Identifying molecular keys regulating phenotypic heterogeneity of *Stenotrophomonas maltophilia* K279a β-lactamase $bla_{L1}$ and $bla_{L2}$ gene expression.

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English language declaration

I have read and hereby declare that this doctoral dissertation "Identifying molecular keys regulating phenotypic heterogeneity of Stenotrophomonas maltophilia K279a β-lactamase $bla_{r,1}$ and $bla_{r,2}$ gene expression" is sufficient in terms of grammar and accuracy of language used. This dissertation is being presented to the University of Hamburg by Ebrahim Mama

Sincerely,

Hari Krishnan
Research Molecular Biologist
Publications

At time of submission, the results of this desertion have been published as:


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Summary

The Gram-negative bacterium *Stenotrophomonas maltophilia* is considered as an emerging pathogen. It is often associated with cystic fibrosis patients but can also be found in any environment such as soil, plants, water and healthy human individuals. The microorganism carries many genes coding for multiple antibiotic resistance mechanisms. Among these are the two β-lactamase genes *bla*$_{L1}$ and *bla*$_{L2}$ that are the major weapons to degrade β-lactam antibiotics. Within this thesis, I was especially interested in identifying non-genetic mechanisms interfering with the expression of the two β-lactamase genes. Thereby, I paid special attention to the phenomenon of phenotypic heterogeneous expression of the β-lactamase genes *bla*$_{L1}$ and *bla*$_{L2}$. Phenotypic heterogeneity is a widely described cell-to-cell variation in bacteria that enables clonal populations to adapt to changing environments including antibiotic therapy. With current antibiotic treatment strategy that bases itself on the traditional minimum inhibitory concentration, a subpopulation of bacteria escapes drug therapy and is implicated in recurrent infections. The underlying mechanism that modulates phenotypic heterogeneity is diverse and needs to be better understood to optimize antibiotic treatment strategies. Thus, the phenotypic responses of *S. maltophilia* K279a were studied first by exposing bacterial cells with variable levels of ampicillin. In an ampicillin challenged model, *S. maltophilia* K279a diverges into cellular subpopulations with distinct but reversible morphotypes of small and big colonies. To verify that the colony morphotypes were not caused by current mutations such as SNPs, the genotypes of 24 colony variants were sequenced and a significant number of SNPs and in/dels were identified. Remarkably, these mutations were not primarily associated with the bacterial resistome and also were not located in the genes essential for growth. When the transcriptomes of big and small colony variants that formed after β-lactam treatment and showed reversible phenotypes were assayed, 12 genes were identified as differentially expressed in big versus small colonies. Among the differentially expressed genes, *bla*$_{L1}$ and *bla*$_{L2}$ were 15.3- and 6.9-fold transcriptionally strongly up-regulated in big colony variant in comparison to cells forming small colonies. In subsequent studies, β-lactamase expression analysis at the single cell level using the promoter fusions *bla*$_{L1}$ and *bla*$_{L2}$ genes showed high levels of phenotypic heterogeneity in batch cultures. Noteworthy, individual cells within filaments (or aggregates) of exponentially growing cultures displayed an "ON" mode, while adjacent cells were in an "OFF" mode. A detailed statistical analysis of several hundred cells for each time point in a batch culture revealed that the majority of cells (95 %) were in the *bla*-OFF during 24 hours period. However, after 32 hours, the majority of cells expressed the red fluorescent protein and were in the *bla*-ON mode.
mode. This response was independent from the presence of ampicillin. Additionally, the addition of sterile-filtered *S. maltophilia* K279a supernatants strongly altered the levels of phenotypic heterogeneity of *bla*<sub>L2</sub> expression in exponential cultures. This response was highly reproducible and could be quenched by heat treatment of cell-free supernatants, suggesting that a heat-labile but yet unidentified factor involved in modulating heterogeneous *bla*<sub>L2</sub> expression at single cell level. To further uncover possible molecular switches determining heterogeneity, the transcription profiles on a genome wide level were analyzed from cells that grew for 27 hours and 32 hours using RNA-seq. Thereby, the *comE* homologue *smlt1134* and two putative transmembrane efflux genes (*smlt2851* and *smlt2852*) were found to be differentially expressed in homogenously versus heterogeneously *bla*<sub>L2</sub> expressing cells. Overexpression of *comE* homologue in *S. maltophilia* K279a reduced the level of cells that were in a *bla*<sub>L2</sub>-ON mode to 1% or lower. However, phenotypic heterogeneity was unaffected by overexpression of multidrug transporter proteins. Therefore, the data implied that the *ComE* homologue affected heterogeneous *bla*<sub>L2</sub> expression but its effect could be disrupted by unidentified signal molecules released into the medium. Furthermore, in the newly constructed SMK279aΔ*smlt3723* (*ampR*) mutant bacterial cells were unable to grow in the presence of ampicillin indicating that both basal and inducible expression of *bla*<sub>L1</sub> and *bla*<sub>L2</sub> depends on AmpR activatory ligand in *S. maltophilia*. While basal expression of β-lactamase led to a reduction in colony size, it was still sufficient to overcome antibiotic stress in the cells forming small colonies. Together with whole-genome sequence analyses of different colony morphotypes, the data presented in this study imply that phenotypic heterogeneity of *S. maltophilia* K279a is a result of mostly non-genetic variations in individual cells involving the gene products of *bla*<sub>L1</sub>, *bla*<sub>L2</sub> and *comE* homologue. Further, phenotypic heterogeneity is the key determinant affecting the expression and the functional outcomes of the β-lactam resistance and diverse genes involved in bacterial virulence, motility and adhesion, and biofilm formation. Altogether these findings indicate that phenotypic heterogeneity is an important non-genetic based property to enhance fitness of *S. maltophilia* K279a.
Zusammenfassung

Zusammenfassung

Zusammenfassung

24 Genome von unterschiedlichen Kolonie-Morphotypen, führen die ausgewerteten Daten der Studie zu der Schlussfolgerung, dass die phänotypische Heterogenität bei S. maltophilia aus überwiegend nicht genetischen Variationen in individuellen Zellen unter Beteiligung der Genprodukte von bla\(_{L1}\), bla\(_{L2}\) und comE-Homologen resultiert. Darüber hinaus stellte sich heraus, dass die phänotypische Heterogenität der entscheidende Faktor ist für die Expression und die Funktion der β-Laktam-Resistenz sowie diverser Gene, die an der bakteriellen Virulenz, Motilität, Adhäsion und der Biofilmbildung beteiligt sind. Zusammenfassend kann gesagt werden, dass phänotypische Heterogenität eine wichtige, nicht genetische Fähigkeit ist, welche die Fitness von S. maltophilia K279a steigert.
1 Introduction

1.1 Occurrence, phylogeny and virulence of *Stenotrophomonas maltophilia*

*Stenotrophomonas maltophilia* is a Gram-negative, rod-shaped ubiquitous Gammaproteobacterium which has been frequently isolated from immunocompromised patients, but also from a diverse range of environmental samples (Figure 1) (Brooke, 2012). It is motile aerobe adapted to grow in a range of temperature from 5°C to 40°C, generally with optima between 30°C to 37°C. *S. maltophilia* was first isolated in 1943 and named as *Bacterium bookeri*. Then it was renamed as *Pseudomonas maltophilia* (Hugh and Leifson, 1963) and later as *Xanthomonas maltophilia* (Swings *et al.*, 1983). Owing to the advent of advanced molecular and genomic characterization it was finally renamed as *Stenotrophomonas maltophilia* with its own genus name (Palleroni and Bradbury, 1993). In samples of environmental origin the strain has been commonly isolated with *Burkholderia* as both are resistant to the same spectrum of antibiotics used for screening. It has been implicated in nosocomial infections causing bacteremia, endocarditis, pneumonia, pyelonephritis, cellulitis, and meningitis among others (Elting and Bodey, 1990, Gutierrez Rodero *et al.*, 1996, Elsner *et al.*, 1997, Al-Hilali *et al.*, 2000, Agger *et al.*, 1986, Nguyen and Muder, 1994). These infections have been usually associated with high morbidity and mortality in immunocompromised patients (Denton and Kerr, 1998). On the other hand, the species has potential biotechnological applications in bioremediation, biocontrol, and production of secondary metabolites (Ryan *et al.*, 2009).

1.1.1 Virulence properties of *Stenotrophomonas maltophilia*

*S. maltophilia* is considered as emerging pathogen causing mainly respiratory and nosocomial infections notably among cystic fibrosis (CF) and intensive care patients. Treatment in these patients is also difficult due to the resistance of the bacteria to several of commercially available drugs such as cephalosporins, macrolides, fluoroquinolones, aminoglycosides, carbapenems, chloramphenicol, tetracyclines, polymyxins and aminoglycosides (Crossman *et al.*, 2008, Ryan *et al.*, 2009, Brooke, 2012). The genotypic and phenotypic adaptions that contributed to virulence of *S. maltophilia* includes efflux pumps, fimbriae, flagella, secretion systems, surface exopolysaccharides, proteases and other secreted enzymes, quorum sensing systems and the ability to form biofilms. Efflux pumps that mediate the extrusion of drugs and other toxic chemicals have been identified and characterized in *S.maltophilia*, including the well-known
Figure 1: Microscopic images of *S. maltophilia* isolates. Scanning electron microscope images of a patient isolate *S. maltophilia* K279a (A) and an environmental isolate CL5 (B). *S. maltophilia* K279a was isolated from the blood of a cancer patient while CL5 from soil samples taken in the Botanical garden of the University of Hamburg. Scanning electron micrographs were obtained after the strains were cultured overnight at 37°C in the absence of antibiotics. (C) A confocal microscopy of green fluorescent protein (GFP) tagged *S. maltophilia* on murine lung epithelial cell line, LA4, co-cultured for 16 h. Cells of *S. maltophilia* formed dense biofilm indicated with an arrow. Images were recorded as described in the Material and Methods section of this work. Murine lung epithelial cell line for (C) was obtained from Ulrich E. Schaible and colleagues at Research Center Borstel, Germany.
resistance-nodulation-cell-division family, the ATP binding cassette (ABC) family, the major facilitator superfamily (MFS) and a fusaric acid extrusion efflux pump (FuaABC) (Li et al., 2002, Lin et al., 2014, Crossman et al., 2008, Huang et al., 2013, Alonso and Martinez, 2000, Hu et al., 2012). *S. maltophilia* is capable of forming biofilms on abiotic surfaces as well as epithelial cells (Brooke, 2012). Bacterial biofilms have been found to protect the microbial communities from environmental stresses, are associated with the majority of hospital acquired infections and persistence in the host (Bjarnsholt, 2013, O’Toole et al., 2000, Wu et al., 2015a). In addition to being able to form biofilms (Figure 1C), which is common in human pathogenic bacteria, *S. maltophilia* produces various extracellular enzymes: protease, lipase, DNase, gelatinase and haemolysin (Thomas et al., 2014). In particular, the proteolytic activities of the protein StmPr1 on collagen, fibronectin, and fibrinogen can contribute to local tissue damage and pulmonary hemorrhage (Windhorst et al., 2002). Bacterial flagella also allow deeper colonization and dissemination into tissue and organs of the human body. The strain can adhere to inanimate and living objects and this is further enhanced by the presence of fimbriae. Many of these virulence factors are also modulated via the quorum sensing regulon.

### 1.1.2 Quorum sensing in *Stenotrophomonas*

The ultimate vital ability of living cells is to multiply and reproduce under challenging environmental conditions requiring coordination of group behavior from single cell to higher levels of kingdom. Bacteria achieve this by using small molecules called autoinducers, whose production increases as a response to changing environmental conditions and as a function of cell density. When the autoinducer production reaches a certain threshold value recognized by membrane-bound receptors, bacteria are able to synchronize gene expression at population level and regulate diverse cellular responses such as biofilm production and extracellular enzyme production.

Cell-to-cell signaling in *S. maltophilia* is mediated by the diffusible signal factor (DSF), a widely conserved quorum sensing (QS) signal in many Gram-negative bacterial species (Huedo et al., 2015, Zhou et al., 2015, Ryan et al., 2015). The DSF based QS is encoded by the rpf (regulation of pathogenicity factors) gene cluster that consists of the two component system RpfGC and a synthase RpFBF. The two component system encodes for a histidine kinase and response regulator that are involved in signal perception. The rpfF gene encodes for an enoyl coenzyme A hydratase, which is essential for synthesis of the main signal factor cis-2-11-methyl-dodecenoic acid. DSF synthesis also partly relies on the gene rpfB (fatty acyl-CoA ligase). This
cell-to-cell signaling system in *S. maltophilia* is known to regulate diverse cellular functions such as biofilm formation, motility and virulence (Figure 2).

**Figure 2: S. maltophilia QS system based on diffusible signal factor.** The QS genes are arrayed as *rpf* clusters consisting of *rpfB* (fatty acyl-CoA ligase), *rpfF* (enoyl coenzyme A hydratase), *rpfC* (histidine kinase) and *rpfG* (response regulator). The product of *rpfF* gene is mainly responsible for catalyzing the reaction leading to the synthesis of the main fatty acid cis-2-11-methyl-dodecenoic acid signal in a cell density dependent manner, a process that is regulated by the sensing of diffusible signal factor (DSF) by the two-component regulatory system-histidine kinase. The response regulator RpfG regulates the cellular content of cyclic-di-GMP and GMP by its HD-GYP domain, which is a cyclic di-GMP phosphodiesterase. Phosphorylation activates RpfG for cyclic di-GMP degradation and thereby the GMP is directly or indirectly influencing the transcription of genes. This cell-to-cell signaling system is known to regulate diverse cellular functions such as biofilm formation, motility and virulence.

*S. maltophilia* further encodes for proteins related to the Rax proteins of *Xanthomonas oryzae*, which is required for the synthesis of the Ax21 homologue protein (McCarthy *et al.*, 2011, Ferrer-Navarro *et al.*, 2013). The Ax21 protein in the rice pathogen *Xanthomonas oryzae* pv. *oryzae* regulates many genes involved in cell motility, biofilm formation and signal transduction.
Introduction

(Park et al., 2014). In the same strain, this Ax21 protein is secreted by the general secretion (Sec) system in association with outer membrane vesicles (OMVs) (Bahar et al., 2014). The Ax21 homologue in S. maltophilia is presumably involved in regulation of motility and biofilm formation though its exact role remains uncertain (McCarthy et al., 2011, Ferrer-Navarro et al., 2013).

1.2 Molecular aspects of β-lactam antibiotic resistance and regulation

β-lactams are a broad class of antibiotics that consist of a β-lactam ring and a variable acyl side chain. They interfere primarily with bacterial cell wall synthesis mainly by inversely inactivating penicillin binding proteins (PBP). As a consequence the growth and cell division of the organism is impaired. While they are still among vital antibiotics to modern day medicine, bacterial resistance is a major challenge facing antibiotic therapy. The use of β-lactam antibiotics to treat bacterial infections for several decades has led to an alarming increase in the frequency of human pathogens that do not respond to it. This includes several of medically important bacterial pathogens, such as species of Legionella, Mycobacterium, Acinetobacter, Pseudomonas, Stenotrophomonas and also many strains belonging to Enterobacteriaceae. Resistance is caused by several factors that arise from misuse of antibiotics, horizontal gene transfer and/or from a mutation (Shaikh et al., 2015, Alekshun and Levy, 2007). However, the production of a family of β-lactamases has emerged as a major mechanism of resistance to clinically important β-lactam antibiotics (Figure 3). These bacterial enzymes hydrolyze the β-lactam ring of the antibiotics rendering them inactive. In addition, other resistance mechanisms such as modification of PBP and a reduced transport of antibiotics due to modification of porin have resulted in resistant phenotypes to β-lactam antimicrobial agents (Figure 3).

β-lactamases are a diverse group of enzymes classified based on functional and molecular properties, a classification that was first proposed by Bush et al. and Almber et al., respectively (Bush et al., 1995, Bush and Jacoby, 2010, Ambler, 1980, Hall and Barlow, 2005). The functional classification scheme is based on physical and biochemical properties and groups the enzymes in correlation with their phenotype. The amino acid sequence homology is used to classify β-lactamases into the classes A, B, C, and D of the molecular classification scheme. Classes A, C and D utilize serine for β-lactam hydrolysis while class B is metalloenzymes. And finally, β-lactamases can be encoded on plasmid or chromosomal DNA.
Figure 3: Mechanisms of β-lactam resistance in Gram-negative bacteria. They involve the proteins β-lactamase, PBPs and efflux pumps. Shown above are the ribbon representation of the atomic structure of a *Klebsiella pneumoniae* β-lactamase and a *Pseudomonas aeruginosa* penicillin binding protein 3 and the tripartite drug efflux complex proteins. Genetically encoded β-lactamases are the major source of resistance that inactivates the β-lactams in clinically important bacteria. Reduced affinity to PBPs and the extrusion of antibiotics are also additional mechanisms conferring resistance. Figure modified after Wilke, *et al.* 2005 (Wilke *et al.*, 2005).

1.2.1 Regulation of β-lactamase expression in Gram-negative bacteria

Expression of β-lactamase in Gram-negative bacteria can be constitutive and/or inducible. Where it is inducible, it does not usually involve a single-step protein regulation. Several proteins are involved in regulation to ensure that the integrity of the cell wall is maintained upon challenge with β-lactam antibiotics. This process is further linked to the cell wall metabolism in that during each cell division cycle the cell has to constantly break the old peptidoglycan (PG) and synthesize a new PG (Zeng and Lin, 2013, Typas *et al.*, 2012). This multi-stage enzymatic activity is catalyzed by several enzymes, mainly involving transpeptidases for PG biosynthesis and lytic transglycosylases (LT) for PG recycling (Typas *et al.*, 2012). Most of the current knowledge on inducible β-lactamase production relies on studies conducted with strains belonging to Enterobacteriaceae and a few other genera. The following section summarizes the most widely described mechanism of induction: the AmpR–AmpC pathway (Figure 4).
**AmpR–AmpC Pathway**

In this pathway, muropetides, i.e. cell wall degradation products are transported to the cytosol via cytoplasmic inner membrane permease AmpG (Figure 4). They are processed by β-N-acetylglucosaminidase (NagZ) and an anhydro- N-acetylmuramyl-L-alanine amidase (AmpD) (Figure 4). One of the major intermediate products in the recycling of peptidoglycan precursor is UDP-MurNAc-pentapeptide. This product is either further processed and can be eventually transported to the periplasm where PG synthesis takes place or it binds to AmpR. AmpR is a LysR family transcriptional regulator that consists of a DNA binding motif and an effector binding domain. The motif has been more or less conserved across all bacteria whereas the effector binding domain is less conserved allowing binding flexibility.

