids in the dissemination of pandemic multi-resistant *E. coli* are ESBL-plasmid-"cured"-variants (PCVs) and their comparison to ESBL-wild-type strains. The purpose of this study was to construct PCVs of ESBL-wild-type *E. coli* strains despite the presence of genes for TA systems.

Findings: Using enhanced temperatures and brain-heart-infusion broth it was possible to construct viable PCVs of wild-type ESBL-*E. coli* strains. The occurrence of TA system-genes including *hok/sok, srnB/C, vagC/D, pemI/K* on ESBL-plasmids of replicon types FIA or FIB was demonstrated by bioinformatic analyses. The loss of the plasmid and the genetic identity of PCV and corresponding wild-type strain was confirmed via different methods including plasmid-profile-analysis, pulsed-field gel electrophoresis and bioinformatics using generated whole genome data of the strains.

Conclusions: This short report describes the successful construction of viable PCVs of ESBL-wild-type *E. coli* strains. The results are hence surprising due to the fact that all "cured" ESBL-plasmids contained at least one complete toxin-antitoxin system, whose loss would normally mean the death of bacterial cells.

Keywords: Plasmid, ESBL, *E. coli*, Toxin-antitoxin system, Plasmid-"cured"-variant

Introduction

Pathogenic *Escherichia coli* cause a wide range of infectious diseases in various animal species and humans, including diarrhoea [1], meningitis, urinary tract and, soft tissue infections [2]. Many *E. coli* produce extended-spectrum beta-lactamase (ESBL)-enzymes, which- in addition to penicillins and others- hydrolyse newer, third-generation cephalosporins and monobactams [3], and limit antimicrobial therapy. Several ESBL-gene families ($bla_{\text{CTX-M}}$, bla_{SHV}, bla_{TEM} and bla_{OXA}) are encoded on plasmids [4]. Prior research has demonstrated that some of the ESBL-carrying plasmids influence factors other than resistance, like the ability of *Klebsiella* strains to invade epithelial cells [5]. In these ESBL-plasmids, addiction models such as toxin-antitoxin (TA) systems have been described [6], which prevent the cell from "post-segregational killing", therefore ensuring the maintenance of the plasmid in the bacterial cell during replication [7]. Several TA systems have been discovered in Gram-negative and Gram-positive bacteria differing basically in functionality and type of the antitoxin. Type I system antitoxins are small antisense RNA molecules, which mostly inhibit toxin mRNA translation or degrade toxin mRNA while type II system protein antitoxins interact post-translationally with protein toxins. Other systems are type III to type V TA systems [8-10]. One of the first [11] and most studied is type I *hok/sok* TA system in *E. coli*. Following replication, in plasmid-free daughter cells type I unstable RNA antitoxin molecules (e.g. sok) degrade rapidly, while stable toxins (e.g. hok) induce cell membrane porins, therefore impairing ATP

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Table 1 Origin and genotypical characteristics of the ESBL-wild-type strains

Strain designation	Host	Origin	Sequence-type	ESBL-type	Plasmid-replicon- type	Toxin-antitoxin system	Other epigenomic resistance genes
VB977549	Dog *(C. lupus familiaris)*	Urinary tract infection	131	CTX-M-14, CTX-M-15	FIA/FIB	*pemI/K, vagC/D, hok/sok*	bla_{TEM-1}, bla_{OXA-1}, *tet*(A), *tet*(R), *aadA, aac(6')-ib-cr*
IMT19205	Brown rat *(R. norvegicus)*	Feces	131	CTX-M-9a, CTX-M-14, CTX-M-15	FIA/FIB	*hok/sok*	bla_{TEM-1}, *tet*(A), *sul2, strA, aac(3)-IV, aac(6')-Ib-cr.*
IMT27685	Raven *(C. corax)*	Feces	131	CTX-M-15	not typed	*pemI/pemK, vagC/D*	bla_{OXA-1}, *tet*(A), *sul1, strA, strB aac(6')-Ib-cr*
IMT16316	Blackbird *(T. merula)*	Feces	648	CTX-M-14, CTX-M-15	FIA/FIB	*pemI/K, vagC/D, srnB/C*	*tet*(A), *tet*(R), *sul1, sul2, strA, strB, aadA, aac(3)-II mph(A), mrx, mphR, dhfrVII,*
VB964041.2	Horse *(E. ferus caballus)*	Soft tissue/ wound infection	648	CTX-M-15	FIA/FIB	*pemI/K, vagC/D, srnB/C*	*tet*(A), *tet*(R), *sul1, sul2, strA, strB, aadA, mph(A), mphR, dhfrVII*
IMT21183	Human *(H. sapiens)*	Urinary tract infection	648	CTX-M-14, CTX-M-15	FIA	*vagC/D, srnB/C*	*tet*(A), *tet*(R), *sul1, sul2, strA, strB, aadA, aac(3)-II, mph(A), mphR, dhfrVII*
IMT23463	Monk vulture *(A. monachus)*	Feces	648	CTX-M-9	FIB	*PemI/pemK, srnB/C, hok/sok*	bla_{TEM-1}, bla_{OXA-1}, *tet*(A),*sul2, strA, strB, aac(6')-Ib-cr*

synthesis and subsequently causing bacterial cell death [12]. In ESBL-plasmids most frequently represented systems not only include *hok/sok* but also *pemK/I* and *ccdA/B*, which seem to be associated with CTX-M-15 and CTX-M-9 encoding plasmids of IncF replicon type [13]. To investigate, which important role ESBL-plasmids play concerning both resistance and factors not related to resistance it is necessary to construct ESBL-plasmid-"cured"-variants (PCVs) and compare those pheno- and genotypically to ESBL-wild-type strains. Different methods are known to "cure" bacterial plasmids, most of them use chemical treatment like ethidium bromide or acridine orange in different concentrations added to bacteria in Luria-Broth (LB) [14]. These methods often involve the problem that "curing" the ESBL-plasmid of the wild-type strain causes the death of the bacterium due to the loss of an operating TA system. In this study we "cured" TA systems-containing ESBL-plasmids from wild-type *E. coli* testing an established method [15] using enhanced temperatures, brain-heart-infusion (BHI) medium and several weeks of continuing sub-cultivation protocols.

Material and methods

To force the loss of the ESBL-plasmid, a heat technique was performed [15]. Single colonies of seven wild-type ESBL-*E. coli* strains (VB977549, IMT19205, IMT27685, IMT16316, VB964041.2, IMT21183, IMT23463 of successful and pandemic sequence types [STs] ST131 and ST648 and different hosts including humans, companion and wild animals (Table 1)) were picked and inoculated in 5 ml BHI

Table 2 Results of agar disc diffusion testing

Strain designation	Cefotaxime	Chloramphenicol	Enrofloxacin	Gentamicin	Streptomycin	Tetracycline	Sulfonamid-trimethoprim
VB977549	R	S	R	S	R	R	S
PCV977549	S	S	R	S	S	S	S
IMT19205	R	S	R	R	I	S	S
PCV19205	S	S	S	R	S	S	S
IMT27685	R	R	R	S	R	R	R
PCV27685	S	S	R	S	S	S	S
IMT16316	R	S	R	R	R	R	R
PCV16316	S	S	R	S	S	S	S
VB964041.2	R	S	R	R	R	R	R
PCV964041.2	S	S	R	S	S	S	S
IMT21183	R	S	R	R	R	R	R
PCV21183	S	S	R	S	S	S	S
IMT23463	R	S	R	S	R	R	R
PCV23463	S	S	R	S	R	R	R

(R = resistant, S = sensitive, I = intermediate).

broth. BHI tubes were incubated at 45°C for 24 hours. Ten microliters of the overnight culture were spread on CHROMagar™ plates. Plates were incubated at 37°C overnight. Replicate CHROMagar™ plates containing an identical numbered grid on the backside of the plate were then prepared. One contained cefotaxime (4 μg/ml cefotaxime) and the other was prepared without supplementation of antibiotics. Twenty single colonies of each strain were randomly

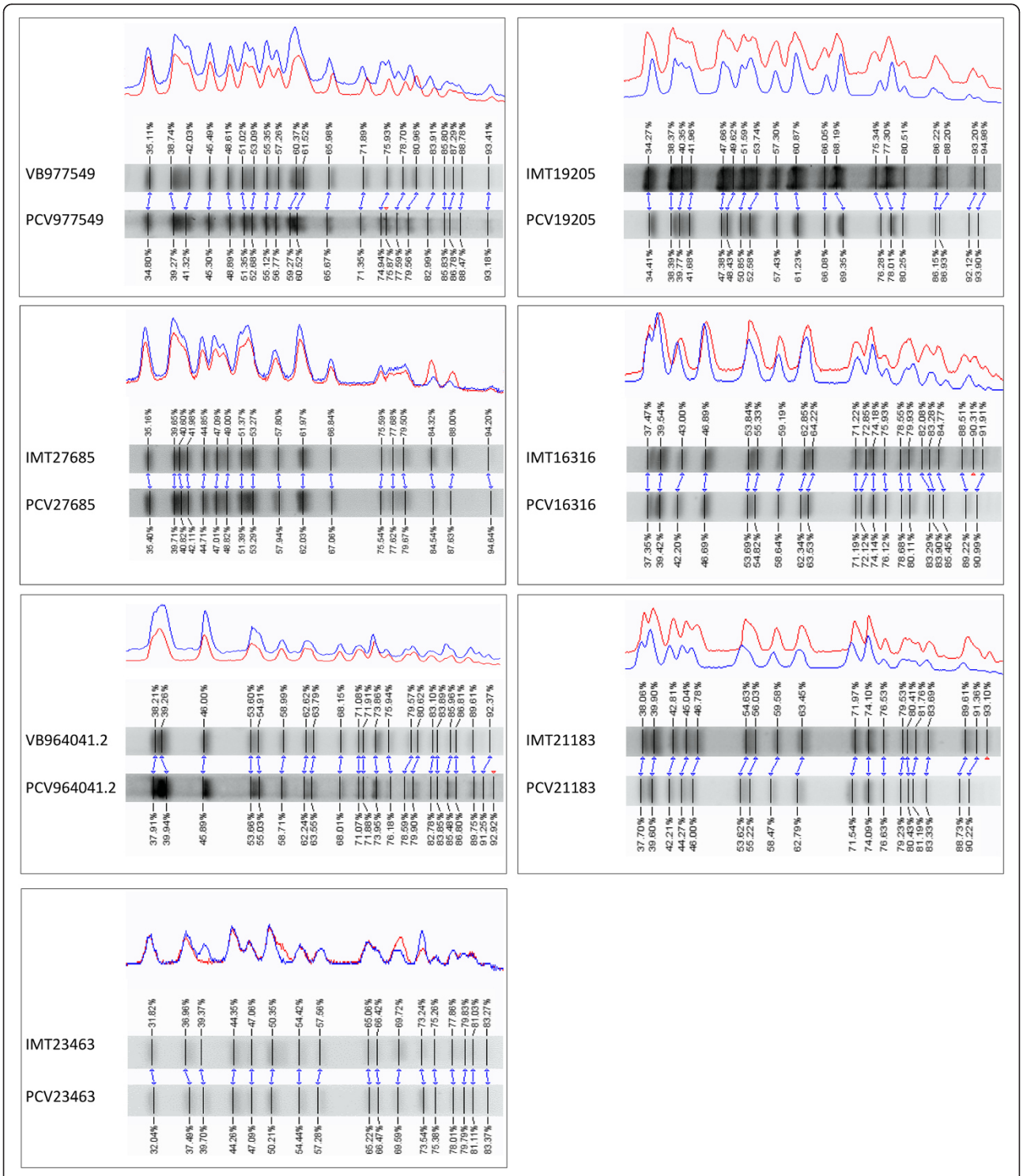

Figure 1 Dendrograms of the pulsed-field gel electrophoresis. Wild-type ESBL-*E. coli* and PCV strains after macrorestriction with *Xba*I and PFGE (dice similarity value >98% for all strains). Software: Bionumerics (Applied Maths, Belgium).