*ampR* is transcribed in an opposite direction to the *ampC* gene which encodes for AmpC, the first bacterial enzyme discovered being able to destroy penicillin. AmpR binds to the intergenic region of *ampR* and *ampC* genes and regulates the expression of chromosomal β-lactamase in Gram-negative bacteria. Under the condition where bacterial cells are not challenged with β-lactam, AmpR maintains an inactive conformation owing to binding to certain effector molecules, such as UDP-MurNAc-pentapeptide (Figure 4). However, upon β-lactam challenge the actions of transpeptidases are blocked or slowed down while lytic transglycosylases are still actively degrading PG leading to increased accumulation of anhydro-MurNAc-oligopeptides. The anhydro-MurNAc-oligopeptides (1,6-anhydroMurNAc-tripeptide or pentapeptide) could displace the AmpR-associated UDP-MurNAc-pentapeptide, triggering conformational change of AmpR, and subsequently activating the transcription of *ampC* (Balcewich *et al.*, 2010, Dietz *et al.*, 1997, Jacobs *et al.*, 1997). It has been further suggested that free pentapeptide could also bring activation as loss of *dacB* (coding for PBP4) function could lead to hyper inducible phenotype (Moya *et al.*, 2009). PBP4 is a non-essential penicillin binding protein with peptidase activity and presumably cleaves the stem amino acid from anhydro-MurNAc-oligopeptides. The expression of inducible β-lactamase is gradually arrested as AmpR undergoes conformational change upon binding of UDP-MurNAc-pentapeptide and with reduction of the antibiotics owing to degradation by the produced β- lactamase (Vadlamani *et al.*, 2015).
Introduction

Figure 4: The AmpR–AmpC pathway of β-lactamase induction in Gram-negative bacteria.
Expression is controlled by the activities of several proteins including AmpG, NagZ, AmpD and the transcriptional regulator AmpR among others. Upon β-lactam challenge the actions of transpeptidases are blocked or slowed down while lytic transglycosylases are still actively degrading PG. This leads to accumulation of one of the major cell wall degradation products - anhydro-MurNAc-oligopeptides - in the cytosol. The anhydro-MurNAc-oligopeptides will then displace AmpR-associated UDP-MurNAc- pentapeptide. This leads to a conformational change of AmpR and a subsequent activation of the transcription of the β-lactam resistant genes. In this particular case, the β-lactamase is transported to the periplasm where it could inactive β-lactams. Source of protein structure (Drawz and Bonomo, 2010). Abbreviations: IM, inner
membrane; PP, periplasm; PG, peptidoglycan; OM, outer membrane; LT, lytic transglycosylases. See text for the detail.

1.2.2 β-lactamase of S. maltophilia K279a

The strain used for this work, S. maltophilia K279a (SMK279a), was isolated from a blood sample of a hospitalized patient and has a genome size of 4,851,126 bp (Crossman et al., 2008). Besides several other resistance genes, its genome encodes two types of β-lactamases designated L1 (blaL1) and L2 (blaL2). The product of blaL1 is an Ambler class B Zn²⁺-dependent metalloenzyme that hydrolyses all classes of β-lactams except the monobactams. The blaL2 encodes for an Ambler class A serine active site β-lactamase, an extended spectrum β-lactamase (ESBL), that inactivates many penicillin and cephalosporin antibiotics but is inhibited by clavulanic acid. Both enzymes are inducible with β-lactams and are controlled by the activities of AmpG, NagZ, AmpD, and the transcriptional regulator AmpR (Hu et al., 2008, Okazaki and Avison, 2008). A homologous ampR-blaL2 module based on an induction mechanism involving the AmpR–AmpC pathway has been identified earlier in S. maltophilia (Lin et al., 2009). In SMK279a, ampR is physically linked to blaL2 and it is one of the few cases that its product AmpR controls unlinked blaL1 expression. Further, it was suggested that the blaL2 and blaL1 genes are differentially regulated during induction, but ‘how’ was not investigated (Hu et al., 2008). In contrast, the AmpR type regulator is necessary for the induction of both, the blaL1 and blaL2 expression in S. maltophilia, yet blaL1 induction requires more activation of AmpR than blaL2 induction does (Okazaki and Avison, 2008).

1.3 Phenotypic heterogeneity and bacterial individuality

Bacteria utilize a diversity of mechanisms in natural environments for successful colonization and reproduction in various habitats. These mechanisms may include smaller genetic variations such as single nucleotide polymorphism (SNPs) and non-genetic variations. While the genetic background determines the ability of a bacterial strain to overcome stress, non-genetic, physiological and behavioral adaptations could also enable bacteria to respond and dwell in natural environments and in homogenous laboratory cultures. Non-genetic mechanisms may result in cell-to-cell variations, often known as bacterial individuality, in a population. They are essential for rapidly addressing the sudden encounter of stressful challenges. Phenotypic heterogeneity is therefore described as individual difference that existed in a clonal population cultivated in an otherwise homogenous environment. It is a common phenomenon in bacterial species and has been demonstrated in a number of model organisms such as Vibrio harveyi, Bacillus subtilis, Sinorhizobium fredii and others (Anetzberger et al., 2009, Deris et al., 2013,

Phenotypic heterogeneity may result from variations in gene expression at single cell level, periodic oscillations in cellular functions (Levine et al., 2013, Lenz and Søgaard-Andersen, 2011) or cell–cell interactions mediated by diffusible molecules or through physical contact between individual cells (Grote et al., 2014, Reuven and Eldar, 2011). It also includes phase variation, a reversible switching of phenotype that arises from a variation in the level of expression of one or more proteins between individual cells within isogenic populations. Phase variation occurs at higher frequencies than classical mutation rates and contributes to virulence by generating heterogeneity. Phenotypic heterogeneity allows division of labor and bet-hedging in the population in the same environment. This benefit of phenotypic heterogeneity is exemplified by Salmonella enterica subspecies 1 serovar Typhimurium (Sturm et al., 2011). ttss-1 expression at the single cell level was reported to be bistable with phenotype ttss-1 ON and ttss-1 OFF. Whereas ttss-1 ON mode results in reduced growth and triggers the immune response of the host, ttss-1 OFF phenotype allows cells to be disseminated to the target site of infection (Sturm et al., 2011). Consequently, phenotypic heterogeneity is a way of task sharing within bacterial populations to enhance the ability to adapt to changing environments.

Non-genetic individuality has been observed in seemingly isogenic populations of bacteria that have been exposed to a variety of antibiotics. A classic example includes the formation of persisting subpopulations that arise from an antibiotic sensitive population. The persister cells form a population of antibiotic resistant and susceptible phenotypes with re-introduction of the antibiotics. The persister phenotype depends on various factors such as the level of signaling nucleotide (p)ppGpp, and various metabolic activities (Maisonneuve et al., 2013). Thereby it is well accepted that toxin-antitoxin (TA) systems play a key role in the regulatory network of persisters. These systems consist of a ‘toxin’, which is normally a stable protein that interferes with vital cellular functions and a cognate ‘antitoxin’, an unstable protein or RNA molecule, which regulates the toxin level. The most prominent example for a TA system controlling persistence is the Escherichia coli hipAB TA system. However additional systems have been described and ranked in the order of their importance (Keren et al., 2004, Lewis, 2007, Wu et al., 2015b).

Other response of bacterial populations against antibiotics includes the phenomenon known as heteroresistance. This term describes heterogeneous response of bacterial populations exposed to antibiotics in a homogeneous environment as represented by laboratory culture conditions. A subpopulation usually has higher antibiotic tolerance than the rest of the population and is capable of growth. Heteroresistors are widely observed among both Gram positive and negative bacteria to an array of antibiotics that includes β-lactams, fluoroquinolones, fosfomycin, and
rifampicin (Morand and Mühlemann, 2007, Wang et al., 2014, Kao et al., 2014, Engel et al., 2013, El-Halfawy and Valvano, 2013). The mechanisms of heteroresistance are generally known to be diverse and might be caused by non-genetically or epigenetically controlled processes and/or acquired after initial exposure to antibiotics (El-Halfawy and Valvano, 2015).

1.4 Aims of the research

β-lactam antibiotics have been universally prescribed to treat bacterial infection and many bacteria secrete β-lactamase to resist the application of β-lactam in their system. In many bacterial species, it is known that in response to an exogenous stress (especially antibiotics), there is a subpopulation that displays cellular variations beneficial for survival despite the sensitivity to stress of the rest of the population. While this is essential for the survival and fitness of the population, the mechanism remains to be uncovered especially if the bacterial cells secrete an enzyme that allows growth within a certain drug range. The aim of this study was therefore to uncover novel mechanisms involved in β-lactam resistance of SMK279a at single cell level. The strain was challenged with β-lactam antibiotic and subsequently investigated for cellular changes using single cell technologies and deep sequencing of transcriptome. To investigate the link between bacterial features and colony morphotypes, transcriptomes samples were also prepared from bacterial colonies that were cultured on agar plates. Furthermore, the genotypes of the variants were investigated using next generation whole-genome sequencing (NGS) and finally the genes identified by deep sequencing were analyzed for their potential impact on single cell β-lactamase expression.
2 Material and Methods

2.1 Bacterial strains, vectors and primers

All the bacterial strains, plasmids, constructs and primers used in this study are summarized in Table 1 and Table 2. Oligonucleotide primers (Table 3) were synthesized by Eurofins Genomics based on the information provided by the manufacturer (Eurofins MWG GmbH, Ebensburg, Germany).

Table 1: Bacterial strains and their respective characteristics used for this study.

<table>
<thead>
<tr>
<th>Strain or construct</th>
<th>Description</th>
<th>Reference/source</th>
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<tbody>
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<td><strong>E. coli strains</strong></td>
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<td></td>
</tr>
<tr>
<td>HB101</td>
<td>F&lt;sup&gt;−&lt;/sup&gt;, hsdS20(r&lt;sub&gt;B&lt;/sub&gt;m&lt;sub&gt;B&lt;/sub&gt;−), recA13, ara14, proA2, lacY1, galK, 2, rpsL20 (Sm&lt;sup&gt;R&lt;/sup&gt;) xyl-5, mit-1, supE44, λ&lt;sup&gt;−&lt;/sup&gt;,</td>
<td>(Figurski and Helinski, 1979)</td>
</tr>
<tr>
<td>SY327</td>
<td>Δ(lac pro) argE(Am) recA56 rif&lt;sup&gt;R&lt;/sup&gt; nala λ pir</td>
<td>(Miller and Mekalanos, 1988)</td>
</tr>
<tr>
<td>DH5α</td>
<td>F&lt;sup&gt;−&lt;/sup&gt; Φ80lacZAM15 Δ(lacZYA-argF) U169 recA1 endA1 hsdR17(r&lt;sub&gt;K&lt;/sub&gt;− m&lt;sub&gt;K&lt;/sub&gt;−) phoA supE44 thi&lt;sup&gt;−&lt;/sup&gt;1 gyrA96 relA1 λ&lt;sup&gt;−&lt;/sup&gt;</td>
<td>(Hanahan, 1983)</td>
</tr>
<tr>
<td><strong>S. maltophilia strains</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SMK279a</td>
<td>Clinical isolate from the blood of a cancer patient</td>
<td>(Avison et al., 2000)</td>
</tr>
<tr>
<td>SMK279aΔsmlt1134</td>
<td>SMK279a lacking the smlt1134 gene, coding for a putative DNA transport competence protein</td>
<td>This study</td>
</tr>
<tr>
<td>SMK279aΔsmlt2851Δsmlt2852</td>
<td>SMK279a lacking the smlt2851 and smlt2852, genes coding for putative transmembrane efflux proteins</td>
<td>This study</td>
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<td>SMK279aΔsmlt3723</td>
<td>SMK279a lacking the ampR (smlt3723) gene; a putative HTH and LysR family transcriptional regulator</td>
<td>This study</td>
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<td>SMK279a carrying a deletion in ORF Smlt0387</td>
<td>This study</td>
</tr>
<tr>
<td>SMK279a EM2</td>
<td>SMK279a carrying pBBR1MCS-5::Pbla&lt;sub&gt;2&lt;/sub&gt;::rfp</td>
<td>This study</td>
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<tr>
<td>SMK279a EM3</td>
<td>SMK279a carrying pBBR1MCS-5::Pbla&lt;sub&gt;1&lt;/sub&gt;::rfp</td>
<td>This study</td>
</tr>
<tr>
<td>SMK279a EM4</td>
<td>SMK279a carrying pBBR1MCS-5::Pno::rfp</td>
<td>This study</td>
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<tr>
<td>SMK279a EM5</td>
<td>SMK279a carrying pBBR1MCS-5::Pbla&lt;sub&gt;2&lt;/sub&gt;::cfp</td>
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<td>SMK279a EM7</td>
<td>SMK279a carrying pBBR1MCS-5::Pbla&lt;sub&gt;2&lt;/sub&gt;::rfp</td>
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<tr>
<td>SMK279a EM8</td>
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<tr>
<td>SMK279a EM9</td>
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<tr>
<td>SM-GFP</td>
<td>GFP chromosomal insert</td>
<td>Research Center Borstel Jörg Steinmann (Universitätsklinikum Essen)</td>
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<td>CF148</td>
<td>Clinical isolate from the respiratory tract of a cystic fibrosis patient</td>
<td>Leibniz institute DSMZ</td>
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<td>DSM-50170</td>
<td>Reference strain from the oropharyngeal region of a patient with cancer</td>
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<td><strong>Sinorhizobium fredii NGR234</strong></td>
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<td>R. fredii NGR234</td>
<td>Wild-type strain, Rf&lt;sup&gt;+&lt;/sup&gt;</td>
<td>(Trinick, 1980)</td>
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<td>R. fredii NGR234</td>
<td>NGR234 PrpoD::rfp promoter fusion in pBBR1MCS-5</td>
<td>(Grote et al., 2014)</td>
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<td><strong>X. campestris</strong></td>
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<tr>
<td>X. campestris 8004</td>
<td>Wild type, Rf&lt;sup&gt;−&lt;/sup&gt;</td>
<td>(Turner et al., 1984)</td>
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**Material and Methods**

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<th>Plasmids or construct</th>
<th>Description</th>
<th>Reference /source</th>
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<tr>
<td>pTZ19R</td>
<td>High copy cloning vector, Cm&lt;sup&gt;+&lt;/sup&gt;</td>
<td>Fermentas (St. Leon-Rot, Germany)</td>
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<td>pTZ19R:: bla&lt;sub&gt;1&lt;/sub&gt;</td>
<td>bla&lt;sub&gt;1&lt;/sub&gt; encoding for Zn&lt;sup&gt;2+&lt;/sup&gt;-dependent β-lactamase cloned in pTZ19R under T7 promoter</td>
<td>This study</td>
</tr>
<tr>
<td>pTZ19R:: bla&lt;sub&gt;2&lt;/sub&gt;</td>
<td>bla&lt;sub&gt;2&lt;/sub&gt; encoding for serine β-lactamase cloned in pTZ19R under T7 promoter</td>
<td>This study</td>
</tr>
<tr>
<td>pRK2013</td>
<td>Kan&lt;sup&gt;R&lt;/sup&gt;; RK2-derived helper plasmid carrying the tra and mob genes for mobilization of plasmids containing oriT</td>
<td>(Figurski and Helinski, 1979)</td>
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<td>pBBR1MCS-5</td>
<td>Broad host range vector, low copy, Gm&lt;sup&gt;+&lt;/sup&gt;</td>
<td>(Kovach et al., 1995)</td>
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<td>pBBR1MCS-5 carrying the rfp gene in the MCS</td>
<td>This study</td>
</tr>
<tr>
<td>pBBR1MCS-5:: Pbla&lt;sub&gt;1&lt;/sub&gt;:: rfp</td>
<td>Pbla&lt;sub&gt;1&lt;/sub&gt;:: rfp reporter fusion in pBBR1MCS-5</td>
<td>This study</td>
</tr>
<tr>
<td>pBBR1MCS-5:: Pbla&lt;sub&gt;2&lt;/sub&gt;:: rfp</td>
<td>Pbla&lt;sub&gt;2&lt;/sub&gt;:: rfp reporter fusion in pBBR1MCS-5</td>
<td>This study</td>
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<td>pBBR1MCS-5:: Pbla&lt;sub&gt;2&lt;/sub&gt;:: yfp</td>
<td>Pbla&lt;sub&gt;2&lt;/sub&gt;:: yfp reporter fusion in pBBR1MCS-5</td>
<td>This study</td>
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<td>pBBR1MCS-5:: Pbla&lt;sub&gt;2&lt;/sub&gt;:: cfp</td>
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<td>Promoterless rfp reporter fusion in pBBR1MCS-5</td>
<td>This study</td>
</tr>
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<td>pBBR1MCS-5:: Pbla&lt;sub&gt;2&lt;/sub&gt;:: rfp</td>
<td>Pbla&lt;sub&gt;2&lt;/sub&gt;:: rfp reporter fusion constructed with the ampR-bla&lt;sub&gt;2&lt;/sub&gt; intergenic region in pBBR1MCS-5</td>
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<td>pBBR1MCS-5:: smlt1134</td>
<td>smlt1134 gene under its native promoter cloned into the pBBR1MCS-5:: rfp reporter fusion</td>
<td>This study</td>
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<tr>
<td>pBBR1MCS-5:: Pbla&lt;sub&gt;2&lt;/sub&gt;:: rfp:: smlt1134</td>
<td>Putative operon of smlt2851 and smlt2852 under its native promoter cloned into the pBBR1MCS-5:: Pbla&lt;sub&gt;2&lt;/sub&gt;:: rfp reporter fusion</td>
<td>This study</td>
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<tr>
<td>pEX18Tc</td>
<td>sacBoriT, Tc&lt;sup&gt;+&lt;/sup&gt;, gene replacement vector</td>
<td>(Hoang et al., 1998)</td>
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<td>pEX18Tc:: Δ0387</td>
<td>pEX18Tc vector with a 950-bp DNA insert of SMK279a containing the flanking region upstream of smlt0387 Cm&lt;sup&gt;R&lt;/sup&gt;, Tp&lt;sup&gt;R&lt;/sup&gt;; mobilizable suicide vector; carries the R6K&lt;sub&gt;y&lt;/sub&gt; origin of replication, the I-Scel recognition site and a xylE reporter gene</td>
<td>This study</td>
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<td>pGPI-Scel-XCm</td>
<td></td>
<td>(Hamad et al., 2010)</td>
</tr>
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<td>pDAI-Scel-SacB</td>
<td>Te&lt;sup&gt;R&lt;/sup&gt;; mobilizable broad host range plasmid; carries the gene for the l-Scel homing endonuclease and the sacB gene</td>
<td>(Hamad et al., 2010, Flannagan et al., 2008)</td>
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<td>pUDK011</td>
<td>PGI-Scel-XCm with a 705-bp SphI/KpnI insert of SMK279a containing the flanking region upstream of smlt1134</td>
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Table 3: Primers used in this study.