picked from the overnight incubated CHROMagar® plate and single colonies were placed on their identical grid locations in the agar of the replicate plates. This was to ensure that colonies from the two different plates could be assigned to the previously selected, single colony. ESBL-plasmid-"cured" single clones should not grow on cefotaxime-containing plates. These visually "cured" single clones were picked from the corresponding CHROMagar™ plate without cefotaxime according to the grid and their phenotypic resistance against cefotaxime and other antimicrobial classes (Table 2) was screened using agar disc diffusion according to the CLSI method [16]. They were further investigated using plasmid-profile-analysis to prove the loss of the plasmid [17]. Clonal identity of the wild-type and the ESBL-plasmid-"cured"-variant was tested via *Xba*I-pulsed-field gel electrophoresis (PFGE) [18] and following comparative bioinformatic analyses. First, the number of orthologous genes in a pairwise comparison of the genome of the wild-type strain and the corresponding plasmid-"cured"-variant was checked using the OrthoMCL pipeline [19]. In a second approach the phylogenetic distances of all strains were tested. The set of genes, which is present in each of all strains, the Maximum Common Genome (MCG), was therefore calculated, the allelic variants of the MCG from the strains was then extracted and a multiple alignment was built (Semmler, personal communication). Verified ESBL-plasmid-free strains were henceforward named PCV (plasmid-"cured" variant: PCV977549, PCV19205, PCV27685, PCV16316, PCV964041.2, PCV21183 and PCV23463). Presence of genes for TA systems on plasmids was investigated evaluating sequence data using bioinformatic methods. Both wild-type strains as well as PCVs were sequenced by an Illumina HiSeq 2000 sequencer. The resulting reads for the PCVs were used for a *de novo* assembly (CLC Genomics Workbench 6.5, CLC Bio, Denmark). The contigs were then used as reference sequences for a reference mapping of the reads from the wild-type strains. All reads from the wild-type strains, which could not be mapped to the PCV sequence are supposed to represent the extracted plasmids and were used for another *de novo* assembly, which resulted in the contigs of the plasmid sequences. Using BLAST for the plasmid and the PCV contigs, the genes for TA systems could be localized. Other plasmid-"curing" methods including treatment of bacteria with acridine orange and ethidium bromide [14] were additionally tested and modified, however, as they turned out not to be successful, data are not included in this manuscript.

Results

Treatment of bacteria with enhanced temperatures was performed to construct viable toxin-antitoxin system-containing ESBL-plasmid-"cured"-variants of wild-type ESBL-*E. coli* strains. Following three to six weeks of daily sub-cultivation, examination of the phenotypical resistance status of the wild-type ESBL-strains revealed seven cefotaxime-susceptible and phenotypically ESBL-plasmid-"cured"-variants. Table 2 shows the results of agar disc diffusion testing of cefotaxime and other classes of antimicrobials. All PCVs lost their cefotaxime resistance along with most of the non-beta lactam resistances. Clonal

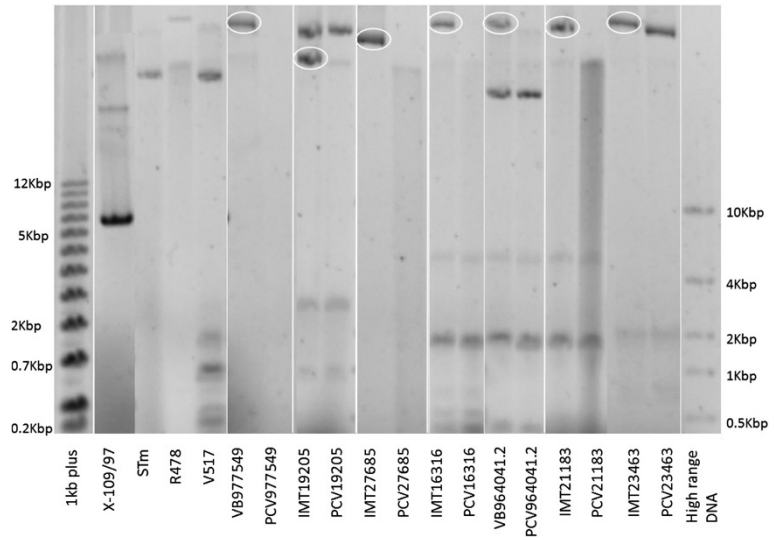

Figure 2 Electropherogram of the plasmid-profile-analysis. Wild-type (IMT/VB, left) and ESBL-plasmid-"cured" variants (PCVs, right) illustrated in pairs. Revolved in white are the big plasmids in the ESBL-wild-type strains, which have been "cured" in the corresponding PCVs. At the beginning and the end are several markers (1 kb plus [Thermo Scientific, USA], X-109/97 [12,5MDa], STm [60MDa], R478 [166MDa], V517 [36.8/4.8/3.7/2.0/1.8/1.4], FastRuler High range DNA [ThermoScientific USA]).

identity of the PCV and its wild-type strain was proven via pulsed-field gel electrophoresis (Figure 1) and bioinformatic analyses. The seven PCV strains showed a highly similar macrorestriction pattern compared to their corresponding wild-type strain. For most of the PCV strains, a small band was missing in the patterns, which might be the "cured" plasmid itself (PCV977549, PCV16316, PCV964041.2 and PCV21183). Comparing orthologous genes, similar results were obtained for all pairs, which showed only one significant excess of genes in the wild-type strain without an ortholog in the "cured"-variant in an amount that corresponds to the size of the plasmid. In case of phylogenetic distances a clustering confirmed the genetic identity for each pair of wild-type and plasmid-"cured"-variant strain (data not shown). The loss of large plasmids (>100 kb) was approved via plasmid-profile-analysis (Figure 2). Plasmid-profile analysis revealed no loss of other smaller plasmids apart from the large ESBL-resistance plasmids. Bioinformatic analyses confirmed the presence of different TA system-genes on plasmids including *hok/sok, srnB/C, vagC/D, pemI/K.* All but one wild-type strain (IMT19205) carried ESBL-plasmids encoding for multiple TA systems, whereat all plasmids belonged either to replicon types FIA or FIB (Table 1).

Conclusions and discussion

As ESBL-plasmid-"curing" of the strains applied in this study did not work treating bacteria with ethidium bromide or acridine orange a previously described method [15] using a heat technique was performed. This method was adequate to construct viable PCVs and to our knowledge we are the first ones to describe a successful "curing" of ESBL-plasmids, which carry genes for toxin-antitoxin systems. The results are surprising because one might think that bacteria cannot survive without this plasmid. Strains used in this study all had at least one complete ESBL-plasmid-encoded toxin-antitoxin system, including *hok/sok, srnB/C, vagC/D* and *pemI/K,* which were encoded on plasmids of replicon types FIA or FIB carrying $bla_{\text{CTX-M-9}}$, $bla_{\text{CTX-M-14}}$ and $bla_{\text{CTX-M-15}}$ or combinations of these three ESBL-genes. The addiction systems and replicon types found have been characterized in ESBL-producing *E. coli* before and certain combinations of ESBL-enzyme, addiction systems and replicon type might contribute to the success and spread of multi-resistant *E. coli* strains [13]. An association of CTX-M-15 with FIA and CTX-M-9 with FIB plasmids has been described before and the same was true for the isolates used in this study (Table 1). Mnif *et al.* also observed that the occurrence of CTX-M-14 correlated with FII replicons, however, FIB replicon type plasmids carrying CTX-M-14 have also been found in this earlier study [13]. No correlation for CTX-M-14 and FII replicons was observed in this study, as this ESBL-type was also present on FIA/FIB plasmids (Table 1). This might be partly due to the fact that $bla_{\text{CTX-M-14}}$ was only detected in combination with other CTX-M enzymes (Table 1). So why did bacteria, whose TA system-containing plasmid was "cured" using a heat technique, survive? One might speculate that some toxins degenerate irreversibly above certain temperatures or that the TA genes are not expressed at 45°C. Or is it that continuing soft stimulation using high temperatures allows a slow adaption to changing environmental conditions and therefore "curing" of the plasmid, while chemicals, like ethidium bromide, together with the loss of a TA system mean too much stress for bacteria? Pulsed-field gel electrophoresis, plasmid-profile-analysis and bioinformatic analyses confirmed that the genetic change is restricted solely to the loss of an ESBL-plasmid and that genetic identity of PCV and wild-type strain remains given. Constructed PCV strains might be important tools to investigate the influence of an ESBL-plasmid on its bacterial host.

Competing interests

The authors declare that they have no competing interests.

Authors' contribution

KS designed and performed the experiments, structured and prepared the manuscript. CE and LHW drafted and revised the manuscript critically for important intellectual content and took part in writing of the manuscript. TS performed bioinformatic analyses. SG participated in the design of the study, revised the manuscript critically for important intellectual content and took part in writing. All authors read and approved the final manuscript.

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ORIGINAL RESEARCH
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Carriage of Extended-Spectrum Beta-Lactamase-Plasmids Does Not Reduce Fitness but Enhances Virulence in Some Strains of Pandemic *E. coli* Lineages

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Pathogenic ESBL-producing *E. coli* lineages occur frequently worldwide, not only in a human health context but in animals and the environment, also in settings with low antimicrobial pressures. This study investigated the fitness costs of ESBL-plasmids and their influence on chromosomally encoded features associated with virulence, such as those involved in the planktonic and sessile behaviors of ST131 and ST648 *E. coli*. ESBL-plasmid-carrying wild-type *E. coli* strains, their corresponding ESBL-plasmid-"cured" variants (PCV), and complementary ESBL-carrying transformants were comparatively analyzed using growth curves, Omnilog® phenotype microarray (PM) assays, macrocolony and biofilm formation, swimming motility, and RNA sequence analysis. Growth curves and PM results pointed toward similar growth and metabolic behaviors among the strains. Phenotypic differences in some strains were detected, including enhanced curli fimbriae and/or cellulose production as well as a reduced swimming capacity of some ESBL-carrying strains, as compared to their respective PCVs. RNA sequencing mostly confirmed the phenotypic results, suggesting that the chromosomally encoded *csgD* pathway is a key factor involved. These results contradict the hypothesis that ESBL-plasmid-carriage leads to a fitness loss in ESBL-carrying strains. Instead, the results indicate an influence of some ESBL-plasmids on chromosomally encoded features associated with virulence in some *E. coli* strains. In conclusion, apart from antibiotic resistance selective advantages, ESBL-plasmid-carriage may also lead to enhanced virulence or adaption to specific habitats in some strains of pandemic ESBL-producing *E. coli* lineages.