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<tr>
<td>Smil3722Rev</td>
<td>CGATGAACTTCGAGCATCGCGGTCTCCTG</td>
</tr>
<tr>
<td>3722rflpFor</td>
<td>CTAGGTACCATCGCGAGTCTG</td>
</tr>
<tr>
<td>3722rflpRev</td>
<td>CTAGGTACCATCGCGAGTCTG</td>
</tr>
<tr>
<td>3722rflp_For</td>
<td>CTAGGTACCCTGCGAGTCTG</td>
</tr>
<tr>
<td>3722rflp_rev</td>
<td>CTAGGTACCCTGCGAGTCTG</td>
</tr>
<tr>
<td>0387_Up_F</td>
<td>CCGAATTCTCGAACAGAGCTGCTG</td>
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<td>0387_Up_R</td>
<td>GAGGACATCTTACGCGGAGCTGCTTACT</td>
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<td>0387_Down_F</td>
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</tr>
<tr>
<td>0387_Down_R</td>
<td>CCAGAAGCTTCACGCGGCTGCTG</td>
</tr>
<tr>
<td>Del_Ch_O_for</td>
<td>CGCTGCTGCAACACATAAG</td>
</tr>
<tr>
<td>Del_Ch_O_Rev</td>
<td>TGTCGAGGCACACCTACG</td>
</tr>
<tr>
<td>Del_Ch_I_for</td>
<td>AAGACCGACGTCGATGGCAT</td>
</tr>
<tr>
<td>Del_Ch_I_Rev</td>
<td>ATGTCGACGCGCTGCTT</td>
</tr>
<tr>
<td>Down_Flank_0387</td>
<td>GGCCTGACTCAACCGTGTTGAA</td>
</tr>
<tr>
<td>Up_flnk_0387</td>
<td>GGCATTGGCAATCGACGGCACTTC</td>
</tr>
<tr>
<td>pex18Tc_for</td>
<td>TCTTGAGTGGTGAATCCCTTT</td>
</tr>
<tr>
<td>pex18Tc_rev</td>
<td>TTGCCTAAGCGGACTGCTGCTT</td>
</tr>
</tbody>
</table>

2.2 Culture media, supplements and solutions

All culture media and heat stable supplements were prepared and autoclaved at 121°C for 20 min according to the manufacturer’s recommendations (Table 4). Then, the media were cooled to 56°C and supplemented with antibiotics or other heat sensitive supplements that were sterile filtered (Rotilabo®-Spritzenfilter (CME, sterile, 0.22 μm), Roth, Karlsruhe, Germany). Prior to autoclaving 1.5 % (w/v) of agar was added into solid culture media. All culture media, solid or liquid was stored at 4°C until used.

Table 4: Antibiotics and supplements used in this study.

<table>
<thead>
<tr>
<th>Antibiotic/supplement</th>
<th>Stock solution (mg/ml)</th>
<th>Final concentration (µg/ml)</th>
<th>Solvent</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>E. coli</td>
<td>S. maltophilia</td>
</tr>
<tr>
<td>Ampicillin</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>Cefuroxime</td>
<td>50</td>
<td>50</td>
<td>45</td>
</tr>
<tr>
<td>Meropenem</td>
<td>4</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td>Gentamicin</td>
<td>10 or 50</td>
<td>10</td>
<td>50</td>
</tr>
<tr>
<td>Kanamycin</td>
<td>25</td>
<td>25</td>
<td>25</td>
</tr>
<tr>
<td>Rifampicin</td>
<td>50</td>
<td>50</td>
<td>50</td>
</tr>
<tr>
<td>Chloramphenicol</td>
<td>25</td>
<td>25</td>
<td>25</td>
</tr>
<tr>
<td>X-Gal</td>
<td>50</td>
<td>50</td>
<td>50</td>
</tr>
<tr>
<td>IPTG</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>Cis -11-2-dodecenoic</td>
<td>(94.2 mM)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
2.3 Cultivation and maintenance of bacterial cultures

Strains of bacteria were cultured with respective growth media under aerobic conditions. Pre-cultures of bacterial strains were made either by streaking out some material of the strain on agar plates or in liquid cultures in Erlenmeyer flasks, test tubes or 96-well microtiter plates and were used accordingly.

Cultures containing *E. coli* cells were inoculated into LB medium or agar plates supplemented with appropriate antibiotics based on the antibiotics cassette of the plasmid and were grown overnight at 37°C. Cultures were agitated constantly at 120 to 200 rpm when grown in liquid media. The plant pathogen *X. campestris* 8004 (Xcc) was grown in nutrient yeast glycerol (NYGB) medium (bacteriological peptone, 5 g/l; yeast extract (Difco), 3 g/l; and glycerol, 20 g/l) at 30°C supplemented with rifampicin (50 µg/ml). *R. fredii* NGR234 was cultivated at 30°C in TY medium (tryptone, 5 g/l; yeast extract, 3 g/l; and CaCl$_2$, 5 mM) supplemented with rifampin (25 g/ml). All strains of *S. maltophilia* were cultured in LB-Lennox medium (tryptone, 10 g/l; yeast extract, 5 g/l; and NaCl, 5 g/l) and incubated at 37°C or 30°C. When required, ampicillin (100 µg/ml) was used as selective pressure for growing pre-cultures of *S. maltophilia*. For single cell gene expression analysis, cultures of SMK279a carrying the reporter plasmid were supplemented with gentamicin (50 µg/ml).

Growth of bacterial cells in liquid cultures was determined by optical density (OD) measurement with an Eppendorf BioPhotometer (Eppendorf, Hamburg, Germany). The OD was measured at a wavelength of 600 nm (OD$_{600}$) using disposable cuvettes (10x4x45 mm, Sarstedt, Nuembrecht, Germany) with a path length of 1 cm. Cell cultures were diluted to ensure the OD of 0.8 was not exceeded. Pure medium was used as reference.

Bacterial cultures were maintained on agar plates for day-to-day routine procedures. Long term storage was carried out by mixing aliquot overnight cultures in 1:1 proportion with 87 % sterile glycerol in screw-cap tubes and stored at -70°C.

2.4 Determination of minimum inhibitory concentration

The minimum inhibitory concentrations (MICs) were analyzed using microdilution technique following the European Committee on Antimicrobial Susceptibility Testing (EUCAST) standard guidelines (European Committee for Antimicrobial Susceptibility Testing of the European Society of Clinical and Infectious, 2003). First, the stock antibiotics solution was prepared as 2-fold maximum concentration desired in the test. Then, overnight cultures of bacteria were diluted in fresh LB to a cell density of $10^4$ to $10^5$ CFU/ml. Using the multipipettor, 100 µl of fresh LB were
pipetted into 96-well microtiter plate. From the 2-fold antibiotic solution, 100 µl was pipetted into all wells in the far-left column (A1-A8). Beginning from far-left column, the concentration of antibiotics decreased with a dilution factor of 1:2 by gradually transferring 100 µl to the next subsequent row and again repeating the same except for all wells in the far-right column (L1-L8). The far-right column (L1-L8) was used as the control. Few microliters (usually 5 µl) of diluted bacterial cultures were added into all wells except the controls and incubated at desired temperature. The optical density was measured at 600 nm after 24 h of growth with Microplate Reader Synergy HT (BioTek, Winooski, Vermont, USA).

2.5 Working with DNA

2.5.1 DNA isolation

A standard technique for extraction of genomic DNA was performed according to the manufacturer’s recommendation. Bacterial cultures were incubated overnight in 5 ml test tubes at 37°C. The cells were pelleted at an OD$_{600}$ of about 1.0 and DNA was isolated with the Aqua Pure Genomic DNA Kit (Bio-Rad Laboratories). For isolation of plasmid DNA with kits, overnight suspensions of bacterial cultures were centrifuged and pelleted from 1 to 5 ml of the cultures. Highly pure plasmids were obtained via High-Speed Plasmid Mini Kit according to the manufacturer’s instructions (Avegene life science, Taipei, Taiwan, China) and eluted in 30 to 50 µl H$_2$O bidest.

Furthermore, plasmid DNA was isolated using alkaline cell lysis method. In brief, 1 to 5 ml of overnight culture was centrifuged in an Eppendorf Cup (E-cup) for 30 sec and the supernatant was discarded. The remaining media was removed by carefully inverting the E-cup on tissue paper to prevent the loss of pellet. The pellet was suspended in 100 µl of P1 buffer and 200 µl of P2 buffer were added and mixed by inverting the tube five to ten times. The mixture was allowed to stand for up to 3 min at room temperature (RT). Then, 200 µl of chloroform were added and mixed well. In order to precipitate proteins, 150 µl of P3 buffer were added and the E-cup was inverted five times and incubated for a min at RT. The mixture was then centrifuged at high speed for 3 min and approximately 500 µl upper phases was transferred to new sterile E-cups. The plasmid DNA was precipitated with 1 ml of ice cold 96 % EtOH and incubated for 30 min at -20°C or for 10 min at -70°C. The plasmid DNA was sedimented by centrifugation at 13,000 rpm, 4°C for 20 min in a centrifuge type 5417R (Eppendorf, Hamburg, Germany). The supernatant was discarded and the pellet was washed using 0.5 ml of ice cold 70 % EtOH. The sample was centrifuged for 2 min (13,000 rpm, 4°C) and the supernatant was removed. After repeating the
washing step, the DNA pellet was dried at 50°C and re-suspended in 20 to 50 μl H₂O bidest and analyzed by agarose gel electrophoresis. Details of buffer preparation for isolation of plasmid DNA is indicated below.

### P1 buffer (sterile filtered)
- **Tris-HCl**: 1.21 g (100 mM)
- **EDTA**: 0.74 g (20 mM)
- **RNase**: 100 μg/ml
- **H₂O bidest**: ad 200 ml

The pH was adjusted to 8.0.

### P2 buffer (sterile filtered)
- **NaOH**: 2.0 g (200 mM)
- **SDS**: 2.0 g (1 % (w/v))
- **H₂O bidest**: ad 200 ml

### P3 buffer (sterile filtered)
- **K-acetate**: 62.73 g (3 M)
- **H₂O bidest**: ad 200 ml

pH 5.5 (adjusted with acetic acid)

All solutions were sterilized by filtration before use. Buffer P1 was stored at 4°C.

### 2.5.2 DNA extraction from agarose gels

In the case, pure DNA of a defined fragment size needs to be eluted from an agarose gel, the desired DNA fragment was excised from the agarose gel followed by purification and concentration of the DNA. Extraction of DNA fragments from an agarose gel was performed after PCR or digestion and subsequent electrophoresis (see Material and Methods, section 2.5.3) using the “Gel/PCR DNA Fragments Extraction kit” (Avegene Life Science, Taipei, Taiwan, China) according to the manufacturer’s instructions. The DNA fragments were eluted with up to 30 μl H₂O bidest. The concentration of small volumes of DNA solutions was carried out in a vacuum concentrator (Concentrator 5301, Eppendorf, Hamburg, Germany) at 45°C for up to 5 min.

The amount and quality of DNA were also estimated with an Eppendorf BioPhotometer or microplate reader employing the Gen 5 software. In the Eppendorf BioPhotometer, the DNA concentration was measured in a disposable micro UV cuvette (Plastibrand®, Brand, Wertheim, Germany) at 260 nm against H₂O bidest as a reference. Alternatively, 2 μl of the extracted DNA was pipetted in the microplate reader and, DNA concentration was measured at 260 nm with Gen5 Software against pure H₂O bidest as a reference. An OD of 1.0 corresponds to 50 μg/ml of double-stranded DNA (Green and Sambrook, 2012). The purity was determined by calculating the ratio of the extinction values at 260 and 280 nm. Pure DNA solutions have a ratio OD : OD of 1.8 to 2.0 (Green and Sambrook, 2012).
2.5.3 Agarose gel electrophoresis for DNA

Agarose gel electrophoresis was done in order to analyze the quantity, size and purity of DNA. Samples were mixed with 1/10 volume of loading dye and loaded on the gel and a current was applied with an electrophoresis power supply EPS 301 (Amersham Biosciences, USA) in 100 Volt for 35-90 min in an electrophoresis gel chamber (Hoefer™ HE-33 mini horizontal submarine unit, Amersham Biosciences, USA) filled with 1 x TAE buffer. The negatively charged DNA moves through the 0.8-2 % agarose gels, with a speed according to the molecular size. Gels were stained for 5 to 15 min in an ethidium bromide solution bath (10 µg/ml) and distained in water to remove excess ethidium bromide. Visualization and documentation of nucleic acids was carried out using UV light in a gel documentation device (Bio-Rad Laboratories, Munich Germany). The gel was documented with Quantity One 1-D analysis software (version 4.6.9, BioRad, Munich, Germany). The size of the DNA fragments was estimated by comparison with marker bands between 100 bp and 10 kb of a GeneRuler™ 1 kb DNA Ladder (Fermentas, St. Leon-Rot, Germany).

---

50x TAE buffer

- **Tris-Ac**: 2 M
- **EDTA (pH 8.0)**: 100 mM
- **H₂O<sub>bident</sub>** ad 1000 ml
- **pH 8.1 (adjusted with acetic acid)**

Loading dye

- **Glycerol (30 %)**: 60 ml
- **EDTA (pH 8.0)**: 50 mM
- **Bromophenol blue (0.25 %)**: 0.5 g
- **Xylencyanol (0.25 %)**: 0.5 g
- **H₂O<sub>bident</sub>** ad 200 ml

---

2.6 Molecular cloning

Type II restriction endonucleases were used to digest plasmids or DNA fragments. All reaction conditions were set according to the manufactures recommendations (Thermo Scientific, Karlsruhe, Germany). Two reactions volumes were generally used based on intended purpose, i.e. either for analytical (10 µl) or preparative analysis (50 µl) (Table 5). The reaction mixtures were mixed by pipetting up and down and spun briefly before incubation at recommended temperature. The reaction time was 1-3 h for the analytical reaction and 3 h to overnight for preparative reaction. When deemed necessary, the mixture was inactivated at 65°C or 80°C for 20 min. Self-ligation of vector was minimized by removing the 5´-end phosphate groups of enzymatically linearized plasmids or vectors with Antarctic phosphatase (New England Biolabs, Frankfurt am Main, Germany) as per the manufacturer’s instructions. For restriction enzyme-based cloning, ligation of restriction digested DNA fragments into vectors was accomplished with T4 DNA ligase as recommend by manufacture’s (Thermo Scientific, Karlsruhe, Germany). PCR fragments obtained after amplification with DNA polymerase were directly ligated into
commercially available vector pDrive (QIAGEN® PCR Cloning Kit, QIAGEN, Hilden, Germany). For TA cloning of the blunt end PCR fragments, A-tailings were introduced to 3’-ends with Taq polymerase. The reaction was set at 70°C for 30 min.

**Table 5:** Typical reaction mixes for the digestion of the DNA template using Type II restriction endonucleases.

<table>
<thead>
<tr>
<th>Reactions mixes</th>
<th>Analytical reaction</th>
<th>Preparative reaction</th>
</tr>
</thead>
<tbody>
<tr>
<td>DNA (template)</td>
<td>0.25-1 µg</td>
<td>1-5 µg</td>
</tr>
<tr>
<td>Restriction buffer (10 x)</td>
<td>1 µl</td>
<td>5 µl</td>
</tr>
<tr>
<td>Restriction enzyme (10 U/µl)</td>
<td>0.5 µl</td>
<td>1 µl</td>
</tr>
<tr>
<td>H_2O bidest</td>
<td>ad 10 µl</td>
<td>ad 50 µl</td>
</tr>
</tbody>
</table>

### 2.7 Polymerase chain reaction

The polymerase chain reaction (PCR) was used for amplification of DNA fragments and was carried out with a Mastercycler personal (Eppendorf Hamburg, Germany), using plasmid DNA, gDNA or PCR products as template. The standard PCR was carried out with the *Pfu* polymerase that produced blunt end PCR products due to the 3’-5’ – exonuclease activity with a very low error rate. Primers used for PCR amplification are listed briefly in Table 3. The primer annealing temperature was calculated based on melting temperature of the primer pair; the lower T_m value from primer pair was used. The melting temperature T_m was calculated according to Chester and Marshak (1993).

\[
T_m \, (^{\circ}C) = 69.3 + 0.41 \times (% \text{ GC}) - (650 / \text{bp}_{\text{Primer}})
\]

\[
T_{\text{ann}} \, (^{\circ}C) = T_m - 5^\circ C
\]

% GC = percentage content of bases guanine and cytosine in primer sequence

bp_{Primer} = base pairs, length of primer

The elongation time was chosen based on the length of the target DNA sequence and considering the elongation efficiency of 500 or 1000 bp per min of the *Pfu* polymerase or *Taq* polymerase, respectively. PCR products which were amplified using the *Pfu* polymerase (Thermo Scientific, Karlsruhe, Germany) were used for further subcloning while PCR products amplified using the *Taq* polymerase (Thermo Scientific, Karlsruhe, Germany) were used as a first control of ligations or for direct cloning into the pDrive vector. Volumes and conditions for the standard reaction are presented below and in Table 6. The PCR reaction was pipetted on ice.
Material and Methods

Standard PCR reaction (50 µl)

DNA (template) 1 µl (corresponding to ~ 1 µg)

_Pfu_ buffer (10 x) 5 µl

dNTPs (10 mM) 1 µl

Primer forward (10 µM) 0.5 µl

Primer reverse (10 µM) 0.5 µl

DMSO 2 µl

_Pfu_ or _Taq_ polymerase 1 µl (corresponding to 2.5 U)

H₂O<sub>bidest</sub> ad 50 µl

Table 6: Thermal cycling conditions for PCR amplification using _Pfu_ DNA Polymerase or _Taq_ DNA Polymerase.