Keywords: ESBL-producing *E. coli*, ESBL-plasmids, fitness costs, enhanced virulence, biofilm formation, plasmid and host interaction

INTRODUCTION

The global emergence of antimicrobial resistance, including extended-spectrum beta-lactamases (ESBL), is driven not only by plasmids encoding for these factors, but is also crucially influenced by pandemic bacterial clonal lineages (Naseer and Sundsfjord, 2011). The success of the pathogenic ESBL-producing *E. coli* clonal lineage of sequence type ST131 and virulence-associated phylogenetic group B2 is particularly noteworthy (Nicolas-Chanoine et al., 2014). B2-ST131 is found worldwide in environments with high antimicrobial selection pressures, including human and veterinary clinics and communities (Nicolas-Chanoine et al., 2008; Ewers et al., 2010). ST131 is also found in remote areas and wildlife (Bonnedahl et al., 2014), where antimicrobial influence is thought to be of lower importance. Despite the recognition of the human clinical reservoir as most abundant, several studies have demonstrated increasing prevalence of ST131 in animals and extra-clinical settings (Ewers et al., 2012). The acquisition of ESBL-genes happened over time, and the initial spread of ST131 most likely evolved from an emergence of chromosomally encoded fluoroquinolone resistance (Nicolas-Chanoine et al., 2014). Besides ST131, several STs, including ST648, ST617, ST167, ST410, ST224, and ST117, appear to be associated with ESBL-production, which demonstrates that ESBL-producing isolates are not equally distributed over all phylogenetic backgrounds (Ewers et al., 2012). This is expected in cases of solely plasmid-driven spread (Ewers et al., 2012). Regarding the success of pathogenic clonal lineages of B2-ST131, similar scenarios might also apply to CTX-M-producing lineages of ST648 belonging to phylogenetic group D, also known for harboring virulent isolates (Pitout, 2012; Ewers et al., 2014). Many strains of ST131 and ST648 carry plasmids encoding ESBL-enzymes, often of the CTX-M-15 type, and have become problematic due to limitations in antimicrobial therapies (Johnson et al., 2010). Besides ESBL-encoding genes, plasmids of these *E. coli* isolates contain antibiotic resistance determinants affecting various antimicrobial classes, which often results in multi-drug resistant phenotypes (Woodford et al., 2011). Prior studies suggest trade-offs between antibiotic-resistance and fitness in such strains (Dasilva and Bailey, 1986; Lenski, 1997; Andersson and Hughes, 2010). However, this does not necessarily apply to ST131 and ST648. In contrast, the combination of multi-resistance, virulence and phylogenetic background is hypothesized to be a recipe for their successful pandemic spread (Johnson et al., 2010; Pitout, 2012; Calhau et al., 2013). In addition to antibiotic resistance genes, ESBL-plasmids harbor non-resistance factors, which are partly unexplored. These include fertility and virulence factors, genes for plasmid maintenance including toxin-antitoxin systems, resistances against heavy metals (Seiler and Berendonk, 2012; Schaufler et al., 2013), and putative protein-coding genes (Smet et al., 2010b).

Why do ESBL-associated STs exist, and why are certain clonal lineages so successful not only in environments with high and moderate antibiotic pressures, but also in antimicrobially isolated areas? It might be due to their ubiquity and frequent detection, whereas rare lineages are found less often, or for reasons beyond antibiotic resistance such as virulence-associated factors. One possibility has rarely been studied: the interaction between ESBL-plasmids and the chromosomal content of particular clonal lineages. The influence of non-resistance genes on the chromosome may be of particular importance. For the closely related species *Klebsiella pneumoniae*, it was shown that acquisition of ESBL-plasmids lead to expression changes of chromosomally encoded fimbriae genes, subsequently affecting the overall invasion ability of tested strains (Sahly et al., 2008). Another main bacterial virulence factor is the chromosomally encoded ability to form biofilms. The subtle interactions between biofilm formation and its counterpart, motility, are mostly regulated by the transcriptional regulator *csgD* (curlin subunit gene D; Hammar et al., 1995; Dudin et al., 2014). Biofilm formation has previously been linked to antimicrobial resistance (Ito et al., 2009); however, the influence of ESBL-plasmids on *csgD*-associated virulence features remains to be investigated.

This study addressed two hypotheses: (i) ESBL-plasmid carriage does not negatively influence the host's growth/metabolic fitness; and (ii) ESBL-plasmid carriage supports the host through the ESBL-plasmid's influence on chromosomally encoded virulence-associated features.

MATERIALS AND METHODS

Strains

Seven wild-type (WT) ESBL-producing *E. coli* strains, their ESBL-"plasmid" cured variants (PCV) (Schaufler et al., 2013), and transformants (T) with the reintroduced large ESBL-plasmid constructed from PCVs (Green and Sambrook, 2012) were analyzed in this study; in total, 21 strains were studied. The WT B2-ST131 and D-ST648 strains originated from different hosts including humans, companion animals, and wild birds (Supplementary Table S1).

ESBL-Plasmid-"Curing" and Transformations

As previously described by Schaufler et al. (2013), large ESBL-plasmids were extracted from seven WT ESBL-carrying *E. coli* strains using a heat technique (Dale and Park, 2004). Loss of the large ESBL-plasmid as well as the clonal character of WT and its corresponding PCV strain were tested via plasmid-profile analysis and *Xba*I-pulsed-field gel electrophoresis (PFGE) (Schierack et al., 2009). To assure genetic identity and to exclude chromosomal changes in the PCV, the whole genome sequences of WT and PCV strains were analyzed using bioinformatics. This included a pairwise comparison of the number of orthologous genes using the OrthoMCL pipeline (Chen et al., 2006) and computation of the phylogenetic distances of all strains based on their Maximum Common Genome (MCG) (Von Mentzer et al., 2014) as previously described (Schaufler et al., 2013). Transformants with the reintroduced large ESBL-plasmid were constructed to verify the observed phenotypic differences (Supplementary Table S1). PCVs functioned as electrocompetent acceptor strains; thus, the respective large ESBL-plasmid was transformed via electroporation (Green and Sambrook, 2012). Transformed

strains were screened for ESBL-production on cefotaxime (4 μg/ mL; Sigma-Aldrich, Taufkirchen, Germany) containing CHROMagar™ (MAST Diagnostica, Reinfeld, Germany) plates for ESBL-enzyme-production. Plasmid profile analysis was used to evaluate the success of electroporation.

ESBL-Plasmid Characteristics

Whole genomes of all WT and their corresponding PCV strains were sequenced using an Illumina HiSeq2000 sequencer in collaboration with the Wellcome Trust Sanger Institute (Cambridge, United Kingdom). The resulting reads were used for a *de novo* assembly (CLC Genomics Workbench 6.5, CLC Bio, Denmark). PLACNET analysis (Lanza et al., 2014) was then performed for all genomes to extract putative plasmid contiguous sequences (contigs) and to assign them to the large ESBL-plasmid and to smaller, additional non-ESBL-plasmids. These were confirmed via BLAST analysis, in which they were compared to reference sequences of known plasmids. All previously defined plasmid contigs showed a high similarity to parts of plasmids that have already been described. Annotation of the contigs was performed using the annotation feature of the program Geneious version 7.1.2 (Kearse et al., 2012) with 100% similarity to an in-house plasmid reference data base (data not shown). ESBL types, relaxase (REL) and plasmid replication initiator (RIP) proteins, incompatibility (Inc) groups, approximate ESBL-plasmid sizes, the number of additional plasmids, and virulence factors were detected based on PLACNET ESBL-plasmid sequences and using VirulenceFinder 1.4 (Joensen et al., 2014) and ResFinder 2.1 (Zankari et al., 2012; **Table 1**). BLAST ring image generator (BRIG) (Alikhan et al., 2011) was used to visualize the annotated PLACNET ESBL-plasmid sequences (pIMT17433, pIMT19205, pIMT27685, pIMT16316, pIMT17887, pIMT21183, and pIMT23463) of the seven WT strains with pEC_L8 as a reference ESBL-plasmid (Smet et al., 2010b; **Figure 1**).

Growth Curves

Growth curves in LB medium were performed in triplicate using standard protocols.

Omnilog® Phenotype Microarray (PM)

Using 96-well microtiter plates spotted with different substrates, growth/metabolic activity in 379 single substrates and sensitivity to 48 antimicrobial and chemical compounds was analyzed for all strains [Omnilog® (Biolog, Hayward, USA), Supplementary Table S2]. Three biological replicates were tested on six PM plates (PM1 and PM2: carbon sources; PM3: nitrogen sources; PM4: phosphorus and sulfur sources [http://www.biolog.com/pdf/pm_lit/PM1-PM10.pdf]; PM13 and PM14: chemical sensitivity [http://www.biolog.com/pdf/pm_lit/PM11-PM20.pdf]). Plates were inoculated with the bacterial suspension according to Omnilog® PM protocols. Plates were incubated for 48 h at 37°C. Respiration was measured by dye (tetrazolium violet) reduction every 15 min. The calculated longitudinal respiration kinetics were analyzed in R (Vaas et al., 2013). In confidence interval plots computed with extract defined by enlisted metadata for the parameter "area under the curve," only wells in which the normalized mean point estimates of WT and T groups showed

TABLE 1 | Characteristics of large ESBL-plasmids of the seven ESBL-producing wild-type strains.

Strain	ESBL type	ESBL-plasmid REL/RIP proteins and Inc group	ESBL-plasmid size (bp)	Number of additional plasmids (sizes in bp)	ESBL-plasmid additional resistance genes	ESBL-plasmid virulence genes
IMT17433	CTX-M-15	MOB_{F12}, RepFIA/FII, IncF	122,823	1 (1600)	bla_{TEM-1}, bla_{OXA-1}, *tet(A), tet(R), aadA, aac(6')-Ib-cr, catB4*	*finO, traT*
IMT19205	CTX-M-27	MOB_{F12}, RepFIA/FIB/FII, IncF	166,151	2 (1500, 5200)	bla_{TEM-1}, *tet(A), sul2, strA, aadA, aac(3)-IId, aac(3)-IV, aac(6')-Ib-cr*	*finO, traT, senB*
IMT27685	CTX-M-15	MOB_{F12}, RepFIA/FII, IncF	133,611	1 (1600)	bla_{OXA-1}, *tet(A), sul1, strA, strB, aadA, aac(6')-Ib-cr, dfrA17*	*traT*
IMT16316	CTX-M-15	MOB_{F12}, RepFIA/FIB, IncF	136,508	4 (2500, 3200, 4100, 7000)	bla_{TEM-1}, *tet(A), tet(R), sul1, sul2, strA, strB, aadA, aac(3)-II, dhfrVII, dfrA17*	*finO, traT*
IMT17887	CTX-M-15	MOB_{F12}, RepFIA/FIB, IncF	143,494	2 (2600, 7100)	bla_{TEM-1}, *tet(A), tet(R), sul1, sul2, strA, strB, aadA, aac(3)-IId, aph(3')-Ia, dhfrVII, dfrA17*	*finO, traT*
IMT21183	CTX-M-15	delta-TraI, RepFIA, IncF	103,420	1 (7100)	bla_{TEM-1}, *tet(A), tet(R), sul1, sul2, strA, strB, aadA, aac(3)-II, aph(3')-Ia, dhfrVII, dfrA17*	*traT*
IMT23463	CTX-M-14	MOB_{F12}, RepFIB/FII, IncF	143,193	1 (4100)	bla_{TEM-1}, bla_{OXA-1}, *tet(A), sul2, strA, strB, aadA, aac(3)-IId, aac(6')-Ib-cr, dfrA17*	*finO, traT, sitABCD, cma*

REL, relaxase; RIP, replication initiator protein; Inc, incompatibility; bp, base pairs.

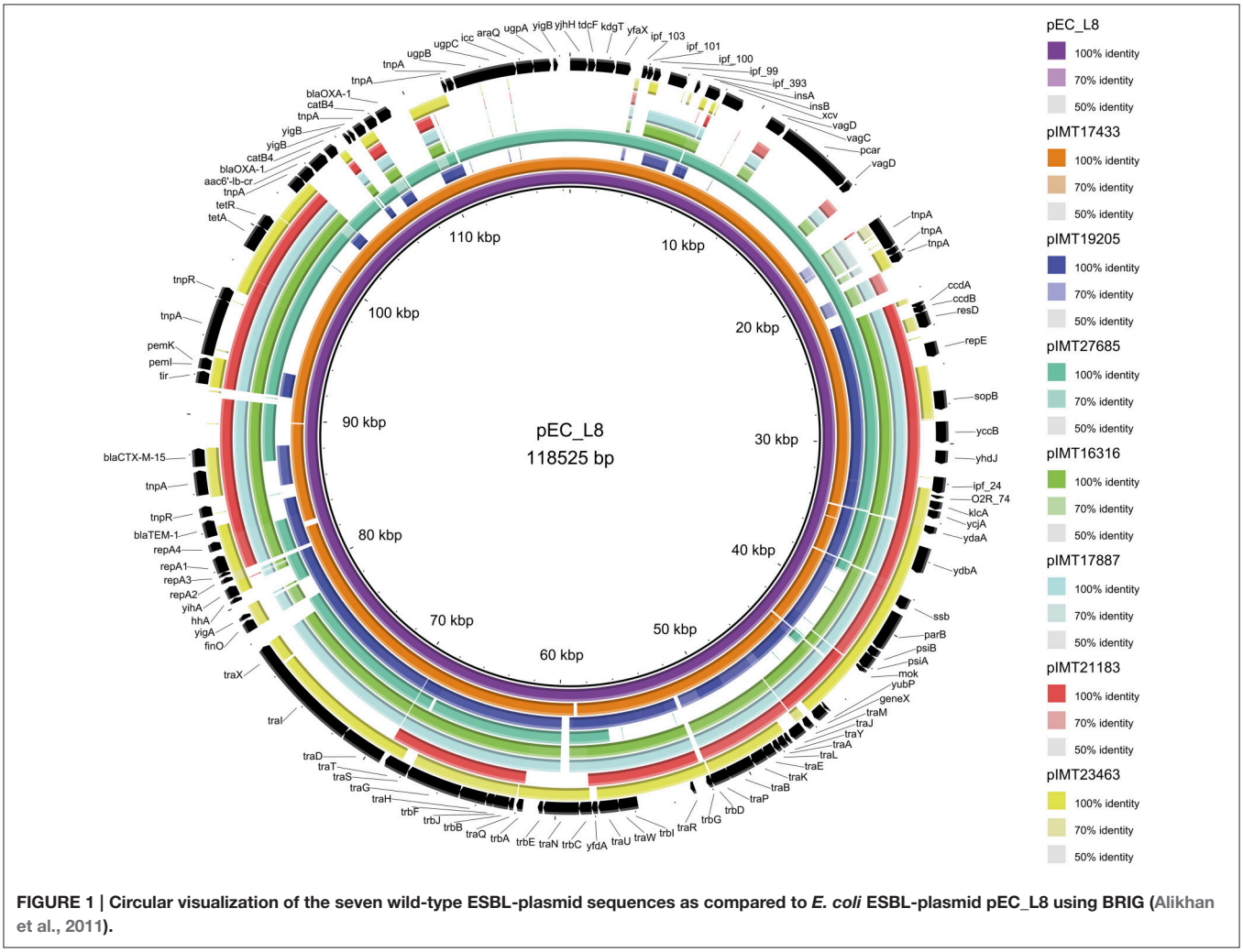

FIGURE 1 | Circular visualization of the seven wild-type ESBL-plasmid sequences as compared to *E. coli* ESBL-plasmid pEC_L8 using BRIG (Alikhan et al., 2011).

no overlapping 95% confidence intervals as compared to those of the PCV group, were considered to be significantly different. The group means of these wells underwent further statistical analysis using Tukey's method for multiple comparisons.

Macrocolonies

Three microliters of overnight culture (from a single colony grown in 5 mL BHI broth) from all strains were dropped on span agar plates (H. Carroux, Germany) with or without sodium chloride (5%) and congo red solution [0.5% congo red (Sigma-Aldrich, Taufkirchen, Germany) and 0.25% coomassie-brilliant-blue (Carl Roth, Karlsruhe, Germany) diluted in ethanol]. Plates were incubated for 5 days at 28°C (Romling, 2005; Richter, 2011). Following an initial comprehensive screening to detect differences between WT and PCV strains regarding their cellulose and/or curli fimbriae production, follow-up runs were performed on plates containing and lacking sodium chloride for all strains (**Table 2**). These follow-up runs were repeated six times. Reference strains (AAEC189, Blomfield et al., 1991, IMT26949, and W3110 Hayashi et al., 2006) were included in all runs for all plates and at all temperatures.

TABLE 2 | Cellulose and curli fimbriae expression by 17433 and 16316 strains on span agar plates with congo red, with or without sodium chloride, incubated at 28°C for 5 days.

Strain	With sodium chloride		Without sodium chloride	
	Cellulose	Curli	Cellulose	Curli
IMT17433	* * * * * *	* * * * * *	* * * * * *	* * * * * *
PCV17433				
T17433	* * * * *	* * * * * *	* * * * *	* * * * * *
IMT16316	* * * * * *	* * * * * *		
PCV16316		* * * * * *		
T16316	* * * * * *	* * * * * *		

*Six repetition runs followed a comprehensive initial screening. *, positive cellulose and/or curli fimbriae phenotype per run; 16316 strains were not tested on plates lacking sodium chloride as they did not show any differences during the initial screening.*

Biofilm Formation Assays

Overnight culture (from a single colony grown in 5 mL LB and minimal medium M63 overnight, Pardee et al., 1959) was set to optical density $OD_{600} = 0.05$. Technical triplicates of

the suspension were added to a 96-well plate, which was then hermetically closed and incubated for 24 or 48 h at 28, 37, or 42°C. Reference strains (AAEC189 and W3110) were included in all runs in triplicate for each medium, at all temperatures and time points. After incubation, OD was measured with an ELISA-reader (Synergy HT, BioTEK Instruments, Bad Friedrichshall, Germany) and bacteria were washed with aqua bidestillata, fixed in 99% methanol, stained with 0.1% crystal violet, and dissolved in 80:20 ethanol:acetone. After dissolution, OD was again measured, then biofilm formation capacity was computed (Martinez-Medina et al., 2009) and statistically analyzed using IBM SPSS Statistics for Windows, Version 20 (Dunn, 2013). Normal distributions of measuring points of all groups (strains) were tested using Kolmogorov-Smirnov (Smirnov, 1948). Based on the non-normal distributions of the measuring points of all groups, the non-parametric Wilcoxon rank-sum test (Mann-Whitney *U*-test; Wilcoxon, 1945) was used to estimate whether the observed biofilm formation capacity differences were statistically significant ($p = 0.05$). This assay was repeated three times in triplicate per temperature, and in two media for all strains.

Motility Assays

Overnight culture (from a single colony grown in 5 mL BHI broth) was set to $OD_{600} = 1$. One milliliter was centrifuged and washed twice with 1 × phosphate buffered saline (PBS). Five microliters of the suspension were dropped onto swim-agar plates (LB and 0.3% agar). Plates were incubated at 28, 37, and 42°C. The strain MG1655 (Hayashi et al., 2006; Richter, 2011) was used as a control for a positive swimming phenotype. After 48 h, colony diameters were measured (Harshey, 2003) and statistically analyzed using IBM SPSS Statistics for Windows, Version 20 (Dunn, 2013). Normal distributions of measuring points of all groups (strains) were tested using Kolmogorov-Smirnov (Smirnov, 1948). Based on the non-normal distributions of the measuring points of all groups, the non-parametric Wilcoxon rank-sum test (Wilcoxon, 1945) was used to estimate whether the observed swimming differences were statistically significant. The significance level for multiple comparisons between WT, PCV and T groups was adjusted to $p = 0.016$. This assay was repeated six times.

RNA Sequencing

The RNA of IMT17433, PCV17433, and T17433 was sequenced. RNA was isolated from two biological replicates from each of two macrocolony and two motility plates. RNA was isolated from cells using the RNASnap method (Stead et al., 2012) and shipped to LGC Genomics (Berlin, Germany) for RNA sequencing with an Illumina HiSeq2000 producing one channel paired end reads. The details of the company's standard protocols for quality control, RNA extraction from the RNASnap technique and rRNA depletion (using Ribo-Zero (Epicentre), Biozym, Hessisch Oldendorf, Germany) can be found on their website (http://www.lgcgroup.com/services). cDNA synthesis, library generation, indexing and cluster generation were performed using Illumina technology (TruSeq RNA Sample Preparation Kit v2). Bioinformatic mRNA differential expression analysis included the following processing steps: (a) generating FastQC reports to check the quality of sequenced reads; (b) clipping Illumina TruSeq sequencing adapters from the 3′ ends of reads; (c) filtering rRNA reads using riboPicker (Schmieder et al., 2012); (d) aligning reads against IMT17433 as a reference using TopHat2 (Kim et al., 2013) and RSEM; (e) counting reads per gene using HTSeq-count, and per transcript and gene using RSEM (Li and Dewey, 2011); and (f) computing differential gene and transcript expression between different groups of samples (including technical and biological replicates) using R/Bioconductor packages DESeq (Anders and Huber, 2010), edgeR (Robinson et al., 2010), and Cuffdiff (part of the Cufflinks software package, Trapnell et al., 2013).

RESULTS

ESBL-Plasmid-"Curing" and Transformations

All PCVs kept their smaller plasmids and lost only the large ESBL-plasmid. Bioinformatic analysis of all WT and PCV genomes assured their genetic similarity, ruling out any changes in the PCVs' chromosomal content during the "curing" procedure (Schaufler et al., 2013; Von Mentzer et al., 2014). To verify detected phenotypic differences between WT and PCV strains, seven transformants containing the reintroduced large ESBL-plasmid were used (T17433, T19205, T27685, T16316, T17887, T21183, and T23463; Supplementary Table S1). These strains showed phenotypic cefotaxime-resistance (CLSI, 2008). Plasmid-profile analysis confirmed transformation of the large ESBL-plasmid.

ESBL-Plasmid Characteristics

Whole genomes of all WT strains were used for plasmid characterization (http://www.sanger.ac.uk/resources/downloads bacteria/escherichia-coli.html#project_2119; IMT17433 [ERR163891], IMT19205 [ERR163889], IMT16316 [ERR163879], IMT17887 [ERR163883], IMT21183 [ERR163880], IMT23463 [ERR163881]; http://www.sanger.ac.uk/resources/downloads/bacteria/escherichia-coli.html#project 2433; IMT27685 [ERR264283]). **Table 1** summarizes the most important ESBL-plasmid characteristics. Their sizes ranged from approximately 100–166 kb. Besides common bla_{CTX-M} types (mostly the CTX-M-15 enzyme), non-beta-lactam resistance genes (e.g., *tet(A)/(R)* and *aac(6′)-Ib-cr*) were found. Virulence-associated genes, mainly *finO* and *traT,* were present. All WT strains harbored at least one smaller non-ESBL-plasmid (**Table 1**). Analysis of PCV genomes showed complete loss of the MOB_{F12}/IncF ESBL-plasmids in six of the seven genomes analyzed. Strain IMT19205 contained two IncF plasmids that were unresolvable by PLACNET. This strain lost its ESBL-phenotype along with one of the IncF plasmids.

Figure 1 displays the annotated ESBL-plasmid sequences (pIMT17433, pIMT19205, pIMT27685, pIMT16316, pIMT17887, pIMT21183, and pIMT23463) as compared to ESBL-plasmid pEC_L8 (Smet et al., 2010a), showing both beta-lactam and non-beta-lactam resistance genes, as well as non-resistance genes, including *icc* (phosphodiesterase) and *hha*

(hemolysin expression modulating protein), which were present on all plasmids. Additionally, this comparison displays typical features of conjugative plasmids (*tra* regions and insertions sites) and plasmid partitioning (toxin-antitoxin systems: e.g., *vagC/D* and *pemI/K*).

In summary, although they contained similar genetic backbones and resistance determinants, ESBL-plasmids were rather diverse.

Growth Curves and Omnilog® Phenotype Microarray (PM)

No differences were detected between the LB growth curves of WT ESBL-producing *E. coli* strains and the corresponding PCVs (data not shown).

Growth/metabolic activity and chemical sensitivity of all strains were then screened using the Omnilog® PM system. Only a minor proportion of strains showed significant differences between the 427 tested compounds (Supplementary Table S2). Exemplary for plate PM1, significant differences were observed for strain combinations 17433 and 17887. Additionally, plates PM2 and PM3 revealed few significant differences (Supplementary Table S2). In contrast, on plate PM4 (sulfur and phosphorus sources), significant differences were observed for 22 wells, of which 18 showed higher values in WT and T strains, while four showed higher values in PCV strains.