<table>
<thead>
<tr>
<th>Steps</th>
<th>Temperatures</th>
<th>Time</th>
<th>Number of cycles</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial denaturation</td>
<td>95°C</td>
<td>5-10 min</td>
<td>One cycle</td>
</tr>
<tr>
<td>Denaturation</td>
<td>95°C</td>
<td>1 min</td>
<td></td>
</tr>
<tr>
<td>Annealing</td>
<td>_T&lt;sub&gt;m&lt;/sub&gt; – 5°C</td>
<td>variable</td>
<td>30 to 35 cycles</td>
</tr>
<tr>
<td>Extension</td>
<td>72°C</td>
<td>2 min/kb or 1 min/kb*</td>
<td></td>
</tr>
<tr>
<td>Final extension</td>
<td>72°C</td>
<td>5-10 min</td>
<td></td>
</tr>
</tbody>
</table>

*Two min per kb for _Pfu_ DNA Polymerase and one min per kb for _Taq_ DNA Polymerase

2.7.1 Direct colony PCR

Direct colony PCR was performed for verification of positive clones with the correct inserts using colonies grown on agar plates. The colonies were picked and suspended in 20 µl of a master mix and therefore serving as DNA templates for PCR amplification with _Taq_ polymerase. Volumes and conditions for the direct colony PCR reaction are as follows:

Direct colony reaction (master mix, 200 µl)

_Taq_ buffer (10x) 20 µl

dNTPs (10 mM) 4 µl

Primer forward (10 µM) 2 µl

Primer reverse (10 µM) 2 µl

_Taq_ polymerase 2 µl

H₂O<sub>bidest</sub> ad 200 µl

The direct colony PCR reactions were prepared and handled on ice until DNA templates (colonies) were added. The PCR temperature conditions are summarized in Table 6. The calculation of _T<sub>m</sub>_ was carried out as described in the section 2.7, Material and Methods.
2.8 DNA transfer techniques

Heat shock and triparental mating were accomplished to transfer the foreign DNA into competent *E. coli* strains or *S. maltophilia* cells. The preparation of heat competent *E. coli* DH5α cell was done according to as previously described (Green and Sambrook, 2012).

2.8.1 Heat shock transformation of *E. coli*

An aliquot of competent *E. coli* DH5α cells was thawed on ice for 5 min. Subsequently the cells were mixed with 5-10 µl ligation reaction mixture (approx 0.1 µg of DNA) by gently tapping the E-cups and incubated on ice for 30 min. The heat shock was performed by incubating the cells at 42°C for 60 sec. After incubation on ice for 5 min, 500 µl of fresh LB medium without antibiotics were added to the mixture and further incubated at 37°C for 30 to 45 min. Afterwards, transformed cells were plated on LB agar plates containing the desired antibiotics and incubated at 37°C.

2.8.2 Conjugation of *S. maltophilia* cells

Triparental mating is used to transform *S. maltophilia* cells. It involves a helper strain (*E. coli* HB101 with pRK2013), a donor strain (*E. coli* DH5α carrying the reporter fusion plasmid) and a recipient strain (SMK279a). All three strains were cultured overnight in LB supplemented with the appropriate antibiotics at 37°C. Cells were pelleted from 1.5 ml bacterial culture and washed twice with fresh LB to remove the antibiotics. The pellet was suspended in 150 µl LB (1/10 volume). The three strains were mixed in the ratio of 3:1:1, (recipient strain: donor strain: helper strain). The mixed culture was spot-plated (65 µl /spot) overnight on LB agar plates without antibiotics at 37°C. To get rid of the ex-conjugants, a serially diluted aliquot were plated on agar plate containing gentamicin (75 µg/ml) and tetracycline (4 µg/ml) and incubated for 3-5 days at 37°C.

2.9 Methods for investigating phenotypic heterogeneity in *S. maltophilia*

2.9.1 Colony morphology heterogeneity on agar plates

For investigation of colony heterogeneity, parent strain SMK279a was cultured overnight in 5 ml test tubes at 37°C with constant agitation at 200 rpm. The occurrence of colony morphology variants in an isogenic bacterial population was investigated by plating aliquots of diluted overnight culture on LB agar plates with or without ampicillin (100 µg/ml). The plates were incubated at 37°C for 48 h. To further investigate if bacterial cells in the single colony types
formed colony heterogeneity in the transfer cultures, serially diluted aliquots were spread on LB agar plates with/without ampicillin (100 µg/ml).

2.9.2 Scanning electron microscopy (SEM)

Bacterial cells were grown overnight in 5 ml LB broth or on LB agar plates for 48 h. Cell suspension was centrifuged at 13,000 rpm for 2 min. The pellets or the colonies were resuspended in 500 µl sterile PBS-buffer and transferred into little cups containing cigarette paper. Cells were fixed in 1 % paraformaldehyde and 0.25 % glutaraldehyde and dehydrated gradually after successive immersions in ethanol solutions of increasing concentrations (30, 50, 70, 90 and 96 %). Each rinsing and dehydrating step lasted 10 min. Finally, cells were dehydrated overnight in absolute ethanol (99.6 %). The drying step was completed by drying pelletized cells at the critical point with Balzers CPD 030 Critical Point Dryer. After coating samples with gold using an SCD 050 sputter coater (Bal-Tec), scanning electron micrographs were taken with a Leo 1525 (Carl Zeiss AG, Oberkochen, Deutschland).

2.9.3 Single cell gene expression studies of β-lactamase

2.9.3.1 Construction of reporter promoter fusions

The activity of the two active β-lactamases, encoded by the \( \text{bla}_{\text{L1}} \) and \( \text{bla}_{\text{L2}} \) genes, was separately verified by cloning and expressing these genes in \( E. \text{coli} \) cells. To further investigate the expression of the two β-lactamases genes at single cell level, reporter gene fusions were constructed. Therefore, the genes \( \text{rfp} \) (DsRed2) (Baird et al., 2000), \( \text{cfp} \) (mCerulean) (Rizzo et al., 2004) and \( \text{yfp} \) (mVenus) (Shaner et al., 2004) were fused to DNA fragments carrying the promoter of the \( \text{bla}_{\text{L1}} \) gene or the promoter and intergenic region of the \( \text{bla}_{\text{L2}} \). The respective promoter region was predicted using PromBase (Rangannan and Bansal, 2011) and accordingly there is a higher probability that the respective promoter region lies within 300 bp upstream of the start codon of the \( \text{bla}_{\text{L1}} \) or \( \text{bla}_{\text{L2}} \) genes (Figure 5). Two different potential promoter regions of \( \text{bla}_{\text{L2}} \) were amplified (318 bp and 180 bp). Separately, a 417 bp fragment for \( \text{bla}_{\text{L1}} \) were obtained. In subsequent steps, the amplified DNA fragments were fused into the vectors containing the \( \text{rfp} \), \( \text{cfp} \) and \( \text{yfp} \) genes. The resulting constructs were then used to transform \( E. \text{coli} \) DH5α cells. The correctness of the construct was analyzed by restriction analysis and via sequencing. The construct was introduced into SMK279a-cells via triparental mating (for methodological details see section 2.8.2)

For selection of SMK279a transconjugants with a functional fluorescent protein, colonies obtained after triparental mating were streaked on agar plates containing ampicillin (100 µg/ml)
and gentamicin (50 µg/ml). After overnight growth at 30°C, bacterial cells were analyzed with the help of a fluorescence microscope. Cells showing a strong fluorescence due to the induction with ampicillin were chosen for single cell expression studies. Additional confirmation was carried out in 5 ml LB broth in reactions tubes supplemented with antibiotics as above and by analyzing samples accordingly (see Material and Methods, section 2.9.3.2). Here, the fluorescent reporter protein was used as reporter gene to measure the activity of the β-lactamase promoters.

![Diagram of predicted promoter regions](image)

**Smlt2666**: putative TonB-dependent ferric siderophore receptor  
**Smlt2667**: metallo-beta-lactamase class B  
**Smlt2668**: conserved hypothetical exported protein  
**Smlt3721**: putative transmembrane Na+/H+ antiporter  
**Smlt3722**: beta-lactamase class A  
**Smlt3723**: LysR family transcriptional regulator  
**Smlt3723**: putative ABC transport system

**Figure 5: Predicted promoter coding region of β-lactamase resistance genes.** The promoter regions were predicted using PromBase (Ranganna and Bansal, 2011) and were located immediately 300 bp upstream of smlt2667 or smlt3722 genes (black arrows: smlt2667 (bla₁) and smlt3722 (bla₂)). The transcriptional regulator ampR shares a divergent overlapping promoter region with smlt3722 (smlt3723 (ampR), light grey arrow).

**2.9.3.2 Single cell analysis via fluorescence microscopy**

For analysis of single cell expression, cultures were grown overnight for 12 to 15 generations (about 15 h) supplemented with gentamicin (50 µg/ml) and without β-lactam inducer. Cells were then diluted in fresh pre-warmed medium to $10^7$ cells/ml and growth was continued for time
indicated at 30°C or 37°C. Unless otherwise stated, generally induction was carried out at time of inoculation. Aliquots of cultures were taken at appropriate time and diluted 1:100 in the same medium. Usually 5 to 10 µl of this suspension was subsequently placed on glass slides under cover slips and attached by air drying for about 5 to 10 min. Microscopy experiments were conducted with a Zeiss Axio Imager fitted with filter BP546/12 (red), the emission filter 605/75 (red) and a Zeiss Illuminator HXP 120156. Phase-contrast and fluorescence images were obtained from the same area and matched using AxioVision (release 4.8). For single cell analyses a minimum of 400 cells were analyzed per preparation.

2.9.3.3 Potential inducer of phenotypic heterogeneity

For investigation of the induction of β-lactamases, experiments were carried out with SMK279a cells harboring the reporter plasmid. As inducer, β-lactams (ampicillin, cefuroxime and meropenem), culture supernatants, crude extract and diffusible signal factor (native DSF and 11-methyl-2-dodecenoic (Sigma, Heidelberg, Germany) were tested. For tests with culture supernatants, cultures were grown to stationary phase and supernatants were filtered using 0.20 µm filters and used immediately or stored at 4°C until used.

For the extraction of native DSF from SMK279 cultures, the cells were grown in LB at 30°C for 48 h using a shaking apparatus. The supernatant was collected via centrifugation and subsequently extracted with ethyl acetate. Phases were separated using the separation funnel and combined organics were dried with sodium sulfate to remove excess water. The drying agent was removed by Whatman filter paper. The organic phase was evaporated to dryness using a rotary evaporator at 40°C. Finally, the residue was then suspended in 0.5 ml methanol and stored at -20°C. Likewise, commercial DSF 11-methyl-2-dodecenoic acid stock solution was prepared in methanol and stored at -20°C. Freshly diluted DSF was used in the induction experiments.

2.9.3.4 Growth and environmental stress as inducers of β-lactamase

As a nutritional stress, LB with 0.5 M NaCl, 1 M NaCl and 0.5 M KCl were used. As environmental stress, heat stress (41°C) was used. Overnight cells of SMK279a carrying Pbla2::rfp reporter fusion was diluted in fresh LB media and the growth was continued for 7 h aerobically at 30°C. However, for heat stress growth was employed at 41°C as suboptimal incubation growth condition. As needed, cells were collected by centrifugation (5 min, 4°C, and 10,000 rpm) and re-suspended in small volume fresh medium and analyzed.
2.9.3.5 Microplate based fluorescence assays

Overnight cultures of SMK279a carrying an appropriate reporter fusion were diluted in sterile LB, which was supplemented with ampicillin (100 µg/ml). Usually 150 µl of diluted sample was pipetted into black 96-well plates with a transparent bottom and incubated at ambient temperature in a Synergy HT microplate reader (BioTek, Winooski, Vermont, USA). The intensity of fluorescence was measured as relative fluorescence units (RFU) and was registered at intervals of 1 h against pure medium employing the Gen5 Software. The fluorescence was measured with the excitation filter (485/20) for 60 to 80 ms and with the emission filter (530/25).

2.9.3.6 Overexpression of proteins in SMK279a

Differentially regulated genes between bla-ON and bla-OFF mode cells identified using RNA-seq was further studied by expression from low copy plasmids in the parent SMK279a strain. Target genes for overexpression were smlt2851, smlt2852 and smlt1134 (ComE homologue). Therefore, the respective operons with their predicted promoter regions were amplified by PCR and inserted upstream of PblaL2::rfp in the pBBR1MCS-5 vector, resulting in pBBR1MCS-5::PblaL2::rfp::smlt1134; and pBBR1MCS-5::PblaL2::rfp::smlt2851::smlt2852. The constructs were verified by DNA sequencing. The recombinant plasmids were transferred into SMK279a cells and challenged with 100 µg/ml ampicillin to analyze their impact on phenotypic heterogeneous blaL2 expression.

2.10 DNA sequencing

2.10.1 Insert sequencing

Insert sequencing for verification of cloning procedure was carried out based on the information provided by the company (Eurofins MWG GmbH, Ebensburg, Germany).

2.10.2 Whole-genome sequencing (WGS) and variant detection.

Usually a single colony was transferred to fresh LB and cultured overnight to an OD_{600} of 1.0. Genomic DNA was isolated from each individual samples with the AquaPure Genomic DNA Kit (BioRad, Hercules, USA). The amount and quality of the DNA in samples were checked after reading with bioanyalyzer. Libraries were generated following the Nextera XT DNA Library Preparation Kit (Illumina, San Diego, CA, USA) and sequenced on the MiSeq (2x310 bp) or NextSeq 500 (2x151 bp) platform (Illumina, San Diego, CA, USA). Respective FASTQ files were submitted to the EMBL-ENA short read archive (http://www.ebi.ac.uk/ena; Acc.no.: ERP011093). Resulting reads were mapped to the SMK279a genome sequence (Acc.no.: NC_010943.1) with
the alignment program SARUMAN (Blom et al., 2011). All isolates were sequenced with a minimum coverage of 50-fold. For variant detection in mapped reads, we employed customized Perl scripts using a minimum threshold of 10-fold coverage and a minimum allele frequency of 75 % (Roetzer et al., 2013). Variant positions were then combined, supplementing the joint list with the respective information from the original mappings. Concatenating all single nucleotide polymorphism (SNP) positions with a reliable base call (10-fold coverage and 75 % allele frequency) in at least 95 % of the isolates yielded a sequence alignment for the construction of a maximum parsimony tree, which was built with the software BioNumerics version 7.5 (AppliedMaths, Sint-Martens-Latem, Belgium).

2.11 Generation of gene deletions

The mutant strain SMK279aΔsmlt0387 was generated according to Hoang et al. (Hoang et al., 1998) and Alavi et al. (Alavi et al., 2013). Briefly, a DNA construct of 950 bp nucleotides consisting of the upstream and downstream flanking regions with respect to the target gene to be deleted was obtained over a two-step PCR using genomic DNA of the SMK279a strain as a template. In the first step, the flanking region of smlt0387 were amplified in two separate PCRs using primer pairs 0387_Up_F/0387_Up_R, and 0387_Down_F/0387_Down_R. PCR primers were designed by incorporating a complementary “tag” region that was added to the 5’ end of each inner primer (0387_Up_R and 0387_Down_F). As a next step, the two amplicons were fused to generate a single DNA fragment using primers 0387_Up_F and 0387_Down_R. The fused DNA fragment was subsequently cloned in the suicide vector pEX18Tc generating pEX18Tc: Δ0387 (Figure 6A-C).

The suicide vector was transferred to SMK279a cells using triparental mating. The first and second crossover events were selected using tetracycline (75 µg/ml) and 10 % (w/v) sucrose, respectively. Generations of the mutant strain was confirmed by performing separate PCRs, each with particular primers designed to confirm the recombination event and the generation of the SMK279aΔsmlt0387 mutant strain. Via sequencing it was further proved that the neighbor’s genes smlt0386 and smlt0389 were intact in the SMK279aΔsmlt0387 mutant strain. The overall scheme of homologous recombination followed for construction of unmarked deletion of smlt0387 is shown in Figure 6A-C.

Markerless SMK279a mutants for SMK279aΔsmlt1134, SMK279aΔsmlt2851Δsmlt2852 and SMK279aΔsmlt3723 were constructed using the pGPI-SceI/pDAI-SceI-SacB system originally developed for bacteria of the genus Burkholderia (Aubert et al., 2014, Flannagan et al., 2008) (Figure 7A-C).
Material and Methods

Figure 6: Schematic representation of the cloning procedure and location of PCR fragments used to generate SMK279aΔsmlt0387. The flanking region of smlt0387 was PCR amplified using primer listed in Table 3 and fused together via cross over PCR, ligated into pEX18Tc suicide vector. A SMK279a cell was transformed with pEX18Tc using E. coli HB101 with pRK2013 as a helper plasmid. The mutant strain was generated via homologous recombination events. Finally, the plasmid pEX18Tc was resolved by sucrose counter-selection.
Figure 7: Physical map of genes mutated in SMK279a. Schematic representation of the cloning procedure and location of PCR fragments used to generate markerless *S. maltophilia* K279a mutants of *smlt*1134 (A), *smlt*2851 & *smlt*2852 (B) and *smlt*3723 (C). Red arrows indicate the target ORFs and their mutated versions; green arrows show the flanking ORFs; primers (Table 2) used for the amplification of PCR fragments (brown bars) are shown as thin, black arrows with labels; the necessary reference base pair numbers are framed.
2.12 Working with RNA

2.12.1 RNA extraction and purification

Cultures of SMK279a were grown in LB medium for 27 h and 32 h at 30°C in the presence of ampicillin (100 µg/ml). Additionally, samples for RNA extraction were taken from colonies that were grown for 48 h in presence of ampicillin (100 µg/ml). At time indicated growth was arrested and total RNA was isolated using the UltraClean Microbial RNA Isolation Kit as per the manufacturer’s instructions (MO BIO Laboratories, Carlsbad, CA, USA). The extracted RNA was stored at -20°C or -70°C till downstream applications. In most cases, the total extracted RNA contained some left over genomic DNA that needed to be eliminated for certain applications such as high throughput sequencing and RT-qPCR. The RTS DNase™ Kit (MO BIO Laboratories, Carlsbad, CA, USA) was used to remove DNA from RNA samples according to the manufacturer’s instruction. Following the removal of residual DNA, RNA was cleaned and concentrated using the RNA Clean & Concentrator™ Kit, as per the protocol (Zymo Research, Freiburg, Germany). Unless otherwise specified a general procedure, which allowed extraction of smaller RNA (including larger than 17 nt) was preferred. Pure RNA was eluted with 20 µl DNase/RNase free H₂O.