The chemical sensitivity plates PM13 and PM14 revealed expected significant differences for ESBL-carrying (WT and T) strains in antimicrobial-containing wells, which showed higher metabolic values than corresponding PCV strains (e.g., PM13, C6: Doxycycline and D4: Cefuroxime; and PM14, G8: Carbenicillin; Supplementary Table S2).

In summary, given the high numbers of substrates tested, only a small proportion of wells showed significant differences in the "area under the curve" parameter between WT, PCV, and T strains. The detected significant differences were bidirectional, meaning that in some cases the PCV strains showed higher growth/metabolic activity values, and in other cases the ESBL-plasmid-carrying strains showed higher growth/metabolic activity values.

Macrocolonies

The expression of the biofilm-associated extracellular matrix components cellulose and/or curli fimbriae was tested using macrocolony assays. **Table 2** shows the results of the six follow-up runs, which were performed for those strains with differences in cellulose and/or curli fimbriae production during the initial screening (combinations 17433 and 16316).

On plates containing sodium chloride, ESBL-carrying WT IMT17433 expressed cellulose and curli fimbriae, whereas its corresponding PCV did not (**Figure 2**). Another mutant (PCV16316) did not show any cellulose expression in any run as compared to its associated WT strain (IMT16316), which produced both cellulose and curli fimbriae. In both cases, in accordance with the corresponding WT strains, the transformants T17433 (**Figure 2**) and T16316 produced curli fimbriae and cellulose in most runs. Only combination 17433 showed the same results on plates lacking sodium chloride (**Table 2**).

In summary, PCV17433 and PCV16316 displayed reduced production of extracellular components as compared to their respective wild-type and transformant strains.

Biofilm Formation Assays

Virulence-associated biofilm assays revealed significant differences among three WT/PCV combinations (IMT/PCV17433, IMT/PCV27685, and IMT/PCV17887) after 24 h and among two combinations (IMT/PCV17433 and IMT/PCV17887) after 48 h. IMT17433 and its corresponding PCV were particularly interesting. At all three temperatures and after both time points, the WT strain showed an enhanced biofilm formation capacity in glucose-containing M63 medium (28°C, 24 h: $p = 0.222$; 37°C, 24 h: $p = 0.008$; 42°C, 24 h: $p = 0.094$; 28°C, 48 h: $p < 0.001$; 37°C, 48 h: $p < 0.001$; 42°C, 48 h: $p = 0.008$) as compared to the PCV strain. In contrast, biofilm formation by PCV17433 was better in LB medium

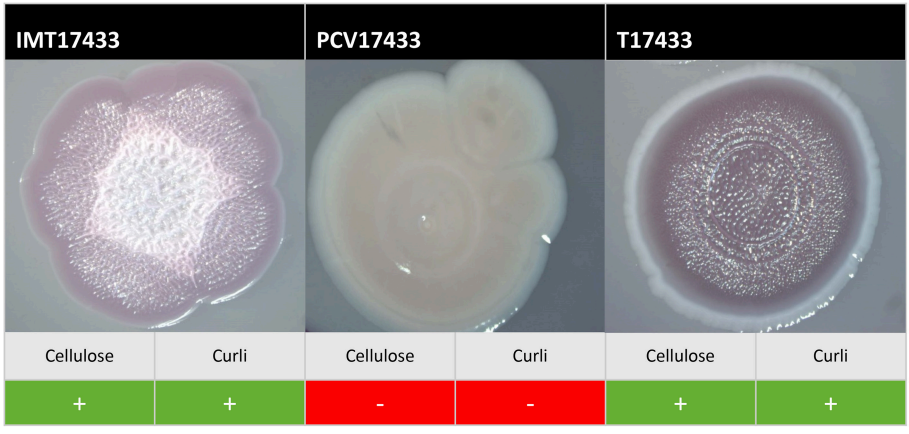

FIGURE 2 | Exemplary macrocolonies of IMT/PCV/T17433. Span agar plates with congo red and sodium chloride, incubated for 5 days at 28°C.

(28°C, 24 h: $p < 0.001$; 37°C, 24 h: $p < 0.001$; 42°C, 24 h: $p < 0.001$; 28°C, 48 h: $p = 0.006$; 37°C, 48 h: $p = 0.004$; 42°C, 48 h: $p = 0.002$; **Figures 3A,B**). Transformants did not show any biofilm formation.

In summary, some WT and PCV combinations showed differences in their biofilm formation capacities in both directions; these results were medium-dependent.

Motility Assays

Motility assays were performed to test the swimming capacity of the strains at different temperatures. Two PCV strains (PCV17433 and PCV17887) showed significantly increased swimming capacity as compared to their corresponding WT strains (IMT17433 and IMT17887) at 28, 37, and 42°C (IMT/PCV17433: 28°C, $p = 0.002$; 37°C, $p = 0.002$; 42°C, $p = 0.006$; IMT/PCV17887: 28°C, $p = 0.002$; 37°C, $p = 0.002$; 42°C, $p = 0.004$). Both transformants (T17433 and T17887) showed significantly reduced swimming capacity as compared to their corresponding PCV strains, with the exception of T17887 at 28°C. (T/PCV17433: 28°C, $p = 0.002$; 37°C, $p = 0.002$; 42°C, $p = 0.002$; T/PCV17887: 28°C, $p = 0.032$; 37°C, $p = 0.004$; 42°C, $p = 0.013$; **Figures 4**, **5A–C**).

In summary, PCV17433 and PCV17887 showed higher swimming capacity than their respective WT and T strains.

RNA Sequencing

To gain insight into differential gene expression, RNA sequencing was performed for IMT/PCV/T17433. Only up- or downregulated genes detected with all three software packages and a threshold of 1.5 fold bidirectional regulation were considered for subsequent analysis.

Cellulose- and curli fimbriae-related differentially regulated genes from the macrocolony assay included: *csgB* (upregulation in WT compared to PCV: 9.8), *csgA* (8.6), *csgE* (6.0), *csgF* (5.5), *csgD* (5.5), *csgG* (4.0), *csgC* (3.6), and *adrA* (2.3). Differentially regulated genes important for swimming in the motility assay were, among others: *fliZ* (downregulation in WT compared to PCV: −1.9), *flgH* (−2.0), *flgF* (−2.5), *flhC* (−2.7), *fliD* (−2.7), *flgL* (−3.0), *flgK* (−3.1), *flgC* (−3.4), *flhD* (−3.5), *fliL* (−4.0), *flgD* (−4.2), and *fliC* (−5.7). Most genes were found to be up- or downregulated in both assays, except for *csgD* and *csgC*, which were only found to be upregulated in the macrocolony assay in the WT strain as compared to the PCV strain. Conversely, downregulation of *flhC* and *flhD* was only observed in the motility assay. All cellulose- and curli-related genes that were upregulated in the macrocolony assay in IMT17433 as compared to PCV17433, were also upregulated in T17433 as compared to PCV17433. Swimming-related genes that were downregulated in the motility assay were not detected in the transformant (e.g., *fliZ*, *flgH, flgA, flgL, flgG, fliC*).

In summary, RNA sequencing verified the observed phenotypes for WT17433, PCV17433, and T17433 at the transcriptional level.

Candidate Genes

The observed phenotypic and transcriptomic differences were only explainable by differences at the genetic level. With only the ESBL-plasmids differing between WT/T and PCV strains and resistance determinants not explaining the results, the next step in this study was to focus on the non-resistance genes encoded by these plasmids. **Table 3** shows candidate genes of the seven ESBL-plasmid sequences based on PLACNET analysis. Previous studies suggest their involvement in biofilm formation and motility. The candidate genes included *hha* (encoding the hemolysin expression modulating protein) and *yihA* (encoding a cell division protein), which were both present on pIMT17433, pIMT19205, and pIMT27685. The genes *icc* (encoding a phosphodiesterase) and *yfaX* (encoding a putative transcriptional factor) were encoded on pIMT17433 and pIMT27685. All ESBL-plasmids carried *tra* genes important for conjugation.

In summary, the detected candidate genes were mostly encoded on ESBL-plasmids of sequence type ST131 (pIMT17433, pIMT19205, and pIMT27685).

DISCUSSION

The success of ESBL-producing *E. coli*, particularly the pandemic pathogenic clonal lineages, cannot be explained by antimicrobial resistance alone. To assess the possibility of interactions between ESBL-plasmids and chromosomal content, we conducted a study using an unconventional approach by constructing PCVs of ESBL-plasmid-carrying WT strains of ST131 and ST648. Based on growth curves in LB medium and detailed growth/metabolic activity under different conditions, WT and PCV strains showed similar behaviors and were thus regarded suitable for analysis in subsequent phenotypic assays. Phenotypic differences between WT and PCV strains were generally verified by T strains.

In PM assays, which are a consolidation of regular LB growth curve tests (Supplementary Table S2), significant differences were detected in select wells (plates PM1-4: 3% among all wells without combination 17433). When the results of PM4 were included for 17433, 15% of the wells showed differences; however, these results need to be treated with caution since the negative control (A01) had high values for PCV17433 even after several repetitions of the assay. High values for negative controls in PM assays have previously been described for *E. coli* (Vaas et al., 2012). Differences in antimicrobial sensitivity assays on plates PM13 and PM14 between ESBL-plasmid-carrying strains and PCVs are explainable by loss of ESBL-plasmids in PCVs, which, besides ESBL-genes, carry additional antimicrobial resistance genes (**Table 1**; e.g., PM13, C6: Doxycycline and D4: Cefuroxime; and PM14, G8: Carbenicillin). Overall, significant differences were bidirectional, meaning that each of the ESBL-plasmid-carrying strains and the PCVs showed higher values in select wells. This indicates, as underscored by LB growth curve results, that ESBL-plasmid-carriage does not necessarily lead to fitness loss. This contradicts the hypothesis that considerable trade-offs exist between antimicrobial resistance and fitness (Dasilva and Bailey, 1986; Lenski, 1997; Andersson and Hughes, 2010). Our finding applied to all seven strain combinations irrespective of origin, ST, or ESBL-plasmid characteristics.

Biofilm-related macrocolonies result from bacterial incubation over several days, where the biosynthesis of the

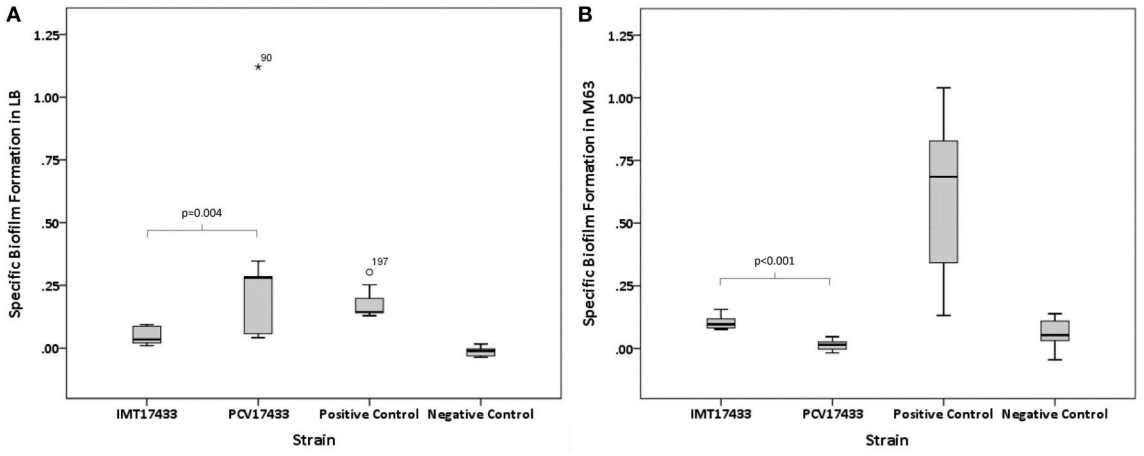

FIGURE 3 | (A) Box plots of the distributions of the specific biofilm formation capacities of IMT17433 and PCV17433 at 37°C after 48 h in LB medium. Plots were generated using IBM SPSS Statistics for Windows, Version 20. Positive control, AAEC189; Negative control, W3110. **(B)** Box plots of the distributions of the specific biofilm formation capacities of IMT17433 and PCV17433 at 37°C after 48 h in M63 medium. Plots were generated using IBM SPSS Statistics for Windows, Version 20. Positive control, AAEC189; Negative control, W3110.

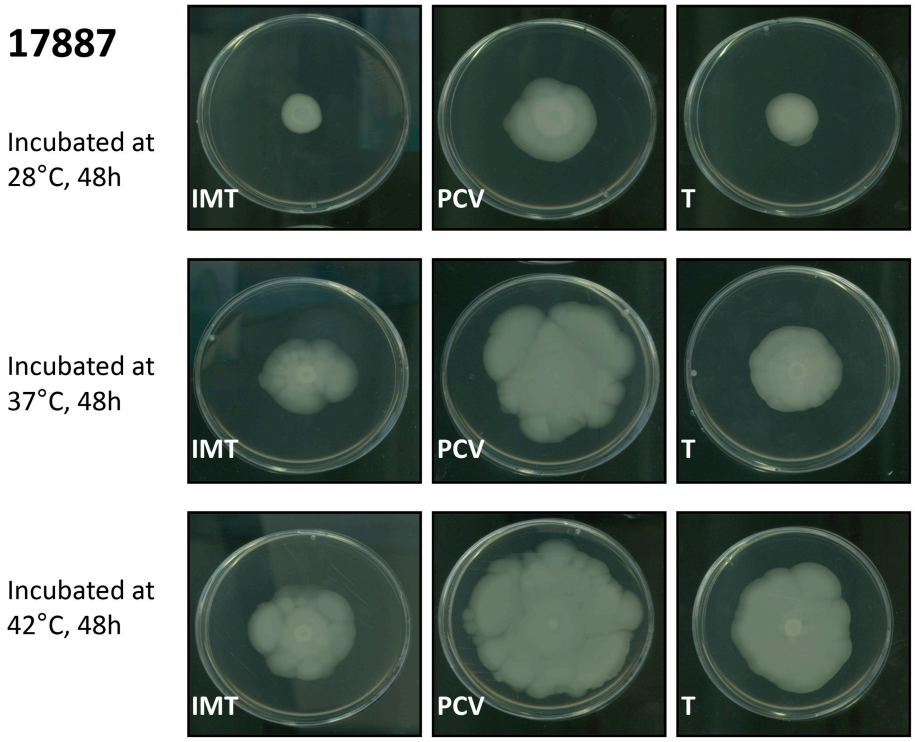

FIGURE 4 | Exemplary swimming motility of IMT/PCV/T17887. LB plates wih 0.3% agar. Incubation at 28, 37, and 42°C for 48 h.

important virulence-related extracellular matrix components cellulose and curli fimbriae typically occurs below 30°C (Bokranz et al., 2005; Richter et al., 2014). Curli fimbriae promote adhesions to abiotic surfaces (Zogaj et al., 2001) and are associated with virulence, as they play key roles during internalization into epithelial cells (Gophna et al., 2001) and persistence in avian guts (La Ragione et al., 1999). Bacteria produce cellulose mainly as protection from both chemical and mechanical influences (Ross et al., 1991). Considering the results of the macrocolony assays for those combinations with differences, the enhanced ability of ESBL-plasmid-carrying WT and T strains to synthesize curli fimbriae and/or cellulose

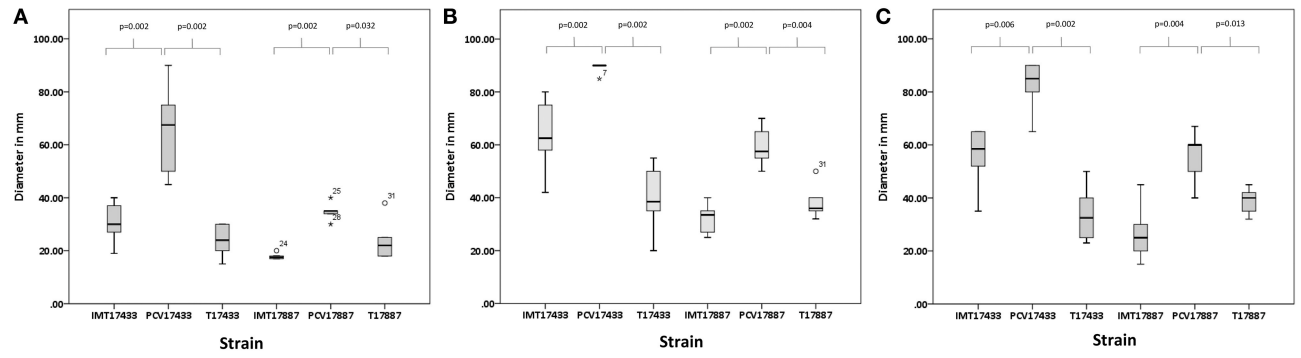

FIGURE 5 | (A) Box plots of the distributions of the diameters, in millimetres, of the swimming capacities of IMT/PCV/T17433 and IMT/PCV/T17887 at 28°C. Plots were generated using IBM SPSS Statistics for Windows, Version 20. **(B)** Box plots of the distributions of the diameters, in millimetres, of the swimming capacities of IMT/PCV/T17433 and IMT/PCV/T17887 at 37°C. Plots were generated using IBM SPSS Statistics for Windows, Version 20. **(C)** Box plots of the distributions of the diameters, in millimetres, of the swimming capacities of IMT/PCV/T17433 and IMT/PCV/T17887 at 42°C. Plots were generated using IBM SPSS Statistics for Windows, Version 20.

TABLE 3 | Candidate genes based on annotated PLACNET ESBL-plasmid sequences, literature survey, and RNA sequencing results.

Gene	Protein	Predicted function	Presence	References
hha	Hemolysin expression modulating protein	Involved in biofilm formation and motility	pIMT17433, pIMT19205, pIMT27685	Barrios et al., 2006
icc	Phosphodiesterase	Involved in biofilm formation	pIMT17433, pIMT27685	Kalivoda et al., 2013
yfaX	Predicted DNA-binding transcriptional regulator	Putative HTH-type transcription factor	pIMT17433, pIMT27685	Perez-Rueda and Collado-Vides, 2000
yihA	Cell division protein, predicted checkpoint GTPase	GTP binding	pIMT17433, pIMT19205, pIMT27685	Lehoux et al., 2003
tra	Transfer regions of the F-conjugative plasmid	Involved in biofilm formation	all seven pIMT ESBL-plasmids	Ghigo, 2001

in contrast to their respective PCV strains was particularly notable. This indicates that ESBL-plasmid carriage confers benefits in terms of biosynthesis of virulent- and survival-associated extracellular matrix components to some bacterial strains. Several prior studies have described an influence of conjugative plasmids on biofilm formation (Ghigo, 2001; Yang et al., 2008). May and Okabe (May and Okabe, 2008) investigated the influence of natural IncF F-plasmids on biofilm formation and maturation. In this study, both conjugative (*tra* regions) and non-conjugative plasmid genes seemed to play a role. In our study, all seven ESBL-plasmids belonged to incompatibility group IncF. Furthermore, all encoded different *tra* genes are important for conjugation; however, since not all WT strains showed differences in extracellular matrix component production as compared to PCV strains, these factors are probably not solely responsible for our observed results.

Biofilm results from IMT/PCV17433 underline the complexity of biofilm formation, which is also dependent on nutrient availability. The enhanced ability of IMT17433 to form biofilms in M63 medium as compared to PCV17433 may point toward plasmid encoded features that enable the WT strain to use limited nutrients efficiently, perhaps via phosphate-dependent pathways. PM results revealing that select phosphate-containing wells in which IMT17433 showed higher respiration values than PCV17433 reinforce the latter hypothesis. Explanations of the observed enhanced ability of the PCV strain to form biofilms in LB medium, however, remain speculative. There may have been no need for planktonic IMT17433 to transform into a biofilm due to optimal utilization of the rich LB medium. A switch from a sessile (multicellular behavior, biofilm) to a planktonic (motility) way of life, and vice versa, underlies subtle interactions at a molecular level that include numerous complex cascades. Flagellar biosynthesis, for instance, seems not only crucial for bacterial swimming but also leads to surface colonization and subsequent biofilm formation. Insufficient nutrient supply leads to detachment of the cells from the biofilm and adoption of a planktonic lifestyle (Harshey, 2003).

Explanations of the observed enhanced swimming capacity among some PCV strains as compared to their corresponding WT and T strains in the motility assays remain similarly speculative. Flagellar synthesis has been shown to be energetically costly, and ESBL-plasmid-carrying strains might use their energy more strategically (Zhao et al., 2007). Alternately, due to better nutrient utilization, ESBL-plasmid-carrying strains may not have to swim as well as PCV strains to reach peripheral zones with, presumably, richer nutrition supplies.

Phenotypic differences in cellulose and curli fimbriae production as well as swimming capacity, particularly those

observed between the 17433 strains, were confirmed by RNA sequencing results showing upregulation of chromosomally encoded extracellular matrix-related genes and downregulation of chromosomally encoded flagellar-related genes in IMT17433 as compared to PCV17433. Reversible RNA sequencing results for T17433 strengthen the reliability of the differences seen at phenotypic and transcriptomic levels. The key role of the transcription factor *csgD* is notable in biofilm formation and motility capacity. *csgD* regulates not only the second curli fimbriae operon *csgBAC* and *adrA,* whose product is not only accountable for several other genes involved in cellulose production, but also influences *flhDC*, the main operon for flagellar synthesis and thus motility (Ogasawara et al., 2011; Chambers and Sauer, 2013). In our study, RNA sequencing results revealed upregulation of *csgD, csgBAC,* and *adrA* and downregulation of *flhDC* in IMT17433 as compared to PCV17433. These results not only confirm the phenotypic data, but also emphasize the central importance of the CsgD protein in regulating the subtle interaction between bacterial sessile and planktonic ways of life.

Overall, the phenotypic tests performed for all seven strain combinations did not show a consistent pattern. Although, this is expected due to high diversity among the group of ESBL-plasmids as well as in the origins of host strains and their phylogenetic backgrounds, such inconsistencies weaken the generalizability of our conclusions. Rather, our results should be seen as an impetus for prospective studies. Interestingly, despite ESBL-plasmid diversity, each of pIMT17433, pIMT19205, and pIMT27685 carried different candidate genes, including *hha* and *icc*, which both have been described to be involved in biofilm formation and motility (Barrios et al., 2006; Kalivoda et al., 2013). Only further experiments that include cloning and knockout methods will provide more information on underlying molecular mechanisms.

In conclusion, this pilot study showed that ESBL-plasmid carriage does not necessarily lead to a growth/metabolic fitness loss. Some ESBL-plasmids in select strains may possess the potential to influence chromosomal gene expression, particularly of those genes that are important for the subtle interactions between the sessile and planktonic ways of life, such as *csgD*. We hypothesize that this may contribute to their virulence potential and pandemic success in different habitats, although underlying mechanisms remain to be identified and characterized.

AUTHOR CONTRIBUTIONS

All authors gave their final approval of the version to be published. Conception and Design: SG, CE, KS, LW, FD. Data analysis: KS, SG, TS, DP, MD. Data acquisition: KS, SG, TS. Writing: KS, SG, LW, CE, DP, TS, MD, FD.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fmicb.2016.00336

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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4. DISCUSSION

The main aim of this thesis has been to contribute to the understanding of why certain bacterial lineages are successful in numerous global settings, whereas others are restricted to certain habitats and hosts. In an era where AMR microorganisms have become increasingly difficult to treat [225] the focus must be placed on pathogenic and zoonotic bacteria that spread rapidly, and simultaneously propagate AMR determinants.