2.12.2 RNA electrophoresis

RNA molecules can be analyzed on either native or denaturing agarose or polyacrylamide gels. To assess the quality and the integrity of RNA, formaldehyde agarose gel electrophoresis in MOPS buffer was carried out. Low molecular weight agarose (1 %) was dissolved in 100 ml of 1X MOPS buffer and boiled in a micro oven until it formed a clear suspension. When cooled to around 55°C, 1 ml of 37 % formaldehyde was added and mixed carefully by gently shaking. In chemical fume hood, an agarose gel was casted, the liquid gel poured into the construct and then allowed to thicken for about 30 min. Afterwards the gel was placed in an electrophoresis apparatus containing 1X MOPS buffer and allowed to equilibrate for about 30 min. While the gel was equilibrating, the RNA samples for electrophoresis were prepared. RNA samples were mixed with equal volumes of the 2-fold RNA loading dye in RNA free E-cups. The mixture was then incubated at 70°C for 10 min before chilling on ice for 3 min. The samples were spun down prior to loading on a gel and then loaded within about 10 min. Few microliters of RNA marker were pipetted and handled in similar fashions as RNA samples. The gels were run in an electrophoresis gel chamber (Hoefer™ HE-33 mini horizontal submarine unit, Amersham Biosciences, USA), which was filled with 1 x MOPS buffer, at 120 Volt for 60 min. The solutions and buffers used are indicated below.
Material and Methods

### MOPS buffer (10x)

<table>
<thead>
<tr>
<th>Component</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>MOPS</td>
<td>200 Mm</td>
</tr>
<tr>
<td>NaAc</td>
<td>50 Mm</td>
</tr>
<tr>
<td>EDTA</td>
<td>10 mM</td>
</tr>
</tbody>
</table>

The pH was adjusted to 7.0, using NaOH

### Formaldehyde gel solution

<table>
<thead>
<tr>
<th>Component</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Agarose</td>
<td>1.2 g</td>
</tr>
<tr>
<td>MOPS buffer (10x)</td>
<td>10 ml</td>
</tr>
<tr>
<td>Formaldehyde</td>
<td>1.2 ml</td>
</tr>
<tr>
<td>H$_2$O (RNase free)</td>
<td>ad 100 ml</td>
</tr>
</tbody>
</table>

### 1X MOPS RNA gel electrophoresis buffer

<table>
<thead>
<tr>
<th>Component</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>MOPS buffer (10x)</td>
<td>100 ml</td>
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<tr>
<td>Formaldehyde (37 %, v/v)</td>
<td>20 ml</td>
</tr>
<tr>
<td>H$_2$O (RNase free)</td>
<td>880 ml</td>
</tr>
</tbody>
</table>

### DEPC treated RNA free H$_2$O

<table>
<thead>
<tr>
<th>Component</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>DEPC</td>
<td>1 ml</td>
</tr>
<tr>
<td>H$<em>2$O$</em>{100}$</td>
<td>ad 1 l H$_2$O</td>
</tr>
</tbody>
</table>

Mixed vigorously, incubated for 12 h at RT and then autoclaved.

### 2.13 RNA-seq and data analysis

To prepare the cell material for RNA-seq analyses, SMK279a was grown on LB agar plates with/without ampicillin (100 µg/ml). Two samples from each colony morphotype (small and big colonies) and two samples from uniform colonies were scrapped from agar plates after 48 h of growth at 37°C, pooled and immediately frozen in liquid nitrogen (Figure 8). About 10-15 mg of wet biomass were generally used for each biological replicate, 10 mg of cell material corresponded to an OD$_{600}$ of 6.35. Additionally, for RNA extraction of liquid cultures, the strain SMK279a carrying the P$_{blaL2}$::rfp reporter fusion was grown for 27 and 32 h with 100 µg/ml ampicillin. Total RNA was extracted using the hot-phenol method as described previously (Blomberg et al., 1990). The residual genomic DNA was removed from the total isolated RNA by DNase I treatment. All samples were adjusted to contain equal amounts of extracted mRNAs.

The cDNA libraries for sequencing were constructed by vertis Biotechnology AG, Germany as described by Sharma et al. (Sharma et al., 2010). The transcripts were not fragmented. The cDNA libraries were sequenced using a HiSeq 2500 machine (Illumina) in single-read mode and running 100 cycles. To assure a high sequence quality, the Illumina reads in FASTQ format were trimmed with a cut-off phred score of 20 by the program fastq_quality_trimmer from FASTX toolkit version 0.0.13 (http://hannonlab.cshl.edu/fastx_toolkit/). The alignment of reads, coverage calculation, gene wise read quantification and differential gene expression was performed with READemption ( Förstner et al., 2014 ) version 0.3.5 (DOI:10.5281/zenodo.13926), ‘segemehl’ (Hoffmann et al., 2009) version 0.2.0 and DESeq2 (Love et al., 2014) version 1.6.3. Visual
Material and Methods

inspection of the coverages was done using the Integrated Genome Browser (IGB) (Nicol et al., 2009). The reference sequence for SMK279a was retrieved from the NCBI database (Acc.no.: NC_010943.1). Genes with a fold-change of $\geq 2.0$ and an adjusted $P$ value (corrected for multiple testing using the Benjamini-Hochberg procedure) $\leq 0.05$ were considered as differentially expressed. The raw, de-multiplexed reads as well as coverage files have been deposited in the National Center for Biotechnology Information's Gene Expression Omnibus under the project ID: GSE71735.

![Figure 8: Colonies were scrapped rapidly and collected in E-cups for RNA-seq analysis.](https://en.wikipedia.org/wiki/Liquid_nitrogen)

2.14 Reverse transcription-quantitative PCR

Selected differentially regulated genes identified in SMK279a RNA-seq analyses were verified by RT-qPCR experiments. Therefore, total RNA was extracted from colonies that were grown for 48 h in the presence of ampicillin (100 µg/ml). UltraClean Microbial RNA Isolation Kit (MO BIO Laboratories, Carlsbad, CA, USA) was used to isolate total RNA from SMK279a cells as per the manufacturer’s instructions. The residual genomic DNA was removed by DNase I treatment according to the manufactures instructions (DNase I, RNase-free, Thermo Scientific, WI, USA). The quality and integrity of RNA was checked on a denaturing formaldehyde gels in MOPS buffer. Intact total RNA ran on a denaturing gel will have sharp 23S and 16S rRNA bands. The SuperScript® VILO™ cDNA synthesis kit (Invitrogen™, life technologies, TX, USA) was used to generate cDNA using 1.2 µg RNA as recommend my manufacturer’s. This kit synthesized a single stranded cDNA via first-strand synthesis from a single stranded RNA. To exclude DNA contaminations, the procedure was carried out by replacing reverse transcription enzyme with
equal volume of RNA-free water. Gene-specific primers used for RT-qPCR are shown in Table 7. The RT-qPCR reactions were set up according to the manufacture’s protocol using the SYBR® Select Master Mix for CFX (Applied biosystems® by life technologies, TX, USA) and performed in the MiniOpticon™ real-time PCR detection system (Bio-Rad Laboratories, Munich, Germany). Standard curves of 10-fold serial dilutions of cDNA were generated for each gene to evaluate the primer efficiency and for data analysis. The efficiency, slope and correlation coefficient were determined by the CFX Manager™ software (Bio-Rad Laboratories, Munich, Germany). All RT-qPCR reactions were run in triplicate and repeated at least three times in separate experiments under the same conditions. The expression values were normalized against rpoD and 16S rRNA. Data were analyzed based on the normalized gene expression (2^{-ΔΔC(t)} method).

Table 7: Gene-specific primers used for RT-qPCR.

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence [5'→3']</th>
<th>Length of amplified product/gene</th>
</tr>
</thead>
<tbody>
<tr>
<td>qsmlt4165-Bfor</td>
<td>AAGGGCCTGGAACAGGGCTA</td>
<td>145 bp/rpoD*</td>
</tr>
<tr>
<td>qsmlt4165-Brev</td>
<td>CCACATCCGGCGCAACTTCA</td>
<td></td>
</tr>
<tr>
<td>q16S-Afor</td>
<td>AGAGTTTGATCCTGGCTCAG</td>
<td>240 bp/16S rRNA*</td>
</tr>
<tr>
<td>q16S-Arev</td>
<td>CTAATCCGACATCGGCTCAT</td>
<td>157 bp/bla,1</td>
</tr>
<tr>
<td>qsmlt2667-for</td>
<td>CGGTCACTGTGCTGAGAACA</td>
<td>144 bp/bla,2</td>
</tr>
<tr>
<td>qsmlt2667-rev</td>
<td>CACCGCCGTTTCTCGAATGG</td>
<td>196 bp/smlt3721</td>
</tr>
<tr>
<td>qsmlt3722-Afor</td>
<td>CGTCGCGATTCCTGAGTT</td>
<td>168 bp/smlt0018</td>
</tr>
<tr>
<td>qsmlt3722-Arev</td>
<td>TTCCAGCGCGGCGGAATCAC</td>
<td></td>
</tr>
<tr>
<td>qsmlt3721-for</td>
<td>GAGCCATGAACTGATCTACC</td>
<td>205 bp/smlt0019</td>
</tr>
<tr>
<td>qsmlt3721-rev</td>
<td>GAACAGGAAACAGCGAGGAAA</td>
<td>126 bp/smlt1007</td>
</tr>
<tr>
<td>qsmlt0018-for</td>
<td>CGGAACGTGAGTTCTACTCTT</td>
<td></td>
</tr>
<tr>
<td>qsmlt0018-rev</td>
<td>TGATGTCCGATCTGTTTAGC</td>
<td></td>
</tr>
<tr>
<td>qsmlt0019-for</td>
<td>TAATACTAACCAGCAGCGACCT</td>
<td></td>
</tr>
<tr>
<td>qsmlt0019-rev</td>
<td>GATGCCGCGAAGGAGCTTTC</td>
<td></td>
</tr>
<tr>
<td>qsmlt1007-for</td>
<td>GAAGGCTTCAACAAAGCTCAC</td>
<td></td>
</tr>
<tr>
<td>qsmlt1007-rev</td>
<td>GATGCCGCGAAGGAGCTTTC</td>
<td></td>
</tr>
</tbody>
</table>

*Genes used for normalization of gene expression.

2.15 Methods for the functional characterization S. maltophilia isolates

2.15.1 Biofilm assay

Qualitative assessment of biofilm formation among S. maltophilia isolates was performed according to O’Toole GA (O’Toole, 2011) with the following minor modifications. The microtiter plates (96-well plates, round-bottom, Sarstedt) were pretreated with acetone for 10 seconds to enhance biofilm adhesion. All the S. maltophilia isolates were cultured overnight in 96-well microtiter plates in LB broth. Each isolate was diluted in fresh LB to achieve a cell density equivalent of 10^8 CFU/ml. Diluted cultures of each 100 µl were transferred into the wells and
incubated 37°C or 30°C for 24 h. The microtiter plates were emptied from the medium and dried for an h at 60°C. The quantification of the biofilm was performed by staining samples with 2 % solution of crystal violet and subsequent solubilization with acetic acid. Usually 125 µl of the dye solution were added to each well, incubated at RT for 15 min and carefully washed three times with water to get rid of the excess dye. The plate was dried overnight at RT. Finally, the dye was dissolved with 125 µl of 30 % acetic acid for 15 min and transferred to a new microtiter plate with flat bottoms. Solubilized crystal violet was quantified at OD 595, against acetic acid.

2.15.2 Tributyrin (TBT) agar plate assay

To examine the lipolytic activity of the S. maltophilia isolates, all strains were cultured overnight in 96-well microtiter plates and transferred on LB agar plates containing 1 % tributyrin (TBT). The plates were incubated for 2-4 days at 37°C or 30°C and possible clearing zones around isolate was inspected visually. The media for the assay was prepared by heating LB agar in a microwave and then adding TBT and homogenizing it for 3 min with an ULTRA TURRAX® T18 basic homogenizer (IKA WORKS Inc., Wilmington, NC, USA). The mixed solution was autoclaved immediately and the plates were prepared as soon as possible to avoid TBT drops.

2.15.3 Screening for protease assay

S. maltophilia isolates were grown overnight in LB broth at 30°C or 37°C. Individual bacterial isolates were further screened for proteolytic activity on skim milk agar medium (Casein enzymatic hydrolysate, 5 g/l; yeast extract, 2.5 g/l; dextrose, 1 g/l; skim milk powder, 28 g/l; agar, 15 g/l). The plates were incubated for 24 h at 37°C or 30°C and possible clearing zones around isolate was inspected visually.
3 Results

It is now increasingly known that individual bacteria respond differently in homogeneous environment via a set of diverse genetic and non-genetic mechanisms. Therefore, this thesis describes the mechanisms that modulate the heterogeneous stress response of SMK279a within isogenic population at the single cell level. This opportunist human pathogen (frequently found in cystic fibrosis patients) produces two β-lactamases (L1 and L2) upon challenge of cells with β-lactam antibiotic. Thus, the phenotypic responses of SMK279a are described first by exposing bacterial cells with variable level of ampicillin. Secondly, reporter fluorescent proteins were used to investigate single cell β-lactamase expression. The third and the fourth subsections of this study describe the molecular keys of phenotypic heterogeneity. Additionally, 106 S. maltophilia isolates were genotypically and phenotypically characterized with research collaboration at Research Center Borstel, Germany.

3.1 Phenotypic and physiological responses of SMK279a to β-lactams

The goal of this thesis was to identify novel mechanism linked to the β-lactamase resistance in SMK279a. To address this issue, initially all the possible β-lactamases identified in the SMK279a genome were cloned and tested them for activities on a wide range of β-lactam antibiotics. Table 8 summarizes the putative ORFs assigned to the β-lactamase family in the genome of SMK279a. To verify which genes are indeed responsible for the β-lactamase resistance in SMK279a, the potential bla genes were PCR amplified and cloned on a plasmid carrying an IPTG inducible promoter. Enzymatic activity was verified by overexpression in E. coli DH5α and growth in the presence of ampicillin. The genes belonging to locus smlt2667 and smlt3722 were active in E. coli as indicated in Table 8. Indeed, these are the two functional β-lactamase genes designated bla1 and bla2 known to hydrolyze a wide spectrum of β-lactam antibiotics, such as penicillin G, ampicillin, meropenem and so forth. The MICs for ampicillin, kanamycin and gentamicin were analyzed using microdilution technique following EUCAST guidelines and found to be 256 (µg/ml), 64 (µg/ml) and 16 (µg/ml), respectively. None of the other cloned ORFs and genes appeared to have any activities on antibiotics.
Table 8: Putative and verified ORFs assigned to the β-lactamase family in the genome of SMK279a

<table>
<thead>
<tr>
<th>Locus Tag</th>
<th>Gene product name</th>
<th>Activity in E.coli</th>
</tr>
</thead>
<tbody>
<tr>
<td>Smlt0115</td>
<td>putative β-lactamase precursor</td>
<td>-</td>
</tr>
<tr>
<td>Smlt0347</td>
<td>metallo-β-lactamase family protein</td>
<td>-</td>
</tr>
<tr>
<td>Smlt0580</td>
<td>putative metallo β-lactamase family protein</td>
<td>-</td>
</tr>
<tr>
<td>Smlt0581</td>
<td>putative metallo β-lactamase family protein</td>
<td>-</td>
</tr>
<tr>
<td>Smlt1470</td>
<td>putative β-lactamase</td>
<td>-</td>
</tr>
<tr>
<td>Smlt1815</td>
<td>putative metallo-β-lactamase family protein</td>
<td>-</td>
</tr>
<tr>
<td>Smlt2514</td>
<td>putative β-lactamase</td>
<td>-</td>
</tr>
<tr>
<td>Smlt2563</td>
<td>putative β-lactamase</td>
<td>-</td>
</tr>
<tr>
<td>Smlt2589</td>
<td>putative β-lactamase AmpC protein</td>
<td>-</td>
</tr>
<tr>
<td>Smlt2667</td>
<td>putative metallo-β-lactamase, bla₁</td>
<td>x</td>
</tr>
<tr>
<td>Smlt3114</td>
<td>putative β-lactamase</td>
<td>-</td>
</tr>
<tr>
<td>Smlt3132</td>
<td>putative β-lactamase</td>
<td>-</td>
</tr>
<tr>
<td>Smlt3495</td>
<td>putative β-lactamase</td>
<td>-</td>
</tr>
<tr>
<td>Smlt3652</td>
<td>putative β-lactamase protein</td>
<td>-</td>
</tr>
<tr>
<td>Smlt3722</td>
<td>putative β-lactamase protein, bla₂</td>
<td>x</td>
</tr>
<tr>
<td>Smlt3807</td>
<td>putative metallo-β-lactamase protein</td>
<td>-</td>
</tr>
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<td>Smlt3991</td>
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<td>-</td>
</tr>
<tr>
<td>Smlt4159</td>
<td>putative β-lactamase protein</td>
<td>-</td>
</tr>
<tr>
<td>Smlt4211</td>
<td>putative β-lactamase protein</td>
<td>-</td>
</tr>
</tbody>
</table>

X - activity detected - activity not detected

3.1.1 Colony morphotypes of SMK279a in response to ampicillin

To investigate the phenotypic response to subinhibitory concentrations of ampicillin, overnight grown log-phase SMK279a cells were used. The advantage of using such cells was that the cells did not enter multiresistant state. Thus, bacterial cells were cultured on agar plates with or without ampicillin (100 μg/ml), and growth was continued for 48 h (see Material and Methods, section 2.9.1). It was easy and direct to observe any modification in colony morphology, but also equally important for the design of therapeutic approaches. Bacterial cells formed uniform, grayish-yellow colored and round colonies on agar plates in the absence of antibiotics (Figure 9A and Table 9). Few colonies grew overnight from the same volume of diluted samples cultured on agar plate containing ampicillin (100 μg/ml). However, after prolonged incubation for 48 h more colonies, which differed in size and height, appeared, forming colony heterogeneity (Figure 9B and Table 9). In this study, the colony morphotypes were further classified in three groups based on size and origin, i.e. small and big in the presence of ampicillin and uniform in the absence of ampicillin. Thereby, small colonies constituted up to 80 % of CFU, with measured a diameter range of 1.4 ± 0.26 (mm), while big colonies constituted up to 20 % with a diameter range of 3.2 ± 0.27 (mm) (Table 9). Uniform colonies were grown and expanded to diameter range of 3.0 ± 0.06 (mm) in the absence of ampicillin (Table 9). Furthermore, growth of SMK279a on agar plates containing ampicillin (600 μg/ml) above MIC levels resulted also in the formation of colony
Results

morphotypes after 48 h at 37°C. Among these colonies some were pointed at the center while others appeared to have a flat colony surface with a wrinkled texture, indicating a strong impact of β-lactam treatment on SMK279a growth behavior and colony morphology (Figure 9D).

Serially diluted aliquots were spread on LB agar plates with/without ampicillin (100 µg/ml) in order to further demonstrate whether bacterial cells in the single colony type formed colony heterogeneity in the transfer cultures. In this manner, the colony heterogeneity shown on ampicillin plates were quenched in sister progeny cells if the selective pressure was omitted (Figure 9E and Figure 10). Intriguingly, in the presence of ampicillin, cells from smaller colonies formed colony morphotypes but not for cells from big colonies that formed only the same phenotype as before (Figure 9E and Figure 10). Finally, the use of kanamycin, which is an aminoglycoside bactericidal antibiotic, resulted in colonies that were uniform in appearance at 48 h (Figure 10).

**Table 9**: Morphological description of SMK279a colony morphotypes grown on agar plates after 48 h.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Colony morphotype</th>
<th>% of all colonies</th>
<th>Colony size [diameter, mm]</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amp 100</td>
<td>small</td>
<td>80</td>
<td>1.4 ± 0.26</td>
</tr>
<tr>
<td>Amp 100</td>
<td>big</td>
<td>20</td>
<td>3.2 ± 0.27</td>
</tr>
<tr>
<td>No Amp</td>
<td>uniform</td>
<td>100</td>
<td>3.0 ± 0.06</td>
</tr>
</tbody>
</table>
Figure 9: Phenotypic heterogeneity of SMK279a cells during growth on solid media. (A) SMK279a formed round, uniform colonies when grown without ampicillin on LB agar plates for 48 h at 37°C. (B) Colony heterogeneity on LB agar supplemented with ampicillin showing small and big colonies after growth for 48 h at 37°C. (C) Small colonies (indicated by red dashed circle) and big colonies cultivated on agar plates containing high concentrations of ampicillin (600 µg/ml). (D) Colonies differ slightly in color when cultivated for 48 h on agar plates containing high concentrations of ampicillin (600 µg/ml). (E) SMK279a parent strain colony morphotypes on LB agar plates in the presence of 100 µg/ml ampicillin. Arrows numbered 1 or 2 indicates an additional 24 h incubation of the cultures or the transfer of colonies to fresh agar plates, respectively. Big colonies of SMK279a appeared overnight and the diameter, but not the number of big colonies, increased with prolonged incubation of plates to 48 h. Small colonies dominated SMK279a growth after approximately 48 generations.
Results

3.1.2 Outer membrane vesicles and biofilm formation

As β-lactam interferes with cell biosynthesis, it was expected that bacterial cells would show some degree of membrane stress that led to vesicle formation. Indeed, the degree of vesiculation between colony morphotypes owning to ampicillin treatment could probably reflect the extent of

Figure 10: Schematic diagram showing colony morphologies observed for SMK279a in response to ampicillin or kanamycin treatment on LB agar plates. Arrows indicate the transfer of fresh colonies or liquid culture samples. Small colonies formed both, big and small colonies when recultivated in the presence of ampicillin (AMP; 100 µg/ml). Cells from big colonies formed in the presence of ampicillin again uniform colonies after 48 h at 37°C after transfer to fresh agar plates containing ampicillin. The use of kanamycin (KAN, 25 µg/ml), which is an aminoglycoside bactericidal antibiotic, resulted in uniform colonies after 48 h at 37°C.
disturbance in cell wall structure. Scanning electron microscopy was performed from SMK279a cells growing under various conditions to further reveal cell physiological response in the form of vesiculation (for experimental details see 2.9.2). Since the majority of the small colonies appeared in the visible phenotype between 24-33 h, SEM images were generally taken after 48 h (approximately 48 generations) and with the main findings (outer membrane vesicles (OMVs), filamentation and biofilm formation) are presented below.

### 3.1.2.1 Filamentation and OMVs

Bacterial cells treated with ampicillin formed elongated cells in comparison to untreated cells that mainly occurred as individual bacillary rods (Figure 11A-C). Further, SEM analysis revealed that filament was formed more frequently in cells of small colonies than big colonies. Interestingly, SMK279a cells from both colony phenotypes showed a massive formation of OMVs. Long bacillary chains were heavily shielded by OMVs, rather than individual bacilli in cells of both morphotypes. Intriguingly, the size of these OMVs varied considerably ranging from 100 nm to 677 nm in diameter in some bacterial cells (Figure 11A and B, Figure 12), which altogether indicated that phenomenon of vesiculation is an important physiological response to cell wall-active antibiotics such as β-lactams. On the other hand, untreated cells showed little or no formation of vesicles (Figure 11C).

![Figure 11: Representative scanning electron micrographs of SMK279a cells grown in the presence or absence of ampicillin at 37°C for 48 h on LB agar plates.](image)

SEM images of SMK279a cells were recorded as previously published (Krohn-Molt et al., 2013). Cells were pelleted, fixed, and dried progressively and images were taken with the Zeiss Leo1525 scanning electron microscope. (A) SEM image of cells obtained from big colonies cultured in the presence of 100 µg/ml ampicillin. (B) SEM image of cells obtained from small colonies cultured in the presence of 100 µg/ml ampicillin. (C) SEM image of cells obtained from colonies cultured in the absence of ampicillin. Cells of (A) and (B) predominantly formed long filamentous cells (indicated by letter F) and OMVs (indicated by arrows).
Results

Figure 12: SMK279a cells formed large OMVs in the presence of ampicillin. The size of the OMVs varied considerably, ranging from less than 100 nm (A) up to 677 nm (as shown with an arrow) (B) in diameter in some SMK279a cells.

3.1.2.2 Extracellular polymeric substances and/or biofilm formation

The growth of SMK279a cells on agar plates containing ampicillin (for example, 300 μg/ml) resulted in few colonies, in some cases less or equal to two colonies were observed on a plate. This growth above MIC levels indicated a further heterogeneous response of the bacterial population exposed to antibiotics in a homogeneous environment. A further mechanism, in addition to vesiculation, might therefore be involved that allowed tolerance at higher levels of antibiotic than those of the rest of the population. Thus, colonies from several LB agar plates were pooled and images were done as described in the methods section 2.9.2. Cells showed increased extracellular polymeric substances (EPS)/biofilm formation in comparison to cells treated with 100 μg/ml of ampicillin; however the phenomenon vesiculation was a common response to ampicillin treatment (Figure 13 A and B).
Results

Figure 13: SEM image of cells obtained from big colonies cultured in the presence of 300 µg/ml (A) and 500 µg/ml (B) ampicillin. The cells showed increased extracellular polymeric substances (EPS) and biofilm formation in comparison to cells treated with 100 µg/ml ampicillin (Figure 11A and B).

3.2 Single cell gene expression analysis

In the presence of colony morphotypes, cells of each type massively forming the OMVs, β-lactamase expression might also be subjected to heterogeneous expression on a single cell level. Therefore, the activity of two functional chromosomal β-lactamase genes bla<sub>L1</sub> and bla<sub>L2</sub> was further investigated at the single cell level using fluorescent reporter proteins that were fused to the promoters regions of bla<sub>L1</sub> or bla<sub>L2</sub> and studied on a plasmid-borne vector in SMK279a.

3.2.1 Construction of reporter promoter-fusions and selection of fluorescent proteins for single cell expression studies

For construction of the plasmid-based reporter fusions in SMK279a, firstly the promoter regions for bla<sub>L1</sub> and bla<sub>L2</sub> were predicted using PromBase (Rangannan and Bansal, 2011). Accordingly, the amplified PCR fragments were 417 bp and 318 bp for the bla<sub>L1</sub> and bla<sub>L2</sub>, respectively (Figure 5 and Figure 14). Since the ampR is the transcriptional regulator for both genes sharing a divergent overlapping promoter region with bla<sub>L2</sub>, a truncated upstream region of 180 bp was further amplified to approximately locate the regulatory region of the β-lactamase genes. The physical map of the various promoter fusions and their size and purpose are summarized in Table 10.

The various reporter constructs listed in Table 10 were introduced into SMK279a by tri-parental mating approach using E. coli HB101, carrying the pRK2013 helper plasmid. To induce β-lactamase expression, which would aid selection of those with proper functioning fluorescent
proteins with active promoter, the SMK279a carrying the reporter fusions were grown overnight on agar plates containing gentamicin (50 µg/ml) and ampicillin (100 µg/ml) (for experimental details see 2.9.3.2 and 2.9.3.3). Analysis of cells by fluorescence microscopy indicated that blaL1 and blaL2 were expressed in most reporter strains, but they were not in cells carrying PblaL2::rfp or Pno::rfp (Figure 15A-F). Nevertheless, bacterial cells carrying PblaL2::rfp reporter fusion produced the strongest fluorescent signal with RFP (Figure 15A and B).

Figure 14: A partial physical map of the SMK279a genome region encoding for β-lactamase resistance genes and the transcriptional regulator ampR. Shown above with dashed vertical lines and red boxes are the approximate promoter DNA regions of blaL1 and blaL2 genes. Numbers indicate the size of the respective promoters. The ampR and blaL2 share an overlapping promoter region (indicated with bold grey line).

Table 10: The size and purposes of promoter regions of blaL1 or blaL2 fused to the red (RFP), yellow (YFP) and cyan (CFP) fluorescent proteins.

<table>
<thead>
<tr>
<th>Name</th>
<th>Size(bp)</th>
<th>Partial physical map in pBBRMCS-5</th>
<th>Purpose</th>
</tr>
</thead>
<tbody>
<tr>
<td>PblaL1::rfp</td>
<td>418</td>
<td><img src="image" alt="PblaL1::rfp" /></td>
<td>For monitoring blaL1 gene expression on single cell level</td>
</tr>
<tr>
<td>PblaL2::rfp</td>
<td>318</td>
<td><img src="image" alt="PblaL2::rfp" /></td>
<td>For monitoring blaL2 gene expression on single cell level</td>
</tr>
<tr>
<td>PblaL2::rfp</td>
<td>180</td>
<td><img src="image" alt="PblaL2::rfp" /></td>
<td>A control promoter fusion constructed only from ampR-blaL2 intergenic region.</td>
</tr>
<tr>
<td>PblaL2::cfp</td>
<td>318</td>
<td><img src="image" alt="PblaL2::cfp" /></td>
<td>For verification blaL2 gene expression on single cell level</td>
</tr>
<tr>
<td>PblaL2::yfp</td>
<td>318</td>
<td><img src="image" alt="PblaL2::yfp" /></td>
<td>For verification blaL2 gene expression on single cell level</td>
</tr>
<tr>
<td>Pno::rfp</td>
<td>0</td>
<td><img src="image" alt="Pno::rfp" /></td>
<td>A promoterless rfp construct for monitoring background uninduced fluorescence</td>
</tr>
</tbody>
</table>
Results

Figure 15: Analysis of single cell fluorescence of Pbla_{1} and Pbla_{2} promoter gene fusions. (A) Expression of the bla_{1} promoter fused to rfp in SMK279a. Cells were grown at 30°C for 17 h under aerobic conditions (200 rpm) in LB medium containing 100 µg/ml ampicillin. (B) Expression of the bla_{2} promoter fused to rfp in SMK279a grown under the same conditions as indicated in (A). (C) Cells carrying the Pbla_{2}::rfp construct were in a bla_{2}-OFF mode indicating the lack of either the promoter recognition or the regulatory region that directs the expression of the reporter protein. (D) Phenotypic heterogeneity observed in cells carrying the Pbla_{2}::cfp. (E) and the Pbla_{2}::yfp promoter gene fusion. (F) Control cells of SMK279a were grown under the same condition as described in (A) carrying a promoterless rfp reporter fusion (Pno::rfp). Images were recorded with 63x/1.30 Oil M27 and 100x/1.30 Oil M27 lenses using a Zeiss Axio Imager 2 fluorescence microscope and employing appropriate filters.

Due to an amplification effect of the plasmid in the reporter strain, a trans-effect on native regulatory network could be expected, particularly of those whose functions are modulated via the activator and the repressor ligand. To address this hypothesis, the parent strain and the SMK279a carrying Pbla_{2}::rfp construct were analyzed for altered phenotype. Both strains were susceptible to the same ampicillin MIC level (256 µg/ml) but the susceptibility to gentamicin was scaled up from 16 to 500 µg/ml which was apparently due to the resistance cassette of the plasmid. Therefore, it was assumed that the native AmpR binding ligand remained intact and was probably not affected in trans by the Pbla_{2}::rfp promoter construct of the plasmid. Bacterial cells of the parent and the transformed SMK279a strain divided in about one h in 25 ml of culture volume in a 100 ml Erlenmeyer flask. However, overnight cultures of SMK279a carrying the Pbla_{2}::rfp promoter construct formed homogenous colonies on LB agar plates containing ampicillin (100 µg/ml).
3.2.2 Heterogeneous expression of \( bla_{L1} \) and \( bla_{L2} \) at the single cell level

Since the introduction of ampicillin to the cultures were accompanied with certain morphological changes in bacterial cells, it was therefore of interest to investigate the associated expression of \( \beta \)-lactamase at a single cell level and of actively growing cells (exponential cells). It is noteworthy that treatment of cells with ampicillin resulted in formation of filament and aggregated cells in comparison to untreated cells (Figure 16A and B). Filamentation and aggregate formation were also more frequently observed during the exponential phase of bacterial growth. Therefore, addressing single cell \( \beta \)-lactamase expression in the transient acquired cellular morphology allows a better understanding of the spontaneous reactions of cells to the drug, which are often specific between the individual cells and essential to optimize antibiotic treatment strategies.

Firstly, analysis of single cells by fluorescence microscopy indicated that \( bla_{L1} \) and \( bla_{L2} \) were subjected to a heterogeneous gene expression at the single cell level using the above constructed reporter fusions (Figure 15A and B, Figure 17A-F). Thereby, bacterial cells with \( bla \)-ON mode and \( bla \)-OFF mode were detected in an isogenic population that represent cells with strong and no fluorescence, respectively. Noteworthy, an individual cell within a filament heterogeneously expressed the RFP (Figure 17C). However, the reporter protein was also expressed homogenously in cells forming a filament (Figure 17B). Cells segregated from a parent chain occurred mainly as individual bacilli, often with reduced size with further incubation of the cultures (i.e. late exponential phase) (Figure 17D). It was further noticeable that aggregated cells expressed the \( bla_{L2} \) gene heterogeneously irrespective of their position, i.e. either at the middle or at the periphery of the microcolony (Figure 17A and D).

Secondly, to investigate if there was a transient gene expression in exponentially growing bacterial cells in response to antibiotics, a cyan reporter protein that matures faster than the RFP was used (Figure 17E and F). Therefore, SMK279a carrying \( P_{bla_{L2}}::cfp \) promoter reporter fusion was induced after two h of growth. In these tests, the \( bla_{L2} \) gene expression was heterogeneous after two h of induction and the \( bla \)-OFF mode was the widely observed phenotype in the majority of cells (Figure 17E and F). Taken together, the data demonstrated that phenomenon of heterogeneity was indeed independent of the type and property of the fluorescent proteins used. In further studies the \( P_{bla_{L2}}::rfp \) reporter fusion was used to analyze phenotypic heterogeneity in more detail.
Figure 16: Treatment of cells with ampicillin resulted in formation of long cells and filaments. (A) Very long filament SMK279a observed after 4 h of ampicillin challenge in liquid cultures. (B) SEM images of overnight ampicillin-challenged bacterial cells.

Figure 17: Analysis of single cell fluorescence of Pbla$_{L2}$ reporter gene fusions in exponentially growing SMK279a. (A) Expression of the bla$_{L2}$ gene fused to rfp in SMK279a. Cells were grown at 30°C for 7 h under aerobic conditions (200 rpm) in LB medium containing 100 µg/ml ampicillin. (B) Homogenous bla$_{L2}$ expression in cells forming filament using the same fusion. (C) Phenotypic heterogeneity observed in cells (carrying the Pbla$_{L2}$::rfp fusion) forming long chains. Cells were grown overnight under aerobic conditions (120 rpm) in LB medium supplemented with 100 µg/ml ampicillin. (D) Expression of the bla$_{L2}$ promoter fused to rfp in SMK279a. Cells were grown at 30°C for 17 h under aerobic conditions (200 rpm) in LB medium containing 100 µg/ml ampicillin. (E) Cells were grown for 2 h in the presence of gentamicin (50 µg/ml) and challenged with 100 µg/ml ampicillin. The arrows show cells in a bla$_{L2}$-OFF mode and (F) a fluorescence microscopic image of (E). The arrow in (E) and (F) shows cells in bla-OFF mode.
3.2.3 Heterogeneous and homogenous expression of $bla_{L1}$ and $bla_{L2}$ genes

Single cell count of the $bla$-ON and $bla$-OFF mode cells were carried out from liquid cultures samples that were induced at the time of inoculation. For the bacterial cells carrying $P_{bla_{L2}}::rfp$ construct, samples for microscopy were taken at 7, 24, 30, 32, and 42 h of growth (Figure 18A). At each time point a minimum of 450 cells were analyzed for individual biological replicates (see Material and Methods, section 2.9.3.2). Data analysis showed that only as few as 5 % of the cells were in $bla$-ON mode for the period between inoculation and before 32 h (Figure 18A). During the same period, cells with the basal level of $bla_{L2}$ expression were shown to be less than 0.7 %.

After 32 h, however, the majority of cells expressed the fluorescent reporter RFP and, thus, was in the $bla_{L2}$-ON mode (Figure 18A). Similarly, at the single cell level, $bla_{L1}$, coding for Zn-independent metallo-$\beta$-lactamase with a broad $\beta$-lactam substrate spectrum, was also expressed heterogeneously, with less than 6 % and more than 95 % of the cells being in "ON" mode at time points of 30 h and 34 h, respectively (Figure 18B). During the exponential growth of SMK279a in LB broth, however, the majority (94 %) of cells were in the "OFF" mode (Figure 18B). Finally, the switching from heterogeneous into a homogeneous gene expression was growth-phase dependent and most likely triggered by an environmental signal.
Figure 18: Analysis of bla\textsubscript{L2} and bla\textsubscript{L1} gene expression at single cell level using the RFP. (A) Percentages of fluorescent and non-fluorescent Pb\textsubscript{L2}::rfp reporter strain cells grown for 24, 30, 32, and 42 h in LB medium containing 50 µg/ml gentamicin and ampicillin (100 µg/ml) at 30°C. (B) Percentages of fluorescent and non-fluorescent Pb\textsubscript{L1}::rfp reporter strain cells grown as described in (A). Red bars, fluorescent cells; grey bars, non-fluorescent cells. Data are mean values of four independent experiments and bars represent the standard deviations. For each individual independent experiment at least 452 cells were analyzed.