Successful bacterial lineages and clones that frequently carry AMR determinants on MGEs, such as plasmids, are found within *Escherichia, Klebsiella* [9]*, Staphylococcus* [19]*, Acinetobacter* [18]*, Enterococcus* [226] and other genera. Not only detected in environments with high antimicrobial selection pressures, they are increasingly described in environments and animals thought to be of moderate and low exposure to antimicrobial compounds [12-15, 227]. This deserves closer investigation in search of mechanisms to explain the global success of bacterial lineages.

We hypothesized that the interaction of ESBL-plasmids and the chromosome of bacterial hosts with a specific phylogenetic background leads to a benefit in *E. coli* with no parallel fitness costs [17]. We focused on ESBL-producing ST131 and ST648 *E. coli*, which harbor international high-risk multi-drug resistant clones. Bacterial lineages and clones may benefit from ESBL-plasmid carriage, even in areas without direct antimicrobial influences. This led to the need to identify non-resistance factors associated with virulence, metabolism and fitness on the ESBL-plasmids.

4.1 ESBL-plasmid "curing" and transformation

To better understand whether the carriage of ESBL-plasmids results in a fitness loss in the bacterial host and whether they interact with the host's chromosome, we extracted ("cured") ESBL-plasmids from bacterial wild-type (WT) strains that belong to sequence types ST131 and ST648 (work objectives I) [228]. The "curing" included application of heat stress above the *E. coli*'s normal growth temperature at 37°C in brain-heart infusion (BHI) broth, free of antimicrobials, following prior protocols [123] with necessary methodological adaptations [228]. At first, extracting plasmids may seem straightforward; after applying the heat procedure for several weeks, however we have realized some bacterial strains had stopped growing and died before voluntarily giving up their ESBL-plasmid. Although also interesting, we did not perform the supposed simpler approach of transforming ESBL-

plasmids into a laboratory plasmid-free *E. coli* strain because of the need to reproduce realistic scenarios; by using isolates with phylogenetic backgrounds that have demonstrated their success in clinical and extra-clinical settings and their stable association with the ESBL-plasmid [229]- ST131 and ST648. Other challenges during "curing" included contaminations that were only detected after performing macrorestriction analysis to assure the genetic identity of WT and ESBL-plasmid "cured" variant (PCV). Those contaminated mutants were excluded from the following analyses.

Why some bacterial strains refused to lose their supposed costly ESBL-plasmid in an environment without antimicrobial selection pressures first seems odd but follows in the context of benefits through ESBL-plasmid-carriage that goes beyond AMR, and plasmid-host co-evolution, as discussed later. In addition TA systems prevent plasmid-elimination as they ensure the survival of plasmid-carrying daughter cells whilst inducing the death of those without plasmids [228]. TA systems are most likely the prerequisite to steadily keep plasmids in a bacterial population [230], thus playing a key role in also maintaining plasmid-encoded additional factors that favor the bacterial host, for example AMR. Even more surprising was the successful construction of seven PCV strains from ESBL-plasmid-carrying WT *E. coli* ST131 and ST648 strains that each carried plasmids with TA systems.

Several studies have observed that cost-intensive plasmids are kept in bacterial populations, even in the absence of positive selection [151, 229, 231]. This not only applies to plasmids carrying TA systems but others, which led to the assumption that plasmids co-evolved with their bacterial host [229].

After initial PCV construction screened on cefotaxime-plates it was necessary to evaluate and visualize the loss of the large ESBL-plasmid, made possible through plasmid-profile analysis (PPA). PPA electropherograms of the seven PCV and corresponding WT strains revealed the loss of the large (> 100kbp) ESBL-plasmids as expected [228]. Interestingly, however, smaller plasmids in the PCVs were kept when compared to the WTs. We can only speculate why the large ESBL-plasmids were eliminated as desired. Larger, AMR plasmids might account for higher fitness costs in the bacterial host as demonstrated previously [232], which in our case, however, did not seem to apply as WT and PCVs demonstrated similar growth and metabolic behaviors. The smaller plasmids, which did not confer AMR, might be less heat sensitive or have no heat sensitive replicons than larger representatives and are thus "unimpressed" by the incubation at high temperatures. This could be investigated prospectively through the analysis of promoter/replicon sequences for specific

motifs. Also, their "curing" might be more challenging due to a higher copy number per bacterial cell.

PFGE analysis following macrorestriction first estimated the PCVs and WTs genetic identity, while sequencing of the whole genomes of all strains afforded greater precision. We compared PCVs and WTs at a genetic level and extracted plasmid sequences of the WT WGS (PLACNET [233]) by using bioinformatics, which verified the genetic identities [17].

Finally we had an adequate small set of seven ESBL-plasmid-free bacterial *E. coli* strains that originated from different origins including animal and human sources to cover a high host spectrum [17]. Those strains were compared to their corresponding WTs, representing not only a unique approach to the study of plasmid-associated fitness costs and plasmid-bacterial host interactions but also providing a verification tool for other important scientific issues, such as bioinformatically extracting plasmid-sequences from WGS data (unpublished data, based on PLACNET [233]).

To increase research significance, results were verified by transformant (T) strains. Those were constructed from PCVs through transformation of the respective large ESBL-plasmids (work objectives I). We chose electroporation, which introduces new (or foreign) DNA molecules into recipient cells by applying an electric field to increase the permeability of the cell membrane [234]. While the transformation of the respective plasmid as such was highly successful, the resulting T strains partially lost their smaller plasmids, as described in a preceding study [235], which might be due to the porous effect on the membrane. This plasmid loss and the fact that electroporation partly weakens the transformed strains generally might explain why some phenotypic differences in WTs and PCVs were not completely verified by the T strains. Still, electroporation was the method of choice to retransfer the large ESBL-plasmid into the PCV strains as other methods would have compromised the approach. Natural conjugation, for example, would have meant taking a detour through a different organism with no guarantee of successful plasmid transmission and not provided the traceability of donated additional elements.

4.2 ESBL-plasmids and fitness costs

In order to be widely successful, bacterial lineages and clones must outcompete rivals in selective and non-selective conditions in varying hosts and habitats. While this seems straightforward for AMR plasmid-carrying bacteria in environments with antimicrobial

selection pressures, it is more likely the contrary in habitats without those pressures. Factors that convey benefits for the bacterial host in challenging environments, such as plasmids that carry AMR components, often yield fitness costs for bacteria as soon as the antimicrobial compound ceases.

We have comparatively analyzed WTs, PCVs and Ts in growth and metabolic assays (work objectives I) [17] to study whether ESBL-plasmid-carriage leads to fitness costs in the bacterial host, and to estimate the suitability of the strains. Any difference in the fitness (growth and metabolism) between ESBL-plasmid-carrying and ESBL-plasmid-free strains, arising from either plasmid-associated costs or weakening of PCVs and Ts through the construction process, would have resulted in the aggravated evaluation of results. That is, any differences in the follow-up phenotypic assays could not have doubtlessly been traced back to the actual ESBL-plasmid's influence on the bacterial host's chromosome.

Classic growth curves in antimicrobial-lacking medium were extended by OmniLog PM assays with the advantage that metabolically relevant, single chemical compounds are tested in a high-throughput approach. After having analyzed results arising both from the growth curves and comprehensive PM assays, we concluded that ESBL-plasmid carriage does usually not negatively affect the bacterial host's growth and metabolic fitness [17]. To our knowledge we were the first to compare ESBL-plasmid-carrying WTs to their isogenic, plasmid-free counterparts in the OmniLog system, which demonstrated the similarity of their metabolism and growth in a highly sophisticated manner. Results allowed us to continue with follow-on studies, but also, importantly, to question the possible trade-offs between plasmid-carriage and bacterial fitness. Many studies agree that plasmid-carriage leads to a fitness cost in bacteria at minimum resulting from plasmid replication and partitioning to ensure their maintenance during each generation [236, 237]. The multi-drug resistant appearance of the seven WT strains enhances the evidence, when noted that broad-spectrum resistance yet seems to exacerbate the costs [238]. However, other studies support our findings of no considerable trade-offs. For instance, it was shown that the AMR-bearing R1 plasmid, despite conferring an initial cost to the bacterial host, did not lead to a decreased fitness after several hundred generations, even in the absence of antimicrobial compounds. The "evolved plasmid" was able to increase the bacterial host's fitness [239], as also demonstrated in another study when compared to an isogenic, plasmid-free strain [240]. We did not observe increased fitness in our strains [17]. For *E. faecalis*, it was suggested that a "cured" native plasmid leads to reduced stress tolerance in the mutant [241].

A stable combination of co-evolved ESBL-plasmids and bacterial host with an evolutionary history that results in compensated fitness outcomes regardless of the surrounding conditions might be a key in the global success of ESBL-producing *E. coli*. The above mentioned study [240] demonstrated that the genetic (compensatory) mutations during co-evolution did most likely not occur in the plasmid but in the bacterial host's chromosome implying that certain bacterial genotypes may be more likely responsive to plasmid influences than others [242]. Underlying mutations in specific genes remain mostly unexplained. A recently submitted study suggested compensatory mutations in chromosomally located gene regulatory regions of *E. coli* ST131 in response to AMR plasmid acquisition [243]. Harrison et al. commented on the responsible mutations more specifically: their results investigating *Pseudomonas fluorescens* and a mega-plasmid revealed the *gacA/gacS* global regulatory system as a main mechanism [244]. Mutations in this bacterial regulatory system reduced the expression of chromosomal and plasmid genes, which seemed to have "relieved the translational demand imposed by the plasmid" [244]. Another rare specific example is the mutation in putative helicase and kinase genes in *Pseudomonas aeruginosa*, which downregulated the expression of the costly plasmid's (pNUK73) replication protein gene *rep*. The expression of *rep* strongly influenced the transcriptional profile of the bacterial host, which was restored upon occurring compensatory mutations in the putative helicase and kinase genes [245]. However, plasmids themselves might evolve through mutations toward fixation within certain bacterial populations contributing to shaping the evolution of sublineages of *E. coli* ST131, as suggested recently [246]. Other studies support the finding of mutations in evolved plasmids [151, 239, 247], for example in replication genes (e.g. *trfA1*) [247].

We cannot be sure of the extent to which the ESBL-plasmid domestication and compensatory mutations in our strains had proceeded. They might be, to their capabilities, already highly adapted to their habitat or, still in the process of adaption and/or compensation. Moreover, the plasmid's role as highly diversified and possibly co-evolved associate should not be underrated while trying to understand the underlying mechanisms.

For international high-risk multi-drug resistant clones and lineages of ST131 and ST648, it might be their phylogenetic backgrounds in combination with the specific content of the respective ESBL-plasmids that has enabled their pandemic success in various habitats, as discussed later. Despite the limited sample sizes in our studies, the significance of results might apply to other strains and even bacterial genera, as we detected similar fitness and

metabolic results across the highly diverse study parameters in terms of a variety of ESBL-plasmids and bacterial hosts [17].

4.3 ESBL-plasmids and non-resistance factor-associated benefits

As ESBL-plasmid-carrying strains did not show major differences in their growth and metabolic activity as compared to the ESBL-plasmid-free counterparts, the path was clear to test our second hypothesis: "an interaction of ESBL-plasmids with the *E. coli* chromosome results in non-resistance factor-associated advantages for the bacterial host". Interestingly, a recently published study suggested "that the gain of virulence-associated genes followed by the tandem development of antibiotic resistance primed the successful global dissemination of ST131" [248]. To test this second hypothesis would contribute substantially to the understanding of how and why ESBL-producing *E. coli* of ST131 and ST648 emerge successfully in varying habitats, even those distant from consistently high antimicrobial selection pressures.