3.2.4 Alternative non β-lactam inducers of β-lactamase production

Since the majority of the cells were in bla-ON mode after 32 h and also ampicillin was inactivated by produced enzymes, it was therefore possible that the growth phase-dependent switch was not induced by antibiotics itself. To test this hypothesis, cells were cultured in the absence of β-lactams for a period of 30 h in 5 ml culture volumes. As predicted, the majority of the cells were in bla-ON mode after 24 h (Figure 19). Therefore, homogenous expression of bla\textsubscript{L2} was independent on the presence or absence of β-lactam antibiotics in the cultures.

To further identify the potential cause of homogenous gene expression, a sterile-filtered culture supernatant was obtained by growing the strain SMK279a carrying the Pb\textsubscript{L2}::rfp reporter fusion in LB broth with 100 µg/ml ampicillin and filter sterilization when the culture reached a stationary phase (2.9.3.3). Interestingly, adding increasing amounts of sterile-filtered culture supernatants to the Pb\textsubscript{L2}::rfp reporter strain resulted in switching of up to 90% of the cells into the "ON" mode.
after 7 h of incubation in 25 ml batch cultures, whereas less than 1 % of the cells of control cultures were in an "ON" mode under the same conditions (Figure 20A). Using this trigger, populations of Pbla<sub>L2</sub>::rfp reporter fusion expressing cells were increased from 15 % in "ON" mode to above 90 % in a dose dependent manner (Figure 20A). This observation suggested that a single molecule or several molecules were produced and released into the medium that were involved in triggering the switch from heterogeneous to homogenous bla<sub>L2</sub> expression.

![Graph showing percentage of fluorescent and non-fluorescent cells over time](image)

**Figure 19:** Homogenous expression of bla<sub>L2</sub> triggered by an environmental signal.

Percentages of fluorescent and non-fluorescent Pbla<sub>L2</sub>::rfp reporter strain cells grown under aerobic conditions (200 rpm) in 5 ml reaction tubes at 30°C for 17, 24 and 30 h in LB medium, containing 50 µg/ml gentamicin. Cells were grown without ampicillin. Red bars, fluorescent cells; grey bars, non-fluorescent cells. Data are mean values of four independent experiments and bars represent the standard deviations.

When cultures treated by the addition of sterile-filtered SMK279a supernatants were further cultivated, a decrease in percentage of cells in the "ON" mode was observed over a time period of up to 24 h. At 24 h, less than 5 % of the cells were in the "ON" mode. However, in such cultures the majority of cells were again in the "ON" mode after 32 h (Figure B 3). In addition, the number of CFU/ml remained fairly constant between the two states, and the majority of viable and growing bacterial populations were in an "ON" mode in a time- and growth-dependent manner. Since heat-treated culture supernatants of SMK279a failed to alter bla<sub>L2</sub> gene expression at the single cell level, the responsible signaling compound is most likely a heat-labile molecule (Figure 20B and Table 11). To further characterize this heat-labile factor, different
compounds or supernatants from previously stressed *S. maltophilia* cultures were tested (for experimental details see 2.9.3.4)

To further investigate the effect of environmental signals that possibly affect *bla* expression, overnight cultures of *Pbla*::*rfp* reporter strain were diluted in fresh LB medium supplemented with high concentrations of NaCl (0.5 M) or KCl (0.5 M). After 7 h of growth at 30°C, aliquot of withdrawn sample for image analysis revealed that *Pbla*::*rfp* gene expression was heterogeneous among salt stressed cells. Cells were also curved at the poles (Figure B 2). The effect became more prevalent as the salt concentration was raised to 1 M. Similarly, cells expressed the *bla* gene heterogeneously when cultured at 41°C in LB medium in the absence of antibiotics and salt stress. Thus, it was concluded that SMK279a cells produced a signal molecule under stress (i.e. salt) that has not yet been identified and that triggered homogeneity.

As stated above, conversion of bacterial cells from "OFF" into "ON" was achieved using culture supernatant from stationary-phase *Pbla*::*rfp* reporter strain. On the contrary, the use of sterile culture supernatant from *R. fredii* NGR234 or Xcc did not alter the *bla* gene transcription profile at the single cell level (Table 11). Thereby, the *R. fredii* NGR234 strain also expressed the *rfp* gene in pBBR1MCS-5. These data indicated that it was most likely a *Stenotrophomonas*-specific molecule involved in switching from homogeneity to heterogeneity. As a putative signaling molecule, the diffusible signal factor was tested. However, DSF failed to alter *bla* gene expression at the single cell level independent of the source and structure of the DSF used, *i.e.* extracted DSF as well as a commercially available DSF (*i.e* cis-2-11-methyl-dodecenoic acid). This signal is most likely different from the known autoinducer molecules in SMK279a (Table 11).
**Figure 20:** Cell-free culture supernatants trigger phenotypic heterogeneity in SMK279a cells. (A) Phenotypic heterogeneity of cells treated with increasing amounts of cell-free supernatants from stationary-phase $P_{bla_{L2}}::rfp$ reporter strain cultures after 7 h of growth. Data in (A) are mean values of at least three independent cultures and for each time point and culture a minimum of 408 cells were analyzed. The volume of control cultures were adjusted with 1.5 ml fresh LB at time ‘0’. (B) Heat-treated culture supernatants of SMK279a failed to alter $bla_{L2}$ gene expression at the single cell level, indicating that the responsible signaling compound was most likely a heat-labile molecule.
Results

Table 11: Phenotypic heterogeneous \(\text{bla}_{L2}\) gene expression was stimulated by several kinds of stresses.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Heterogeneity in EM2*</th>
<th>Time (h)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Antibiotics added to SMK279a EM2</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ampicillin (100 μg/ml)</td>
<td>+</td>
<td>17</td>
</tr>
<tr>
<td>Cefuroxime (100 μg/ml)</td>
<td>+</td>
<td>17</td>
</tr>
<tr>
<td>Meropenem (16 μg/ml)</td>
<td>+</td>
<td>17</td>
</tr>
<tr>
<td><strong>QS signalling molecules added to SMK279a EM2</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DFS extract from SMK279a</td>
<td>-</td>
<td>17</td>
</tr>
<tr>
<td>Cis-11-2-dodecenoic acid (94.2 mM)</td>
<td>-</td>
<td>17</td>
</tr>
<tr>
<td><strong>Salt added to, or heat treatment of SMK279a EM2</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>KCl (500 mM)</td>
<td>+</td>
<td>17</td>
</tr>
<tr>
<td>NaCl (1,000 mM)</td>
<td>+</td>
<td>17</td>
</tr>
<tr>
<td>NaCl (500 mM)</td>
<td>++</td>
<td>17</td>
</tr>
<tr>
<td>Growth at 41°C</td>
<td>+</td>
<td>17</td>
</tr>
<tr>
<td><strong>Sterile filtered culture supernatants from</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SMK279a parent strain, ampicillin (100 μg/ml)</td>
<td>-</td>
<td>7</td>
</tr>
<tr>
<td>SMK279a parent strain, ampicillin (100 μg/ml), heat inactivated &amp; NaCl, 500 mM</td>
<td>-</td>
<td>7</td>
</tr>
<tr>
<td>SMK279a, ampicillin (100 μg/ml), NaCl 500 mM</td>
<td>-</td>
<td>7</td>
</tr>
<tr>
<td>SMK279a EM2</td>
<td>+++</td>
<td>7</td>
</tr>
<tr>
<td>SMK279a EM2, w/o ampicillin heat inactivated</td>
<td>-</td>
<td>7</td>
</tr>
<tr>
<td>SMK279a EM4</td>
<td>-</td>
<td>7</td>
</tr>
<tr>
<td>SMK279a EM1</td>
<td>-</td>
<td>7</td>
</tr>
<tr>
<td>Xcc</td>
<td>-</td>
<td>7</td>
</tr>
<tr>
<td>R. fredii NGR234</td>
<td>-</td>
<td>7</td>
</tr>
<tr>
<td>R. fredii NGR234::PrpoD::rfp</td>
<td>-</td>
<td>7</td>
</tr>
</tbody>
</table>

* The number of plus sign indicates the degree of effect on heterogeneous \(\text{bla}_{L2}\) expression at single cell level.

3.2.5 Phenotypic heterogeneity with additional \(\beta\)-lactam antibiotics

Ampicillin has been considered as strong inducer of an inducible \(\beta\)-lactamase production in Gram-negative bacteria. To further study the observed heterogeneity, cefuroxime and meropenem were tested for their potential effects on heterogeneous regulation of the \(\beta\)-lactam gene in SMK279a (Table 11, Figure 21A). At single cell level, \(\text{bla}_{L2}\) was also expressed heterogeneously with these antibiotics, with less than 6% of the cells being in "ON" mode at time point of 24 h (Table 11 and Figure 21A). Interestingly, the observed cell to cell variations were independent of the type of \(\beta\)-lactam antibiotics used (Table 11 and Figure 21A). The replenishment ampicillin after 7 h of growth did not alter significantly the \(\text{bla}-\text{ON}\) and the \(\text{bla}-\text{OFF}\)
mode cells after 24 h (Figure 21B). For fed-batch culture experiments, 25 % of the culture media was removed after 7 h growth and substituted with the same volume fresh LB with ampicillin (100 µg/ml).

Figure 21: Heterogeneity in \textit{bla}_{L2} expression. (A) Comparison of cefuroxime (CXM) and ampicillin (AMP) on single cell \textit{bla}_{L2} expression in batch culture. (B) Comparison of fed-batch and batch growth conditions on single cell expression of \textit{bla}_{L2} gene in SMK279a. For fed-batch culture experiments, after 7 h growth, 25 % of the culture media was removed and substituted with the same volume fresh LB with ampicillin (100 µg/ml). The results shown above were obtained after 24 h of growth.

3.3 Molecular switches of phenotypic heterogeneity

Phenotypic heterogeneity with respect to expression resistant genes might be caused by non-genetically or epigenetically controlled processes and/or acquired after initial exposure to antibiotics. In this study, next-generation sequencing technology was used to uncover possible molecular switches determining heterogeneity.
3.3.1 Mutations did not affect colony heterogeneity formation

After exposure to antimicrobial stress, the bacterial stress response is mostly accomplished without evolving major changes and/or re-organization in the genetic make-up of the cell. However, in few cases the altered phenotypes may result from mutations. To uncover, if genetic mutations were involved in the colony heterogeneity formation in the presence of ampicillin on agar plates, DNA from 24 colonies (representing 9 big, 13 small and 2 uniform colonies) was sequenced and the whole genome sequences established and mapped on the published SMK279a reference genome (Table 12). Libraries were generated following the Nextera XT DNA Library Preparation Kit und sequenced with a minimum coverage of 50-fold (for experimental details see 2.10.2).

Compared with the published SMK279a genome, WGS identified a total of 55 mutations in the analyzed samples. Out of these 28 mutations were found in the intergenic regions, 18 in hypothetical protein coding ORFs, and nine in annotated proteins coding ORFs (Table 13). The majority of these intergenic mutations were within 200 bp of the regulatory regions of proteins of unknown functions. Within sequenced samples, WGS identified per colony two to 15 SNPs, up to seven deletions and four insertions (Table 12). Furthermore, SNPs or smaller deletions occurred in not more than 16 genes and none of these appeared to be linked to a gene that is essential to growth (Table 13). Thereof, only one may be linked to β-lactam treatment: Smlt3855, a putative UDP-N-acetylmuramate--L-alanyl-gamma-D-glutamyl-meso-diaminopimelate ligase. Two other regions in the SMK279a genome were identified as hotspots for mutations. These loci are assigned to genes coding for the hypothetical protein (Smlt1844B) and the two-component regulatory system-sensor histidine kinase (Smlt3944). Smlt1844B and Smlt3944 carried mutations at seven or nine base positions, respectively. These mutations resulted in amino acid substitutions in the respective proteins. The nonsynonymous mutations that occurred in the smlt3944 gene were further located in the DNA region annotated as signal receiver domain histidine kinase (Figure 22).

Furthermore, variants were detected using customized Perl scripts using a minimum threshold of 10-fold coverage and a minimum allele frequency of 75 % (for experimental details see 2.10.2). Therefore, 9 out of 24 samples matched exactly to reference genome that contains small, big and uniform colonies. The remaining variants were matched to reference genome with slight difference of one to three node distances (Figure 23). Taken together, WGS revealed that mutations in the genome of SMK279a did not affect β-lactam resistance phenotypes shown on agar plates.
Table 12: Summary of mutations identified in SMK279a samples by whole-genome sequencing.

<table>
<thead>
<tr>
<th>Isolate ID</th>
<th>Colony morphology</th>
<th>Ampicillin [μg/ml]</th>
<th>Coverage after analysis [%]</th>
<th>SNPs</th>
<th>DEL*</th>
<th>INS*</th>
</tr>
</thead>
<tbody>
<tr>
<td>K279a-1</td>
<td>Big</td>
<td>600</td>
<td>98.4</td>
<td>1</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>K279a-2</td>
<td>Big</td>
<td>600</td>
<td>104.3</td>
<td>1</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>K279a-3</td>
<td>Small</td>
<td>100</td>
<td>52.8</td>
<td>1</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>K279a-4</td>
<td>Small</td>
<td>100</td>
<td>56.9</td>
<td>3</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>K279a-5</td>
<td>Small</td>
<td>100</td>
<td>56.4</td>
<td>3</td>
<td>0</td>
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</tr>
<tr>
<td>K279a-6</td>
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<td>100</td>
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</tr>
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<td>K279a-7</td>
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<td>62.0</td>
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</tr>
<tr>
<td>K279a-9</td>
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<tr>
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<tr>
<td>K279a-11</td>
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<tr>
<td>K279a-12</td>
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<tr>
<td>K279a-15</td>
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<td>109.7</td>
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<tr>
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<td>0</td>
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<td>K279a-22</td>
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<td>68.9</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>K279a-23</td>
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<td>100</td>
<td>62.4</td>
<td>1</td>
<td>0</td>
<td>0</td>
</tr>
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<td>K279a-24</td>
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<td>100</td>
<td>66.6</td>
<td>3</td>
<td>0</td>
<td>0</td>
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<tr>
<td>K279a-25</td>
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<td>100</td>
<td>87.4</td>
<td>1</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>K279a-26</td>
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<td>100</td>
<td>95.3</td>
<td>2</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>K279a-27</td>
<td>Small</td>
<td>100</td>
<td>112.3</td>
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<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

*DEL, deletion; INS, insertion.

Figure 22: Partial physical arrangement of the two component regulatory system-sensor histidine kinase Smlt3944 and flanking ORFs on the SMK279a genome. The black arrow indicates the histidine kinase Smlt3944, grey-shaded arrows indicate flanking ORFs that are possibly under the regulatory effect of this sensor histidine kinase. Colored boxes indicate the...
domains found within the sequence of Smlt3944. All SNPs identified in smlt3944 are located within the signal receiver domain (A) of the histidine kinase and are indicated by a vertical arrow.

Table 13: SNPs observed in different colony morphotypes of SMK279a. Mutations were determined comparing all 24 sequenced/analyzed colonies to our laboratory reference SMK279a genome sequence.

<table>
<thead>
<tr>
<th>ORF/gene</th>
<th>Sum of all SNPs in colony samples*</th>
<th>Number of positions affected within ORF</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>small</td>
<td>uniform</td>
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<tr>
<td>Smlt0301</td>
<td>0</td>
<td>1</td>
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<tr>
<td>Smlt0590</td>
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<td>0</td>
</tr>
<tr>
<td>Smlt0967</td>
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<td>0</td>
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<tr>
<td>Smlt1450</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>Smlt1562</td>
<td>6</td>
<td>0</td>
</tr>
<tr>
<td>Smlt1844B</td>
<td>27</td>
<td>3</td>
</tr>
<tr>
<td>Smlt1958</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>Smlt2315 (flgD)</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Smlt2360</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>Smlt3752 (sucD)</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Smlt3885 (mpl)</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>Smlt3927 (pcm)</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>Smlt3944</td>
<td>22</td>
<td>2</td>
</tr>
<tr>
<td>Smlt4235</td>
<td>4</td>
<td>0</td>
</tr>
<tr>
<td>Smlt4306a2 (cap)</td>
<td>3</td>
<td>0</td>
</tr>
</tbody>
</table>

*Genomes of 13 small, nine big, and two uniform SMK279a colonies were analyzed.
Figure 23: Maximum parsimony tree built from 15 single nucleotide polymorphism positions identified in different colony variants compared to SMK279a. The SNPs were identified in a set of 13 small, nine big and two uniform colony variants compared to the genome sequence of strain SMK279a. Numbers on branches indicate the number of distinct SNP positions between isolates. The tree was calculated with the program suite BioNumerics version 7.5 (AppliedMaths, Sint-Martens-Latem, Belgium).

3.3.2 Differential expression of genes among colony morphotypes

It was shown here that isogenic populations of SMK279a formed colonies that not only differed in growth on agar plates but also acquired mutations that did not affect colony morphotypes formation. Indeed, it can be hypothesized that the colony morphotypes resulted either from gene expression differences on the cellular level within isogenic populations or from the heterogeneous expression of resistance genes on a single cell level. Therefore, the average transcriptional changes among cells forming colony morphotypes were analyzed using RNA-seq. Total RNA was isolated from 10-15 mg cell material of colonies that were grown for 48 h at 37°C. The RNA was converted to cDNA libraries and sequenced as described in the methods section (2.13). A
Results

total of between 7.3 and 12.3 million reads were generated for each biological replicate and more than 98 % of the unique reads were mappable to the reference genome SMK279a (Acc.no.: NC_010943.1) (Table 14).