One would agree that a high tenacity/tolerance or virulence potential is among the key factors for bacteria to prevail in challenging environments. We tested the strains' ability to form biofilms and its counterpart, the motility (work objectives II), which are often part of bacteria's core repertoire and important virulence-associated traits. Summarized, our results demonstrate that biofilm-formation, mostly tested in macrocolony assays, was increased, and motility, tested in swimming assays, was decreased in some of our tested ESBL-plasmid-carrying strains (WTs and Ts) when compared to the PCVs. Those phenotypic results were verified by RNA-sequencing that was performed for one WT, PCV and T combination (17433). Results suggested the *csgD*-dependent pathways as primarily involved [17]. A detailed plasmid-characterization through bioinformatics analyses allowed the identification of several so-called candidate genes, supported by previous studies [174, 249, 250] that might be responsible for the detected differences. Still, further investigations are necessary.

Clearly, plasmids that confer both the required AMR but also other beneficial non-resistance factors constitute an advantage in an antimicrobial-enriched environment. But even more importantly, when the pressure ceases, the AMR-conferring plasmids stick to the bacterial cell. As previously discussed, this is mostly owed to plasmid maintenance-ensuring systems but might be also influenced by favoring non-resistance factors on the AMR plasmids that are either i) co-selected on the plasmid and enhance virulence or other traits [251, 252] or

ii) affect chromosomally-encoded non-resistance features including virulence, metabolism and fitness. Possibly, ESBL-producing *E. coli* have been selected under high selective pressures in the human and animal clinics, from where they found their way into extra-clinical, environmental settings. Being "thrown" into a setting with moderate and/or low antimicrobial selection pressures, as demonstrated in studies that detected ESBL-producers in rather isolated areas [12, 77], some strains might have had to rely on features beyond resistance to adapt to challenging, even hostile environments. TA systems contribute their part in maintaining AMR plasmids in the bacterial host, thus possibly enabling the development of co-evolution or compensatory mutations. One of our recently published studies has demonstrated the occurrence of a sepsis-associated, clinically-relevant ESBL-producing *E. coli* clone of ST410 in diseased humans and dogs, environmental canine feces, and, surprisingly, wild birds [253]. This apparent success of a ST410 clone from phylogenetic group B1 in wildlife with supposed moderate or low antimicrobial selection pressures might be due to non-resistance, virulence-associated or adaption-benefiting factors. However, at this point I would like to mention that the humans influence across ecosystems [254, 255], including those arising from the use of antimicrobial substances, must not be underrated; we cannot conclude just how high the antimicrobial selection pressures in these systems actually are and should consider that even sublethal concentrations of antimicrobials may select and enrich for bacteria that carry AMR determinants [256].

To attribute the detected differences in biofilm formation and motility to chromosomal compensatory mutations seems rather unlikely. Or why would there have been differences in some PCV strains once the ESBL-plasmids were extracted from the host cells? In case of chromosomally-encoded compensatory mutations, an ESBL-plasmid loss would not have affected the host as directly.

The enhanced ability to form biofilms in some of our tested ESBL-plasmid-carrying strains is likely a clue in the puzzle to elucidate the mechanisms important to explain the pandemic success of these bacteria. A study from 2014 suggested that the transformation of UTI-associated *E. coli* with newly acquired AMR plasmids (ampicillin and kanamycin resistance and their combination) influences biofilm formation. Interestingly, dependent on the respective transformed AMR plasmids and the two strains, which differed in the combination of their VF equipment, biofilm formation was either increased or decreased [257]. This might give a hint toward the interpretation of our results, where both bacterial hosts and carried AMR plasmids showed greatly varying characteristics, perhaps explaining

the non-consistency in phenotypic assays among our different strains [17]. Classifying and typing bacteria, such as assigning STs, might allow a first rough categorization but each strain has its own specific chromosomal and episomal characteristics, be it in terms of AMR and virulence determinants or fitness and physiology. This sophisticated combination, maybe co-evolution, of chromosomal and episomal content might be one important explanation for the successful emergence of some international high risk multi-drug resistant clones and lineages.

In summary, I would like to suggest the following as the main underlying mechanisms: i) the occurrence of TA systems on AMR plasmids ensuring the plasmids' maintenance without antimicrobial selection pressures, ii) compensated fitness costs (although not investigated here but suggested in literature), and iii) virulence, adaption, and/or fitness benefits through plasmid-host interactions.

5. CONCLUSIONS AND OUTLOOK

- The successful construction of PCVs and Ts of *E. coli* WTs allowed the investigation of ESBL-plasmid-associated effects on the bacterial host. This epidemiologically valuable set of seven triplets can be used for further analyses, such as their performance in biological test systems, the verification of bioinformatics tools testing the reliability of bioinformatically extracted plasmid sequences from WGS data, and to elucidate how *E. coli* WTs colonize the human intestinal tract differently from their corresponding PCVs. The latter two approaches have been considered by different international research groups (unpublished data).

- The results detected in growth curves and Omnilog PM assays demonstrate that ESBL-plasmid-carriage did not lead to growth and metabolic costs in WT and T strains compared to the PCVs in laboratory conditions. This might apply to other *E. coli* strains and perhaps other bacterial genera, underlined by the diversity of strains and plasmids used in this thesis. Prospective investigations should, however, test these findings with more isolates of different origins and other bacteria with varying plasmids and, most importantly, consider biological test systems including animal infection models to prove the biological reliability of these results. Moreover, further studies should investigate the exact role of plasmid-host co-evolution, including compensatory mutations and plasmid domestication. Generally, hypothesis I ("The carriage of ESBL-plasmids in *E. coli* does not result in fitness-associated disadvantages for the bacterial host") is accepted.

- The results detected in biofilm and motility assays demonstrate that ESBL-plasmid-carriage leads to benefits for some bacterial strains of ESBL-producing *E. coli* of ST131 and ST648 beyond AMR in laboratory conditions. Whether these results apply broadly across *E. coli* strains and bacterial genera remains unanswered. To explain the pandemic success of international high-risk multi-drug resistant clones and lineages poses further difficulties. Most likely, some specific bacterial host and plasmid combinations are more successful than others, which should be explored in prospective studies that focus on the detailed characterization of the bacterial host's chromosome and their respective plasmids. In addition, large-scale studies should employ *in vitro* but also *in vivo* models. Generally, hypothesis II ("An interaction of ESBL-plasmids with the *E. coli* chromosome results in non-resistance factor-associated advantages for the bacterial host") is accepted for some strains.

- The results detected suggest that factors carried by the large ESBL-plasmids do influence certain bacterial host's chromosomally-encoded non-resistance features, such as biofilm formation and motility. In-depth, follow-on studies, including the manipulation of genetic contents through knock-outs and cloning can help identify and validate the exact factors responsible. Subsequent work must also explore so-called plasmid "cargo" genes, those that are suggested to be carried along with more important or essential genetic information, such as certain resistance factors.

6. SUMMARY

Extended-spectrum beta-lactamase (ESBL)-producing *Escherichia (E.) coli* occur frequently all over the world, not only in human and veterinary medicine clinical-associated contexts but also in wildlife and the environment, which are considered to be less affected by antimicrobial compounds. Specifically, international high-risk multi-drug resistant clones and lineages of ESBL-associated sequence type (ST) 131 and, to a lesser extent, 648 seem to be of importance. Their possession of ESBL- and non-resistance-factor-carrying plasmids should be more closely examined; the success of certain bacterial clones and lineages might not depend on antimicrobial resistance (AMR) alone. My thesis aimed at investigating the interaction of ESBL-plasmids with chromosomally-encoded, non-resistance features of ST131 and ST648 *E. coli*. This interaction is important as it might result in benefits for the bacterial host beyond those of AMR such as virulence, fitness and metabolism, yet presents no parallel fitness costs.

ESBL-plasmid-carrying wild-type (WT) *E. coli* strains were compared to a constructed, corresponding ESBL-plasmid "cured" variant (PCV) and to a complementary ESBL-carrying transformant (T) in fitness/metabolic assays, biofilm and motility assays, and RNA sequence analysis. No differences were observed in the strains' growth or metabolic behaviors. Some differed in biofilm and motility assays, however, exemplified by an enhanced curli and/or cellulose production and a reduced swimming capacity of WTs/Ts compared to the corresponding PCV. RNA sequencing mostly confirmed the phenotypic results on a transcriptomic level, revealing the chromosomally-encoded *csgD*-pathway to be a key factor.

The results clearly indicate that ESBL-plasmid carriage does not necessarily lead to a fitness/metabolic disadvantage for the bacterial host. On the contrary, the results suggest that an interaction of ESBL-plasmids with the bacterial host's chromosome in some strains, especially in terms of non-resistance-associated features, presumably contributes to the pandemic success of some isolates of ESBL-producing *E. coli* clones and lineages in various hosts and habitats, also beyond high antimicrobial selection pressures.

7. ZUSAMMENFASSUNG

Funktionelle Plasmidanalyse von ESBL-bildenden *Escherichia coli* der pandemischen Sequenztypen ST131 und ST648

Extended-spektrum beta-Laktamase (ESBL)-bildende *Escherichia (E.) coli* kommen häufig weltweit nicht nur in human- und veterinärmedizinisch klinisch-assoziierten Kontexten, sondern auch in Wildtieren und der Umwelt vor. Bei letzteren wird angenommen, dass diese durch antimikrobielle Wirkstoffe weit weniger beeinflusst sind. Besonders internationale Hoch-Risiko Klone und Linien des ESBL-assoziierten Sequenztyps (ST) 131 und in geringerem Maß 648 scheinen von Bedeutung zu sein. Ihre Trägerschaft von ESBL- und nicht-Resistenzfaktoren-tragenden Plasmiden sollte genauer untersucht werden, da der Erfolg von bestimmten bakteriellen Klonen und Linien nicht allein von einer antimikrobiellen Resistenz abhängig zu sein scheint. Meine Dissertation hatte insbesondere zum Ziel, die Interaktion von ESBL-Plasmiden mit chromosomal-kodierten Nichtresistenzfaktoren von ST131 und ST648 *E. coli* zu untersuchen. Bei dieser Interaktion außerhalb antimikrobieller Resistenzen handelt es sich zum Beispiel um Virulenz, Fitness und Metabolismus, welche in Vorteilen für den bakteriellen Wirt resultieren und keine parallelen Fitnesskosten verursachen.

Die ESBL-Plasmid-tragenden Wild-typ (WT) *E. coli* Stämme wurden mit einer konstruierten, korrespondierenden ESBL-Plasmid „gecurten“ Variante (PCV) und einer komplementären ESBL-tragenden Transformante (T) in Fitness/Metabolismus Assays, Biofilm- und Motilitätstests sowie in einer RNA Sequenzanalyse verglichen. Es wurden keine Unterschiede der Stämme bezüglich ihres Wachstums- oder metabolischen Verhaltens festgestellt, allerdings unterschieden sich einige in den Biofilm- und Motilitätstests, exemplifiziert durch eine erhöhte Curli und/oder Zellulose Bildung und einer reduzierten Schwimmkapazität der WTs/Ts verglichen zur korrespondierenden PCV. Die RNA Sequenzanalyse bestätigte zu großen Teilen die phänotypischen Ergebnisse auf Transkriptomebene und zeigte den chromosomal-kodierten *csgD*-Pfad als Schlüsselfaktor auf.

Die Resultate indizieren klar, dass eine ESBL-Plasmid Trägerschaft nicht notwendigerweise zu einem Fitness/Metabolismus Nachteil für den bakteriellen Wirt führt. Im Gegenteil, bei manchen Stämmen trägt eine Interaktion von ESBL-Plasmiden mit dem Chromosom des bakteriellen Wirts, speziell bezüglich Nichtresistenz-assoziierter Faktoren, vermutlich zum pandemischen Erfolg einiger Isolate von ESBL-produzierender *E. coli*

Klonen und Linien in unterschiedlichen Wirten und Habitaten bei, auch außerhalb eines antimikrobiellen Selektionsdrucks.

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11. SELBSTSTÄNDIGKEITSERKLÄRUNG

Hiermit bestätige ich, dass ich die vorliegende Arbeit selbständig angefertigt habe. Ich versichere, dass ich ausschließlich die angegebenen Quellen und Hilfen in Anspruch genommen habe.

Katharina Schaufler Berlin, 01.12.2016