Table 14: Overall transcriptome metadata for the analyzed SMK279a colony morphotypes samples.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Colony variant</th>
<th>No. of reads generated (x10^6)</th>
<th>No. of uniquely mapped reads (x10^6)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>big colony</td>
<td>8.80</td>
<td>0.69</td>
</tr>
<tr>
<td>2</td>
<td>big colony</td>
<td>9.88</td>
<td>0.80</td>
</tr>
<tr>
<td>3</td>
<td>small colony</td>
<td>9.72</td>
<td>0.63</td>
</tr>
<tr>
<td>4</td>
<td>small colony</td>
<td>12.35</td>
<td>0.98</td>
</tr>
<tr>
<td>5</td>
<td>uniform colony</td>
<td>7.35</td>
<td>0.38</td>
</tr>
<tr>
<td>6</td>
<td>uniform colony</td>
<td>9.60</td>
<td>1.25</td>
</tr>
</tbody>
</table>

For comparative analyses of RNA-seq data, genes with a fold-change of ≥ 2.0, a Likelihood value ≥ 0.9 and an adjusted P-value of ≤ 0.05 were considered as statistically significant and differentially expressed between the colony variants (see Material and Methods, section 2.13). Thereby, genome wide transcription analysis revealed that isogenic population of SMK279a cells regulated various genes differentially among colony morphotypes (Table 15, Figure 24). As described in Table 15, a total of 34 genes were altered transcriptionally when comparing ampicillin challenged colonies to untreated samples. However, only 12 genes were significantly and differentially regulated between big and small colony variants (Table 15). The transcriptional response in small and big colony variants to β-lactam challenge involved a differentially expressed resistant genes blaL1 and blaL2 (Table 15). Therefore, blaL1 (smlt2667) and blaL2 (smlt3722) were 15.3- and 6.9-fold up-regulated in cells forming big colonies in comparison to those forming small colonies, respectively. Up-regulated genes included, among others, the hypothetical proteins assigned to locus smlt0018 and smlt0019, which most likely form an operon. While all transcripts were sense strand a hypothetical exported protein (Smlt2668) which is located downstream to blaL1 was antisense. Furthermore, transcripts of ampR between the different colony morphotypes were analyzed on the basis of the RNA-seq data. Despite the fact that cells forming small colonies were investigated under the selective pressure of ampicillin only slightly upregulated blaL1 and blaL2 gene transcripts were noticed as cells were forming homogenous colonies on agar plates (Figure 25). The transcriptional activation of these genes in the populations of small colonies ranged from 1 to 1.5-fold change compared to uniform colonies that were handled in an antibiotic-free condition. At sub-MIC levels ampR was not differentially expressed in the big and small colonies. In addition to ampR, several other transcripts previously identified in regulation of β-lactamase expression in SMK279a were only slightly differentially
regulated (Table 16). On the other hand, the $\text{bla}_{L2}$ flanking gene $\text{ampH}$ was three-fold up regulated in big colonies. The function of $\text{ampH}$ remains to be determined.

**Figure 24: RNA-seq data analysis for different SMK279a colony morphotypes.** Scatter plot of fold change in transcription of genes among cells of big ($Y$-axis) and small ($X$-axis) colonies relative to untreated samples of uniform colonies grown in absence of ampicillin on LB agar plates. A two-fold change in the genes transcription with adjusted $P$ value of $\leq 0.05$ is considered as significantly differentially regulated. Cells forming big colonies differentially regulated several genes mainly involved in degrading antibiotics; numbers in plot indicate most strongly regulated genes 1, $\text{smlt0018}$; 2, $\text{smlt2667}$; 3, $\text{smlt3772}$; 4, $\text{smlt2668}$; 5, $\text{smlt0019}$; 6, $\text{smlt3721}$; 7, $\text{smlt1760}$ and 8, $\text{smlt0596}$. 
Results

Figure 25: Transcriptome profiles of \textit{bla}_L2 and the flanking genes \textit{ampR (smlt3723)} and \textit{ampH (smlt3721)} among the colony morphotypes. The \textit{bla}_L2 gene was 6.9-fold and 7.4-fold down-regulated in cells forming small colonies in comparison to big and uniform colonies, respectively. Transcriptome profile images of the leading strand (indicated in red) and the lagging strand (indicated in blue) were generated with the IGB software (Nicol et al., 2009), merged and rearranged on the leading strand for a simplified visualization.

Since AmpR is the central regulator for the expression of β-lactamases and a weakly constitutive expression of \textit{ampR} was shown above for \textit{S. maltophilia} where the level of expression was unaffected by β-lactams pressure, the loss of expression of the \textit{ampR (smlt3723)} on colony morphotypes was analyzed by constructing an \textit{ampR}-deletion mutant strain. The SMK279aΔsmlt3723 deletion was constructed using the pGPI-Scel/pDAI-Scel-SacB system (Figure 7C) and was verified by PCRs and DNA sequencing, via next generation sequencing (NGS) whole genome sequencing (Table B 2). With the loss of the activity in the \textit{smlt3723} gene, the bacterial cells were unable to grow on LB agar plates supplemented with ampicillin (100 μg/ml) indicating that both basal and inducible expression of \textit{bla}_L1 and \textit{bla}_L2 depends on AmpR. No more colonies appeared with extended incubation of agar plates to 72 h. The complete absence of especially small colonies in the strain carrying \textit{ampR} gene deletion indicated that the basal level of expression of these enzymes was responsible for the ability of cells forming small colonies to grow under antibiotic stress.
Table 15: Differentially expressed genes for three colony morphotypes in SMK279a. The table shows genes with a fold-change of ≥ 2.0, a Likelihood value ≥ 0.9 and an adjusted P-value of ≤ 0.05.

<table>
<thead>
<tr>
<th>Locus tag</th>
<th>Predicted function</th>
<th>Fold change big vs. small</th>
<th>Fold change big vs. uniform</th>
<th>Fold change small vs. uniform</th>
</tr>
</thead>
<tbody>
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<td>hypothetical protein</td>
<td>+7.6</td>
<td>+11.4</td>
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<td>Smlt0019</td>
<td>hypothetical protein</td>
<td>+2.2</td>
<td>+3.9</td>
<td>-</td>
</tr>
<tr>
<td>Smlt0706</td>
<td>fimbria adhesin protein</td>
<td>-2.3</td>
<td>-2.0</td>
<td>-</td>
</tr>
<tr>
<td>Smlt0709</td>
<td>fimbria adhesin protein</td>
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<td>-</td>
<td>-</td>
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<td>Smlt1735</td>
<td>tRNA</td>
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<td>-</td>
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<td>Smlt1766</td>
<td>hypothetical protein</td>
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<td>-</td>
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<td>Smlt2667</td>
<td>β-lactamase</td>
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<td>Smlt3721</td>
<td>predicted antiporter</td>
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<td>Major facilitator superfamily transmembrane protein</td>
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<td>-</td>
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<td>hypothetical protein</td>
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<td>-</td>
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<td>Smlt3056</td>
<td>transmembrane protein</td>
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<td>-</td>
<td>+2.1</td>
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<td>anti-sigma factor RseA</td>
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<td>-</td>
<td>-2.1</td>
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<td>Dehydrogenase</td>
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<td>-</td>
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<td>two-component system response regulator, AlgR</td>
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<td>endonuclease L-PSP family protein</td>
<td>-</td>
<td>-</td>
<td>+2.0</td>
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</table>
Table 16: Genes and their products known to be involved in the regulation of β-lactamase expression in SMK279a. The transcripts of these genes were not differentially expressed in big colonies that up-regulated β-lactam gene.

<table>
<thead>
<tr>
<th>Locus tag</th>
<th>Predicted function</th>
<th>Fold change (big colonies)</th>
<th>Evidence for activity</th>
</tr>
</thead>
<tbody>
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<td>Smlt3723</td>
<td>AmpR</td>
<td>+1.8</td>
<td>(Okazaki and Avison, 2008, Lin et al., 2009)</td>
</tr>
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<td>Smlt3538</td>
<td>NagZ</td>
<td>+1.6</td>
<td>(Huang et al., 2012)</td>
</tr>
<tr>
<td>Smlt3826</td>
<td>MrcA</td>
<td>+1.0</td>
<td>(Lin et al., 2009)</td>
</tr>
<tr>
<td>Smlt0154</td>
<td>AmpDII</td>
<td>+1.2</td>
<td>(Yang et al., 2009)</td>
</tr>
<tr>
<td>Smlt0413</td>
<td>AmpG</td>
<td>-0.8</td>
<td>(Huang et al., 2012)</td>
</tr>
<tr>
<td>Smlt1562</td>
<td>AmpDI</td>
<td>-0.8</td>
<td>(Yang et al., 2009)</td>
</tr>
</tbody>
</table>

3.3.2.1 Reverse transcription

RT-qPCR experiments were done for selected differentially regulated genes in order to verify data obtained by RNA-seq (for experimental details see sections 2.12 and 2.14). Therefore, cDNA was generated with random hexamer primers using 1.2 µg RNA isolated from colonies that were grown for 48 h in the presence of ampicillin (100 µg/ml) (Figure 26B). Using this cDNA as a template and gene specific primers, internal DNA fragments of the genes between 150 bp and 250 bp were PCR amplified (Table 7, Figure 26A). The expression values were normalized against *rpoD* (Figure 26C). The data was in agreement with RNA-seq in that case both β-lactamase and the Na⁺/H antiporter genes were among the strongly upregulated genes in cells forming big colonies (Figure 26D). Among cells forming big vs. small colonies, RT-qPCR indicated a 10- and 12.45 fold up-regulations in the transcription of *blaL1* and *blaL2* genes, respectively, while 12 fold up-regulation was detected for the flanking *ampH* (Figure 26D). Additionally, the hypothetical proteins which are probably transcribed as operon were also regulated differentially in big vs. small colony variants (Figure 26D).
Figure 26: RT-qPCR data analysis for different SMK279a colony morphotypes. (A) The internal DNA fragments of the PCR amplified candidate genes indicating the specificity of the primer used. (B) Total RNA was isolated from 10-15 mg of wet biomass of the colony morphotypes and displayed on a 1.25 % formaldehyde/MOPS agarose gel. Intact total RNA ran on a denaturing gel indicating a sharp 23S and 16S rRNA bands. (C) RT-qPCR amplification curves of the reverse transcribed samples showing different crossing point C<sub>q</sub> values for bla<sub>L1</sub> and bla<sub>L2</sub> in big vs. small colonies. The C<sub>q</sub> values for the two reference genes (rpoD and 16S rRNA) used do not vary much among the colony morphotypes. Numbers in the (C) indicate amplification curves; 1, 16S rRNA (big and small); 2, rpoD (big and small); 3, bla<sub>L1</sub> (big); 4, bla<sub>L2</sub> (big); 5, bla<sub>L1</sub> (small) and 6, bla<sub>L2</sub> (small). (D) RT-qPCR of genes identified in RNA-seq. The SuperScript<sup>®</sup> VILO<sup>™</sup> cDNA synthesis kit (Invitrogen<sup>™</sup>, life technologies, TX, USA) was used to generate cDNA using 1.2 µg RNA. Both β-lactamase and the Na<sup>+</sup>/H antiporter genes shown above are among the strongly upregulated genes in cells forming big colonies. The expression values were normalized against rpoD and were obtained from three independent experiments.
3.3.3 RNA-seq analysis of \textit{bla}$_{L2}$ ON vs. OFF cells

Detailed statistical analyses of several hundred cells for each time point during growth in LB medium revealed that the majority (>90 \%) of the cells were in the \textit{bla}$_{L2}$-OFF mode during the first 24 h of growth in 25 ml batch cultures on a shaker (200 rpm). After 32 h, however, the majorities of cells expressed the red fluorescent reporter and, thus, were in the \textit{bla}$_{L2}$-ON mode (Table 17). In the light of these observations, cells that were cultured for 27 and for 32 h were harvested and analyzed their transcriptomes using RNA-seq. Samples for RNA-seq analysis were collected at time points of 27 and 32 h representing \textit{bla}$_{L2}$-ON and \textit{bla}$_{L2}$-OFF mode in the majority of cells at the single cell level, respectively. For each time points two independent biological replicates of samples were immediately frozen in liquid nitrogen (Figure 8). Total RNA extraction and cDNA synthesis were accomplished as described before (see Material and Methods, section 2.13). For each sample, a total of 6.2 to 6.8 million of reads were generated and unique reads were mapped to the reference genome SMK279a (Table B 1).

RNA-seq data of \textit{bla}$_{L2}$-ON vs. \textit{bla}$_{L2}$-OFF mode cells identified candidate genes possibly involved in modulating the phenotypic heterogeneity in SMK279a (Figure 27). The identified genes encode for a putative competence protein (Smlt1134) homologous to \textit{comE}, and two putative transmembrane efflux proteins (Smlt2851 and Smlt2852) encoded in an operon. The \textit{comE} gene encodes for a DNA-binding protein that is in part regulated by the RNA-binding protein (Hfq). The Hfq influences cellular responses to antibiotics via interacting with small RNAs (sRNAs) and mRNAs. Additionally, the transmembrane efflux protein is one of the major mechanisms of drug resistance in bacteria. Bacterial efflux transporters use the proton motive force (PMF) to efficiently extrude toxic effects of drugs such as dyes, and antibiotics from the bacterial cell (Putman et al., 2000).

Between "ON" and "OFF" mode cells, the \textit{smlt2851} and \textit{smlt2852} genes were 29- and 7.2-fold up-regulated, respectively, while \textit{smlt1134} was 5.4-fold down-regulated. Additional RT-qPCR data supported the observation made for the \textit{comE} homologue. RT-qPCR indicated a 1.74±0.29 fold down-regulation in the transcription of the \textit{smlt1134} gene among cells that were in the \textit{bla}$_{L2}$-ON vs. \textit{bla}$_{L2}$-OFF mode cells (Figure B 6). For the \textit{smlt2851} and \textit{smlt2852} loci, RT-qPCR data did not confirm the RNA-seq data.
Results

Figure 27: Partial physical arrangement of genes whose fold expression level was significantly altered in "ON" and "OFF" mode cells (32 h vs. 27 h) along with transcription profiles. Between "ON" and "OFF" mode cells, the \textit{smlt2851} and \textit{smlt2852} genes were 29- and 7.2-fold up-regulated, respectively, while \textit{smlt1134} was 5.4-fold down-regulated. Transcriptome profile images were generated with IGB software and merged (indicated with vertical dashed line) for visualization.

3.3.4 Phenotypic heterogeneity in the background of mutants

Phenotypic heterogeneity was also analyzed in the genetic background of newly constructed mutants defective in the synthesis of Smlt1134, Smlt2851, Smlt2852 and Smlt0387 (containing the Ax21 homologue signaling peptide). Since it was indicated that Ax21 homologue acts as a cell-cell signal to regulate a diverse range of functions, including virulence, its effects on heterogeneous \textit{bla} expression was also analyzed by knocking out the functional gene \textit{smlt0387}. Therefore, except for \textit{smlt0387}, markerless SMK279a mutants were constructed using the pGPI-Scel/pDAI-Scel-SacB system originally developed for bacteria of the genus \textit{Burkholderia} (Figure 6 and Figure 7). Via triparental mating, the \textit{Pbla\textsubscript{A2}::rfp} reporter fusion was introduced to the respective mutant strain and the correct transformant was obtained as stated in section 2.8.2 of the Material and Methods.

Single cell expression studies in the background of SMK279a\textDelta{smlt0387} revealed that the majority of the cells were in \textit{bla}-OFF mode in exponential state, while later in \textit{bla}-ON mode in the cultures after 32 h growth. Therefore, it was noteworthy that the growth phase dependent \textit{bla}\textsubscript{A2}
expression remained unaffected by the loss of the activities of the smlt0387 gene. Likewise, the mutants SMK279aΔsmlt1134 and SMK279aΔsmlt2851Δsmlt2852 were analyzed for their effect on single cell bla\textsubscript{L2} expression. However, the loss of neither ComE homologue nor putative multidrug transporter affected the growth phase dependent switch of bla\textsubscript{L2} expression at 32 h of growth (Table 17). This data suggested that the lack of expression of these genes were not linked to heterogeneous expression of bla\textsubscript{L2}.

**Table 17**: Effects of SMK279a Pbla\textsubscript{L2}::rfp expression on a single cell level.

<table>
<thead>
<tr>
<th>Assayed SMK279a strain</th>
<th>Gene copy added</th>
<th>Predicted function</th>
<th>% of cells showing the bla\textsubscript{L2}-ON mode after 32 h of growth</th>
</tr>
</thead>
<tbody>
<tr>
<td>SMK279a EM2</td>
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<td></td>
<td>&gt;90 %</td>
</tr>
<tr>
<td>SMK279a EM8</td>
<td>smlt1134</td>
<td>competence protein</td>
<td>&lt; 1 %</td>
</tr>
<tr>
<td>SMK279a EM9</td>
<td>smlt2851-smlt2852</td>
<td>multidrug transporter</td>
<td>&gt;90 %</td>
</tr>
<tr>
<td>SMK279aΔsmlt1134</td>
<td></td>
<td></td>
<td>&gt;90 %</td>
</tr>
<tr>
<td>SMK279aΔsmlt2851Δsmlt2852</td>
<td></td>
<td></td>
<td>&gt;90 %</td>
</tr>
<tr>
<td>SMK279aΔsmlt0387</td>
<td></td>
<td></td>
<td>&gt;90 %</td>
</tr>
</tbody>
</table>

### 3.3.5 Overexpression proteins in SMK279a

As it is shown above the presence of the SMK279aΔsmlt1134 or SMK279aΔsmlt2851Δsmlt2852 mutation did not alter bla\textsubscript{L2} gene expression at the single cell level when compared to the parent SMK279a carrying Pbla\textsubscript{L2}::rfp reporter fusion. It is noteworthy that this observation partly reconciled with strong downregulation of comE gene in bla-ON cells. To investigate if the overexpressed smlt1134 and smlt2851-52 genes affected heterogeneous expression of the bla\textsubscript{L2} gene on single cell level, the respective operons and predicted promoter coding regions were PCR amplified and inserted upstream of the RFP in the reporter plasmid pBBR1MCS-5::Pbla\textsubscript{L2}::rfp (Figure 28A). These fragments were in size 1095 bp and 1297 bp for smlt1134 and smlt2851-52, respectively. The correctness of the constructs and orientation of ORFs were verified by sequencing. The recombinant plasmids were then transferred into SMK279a cells and were challenged with ampicillin (100 µg/ml).

Initial fluorescence microscopy analysis revealed an altered population-wide bla\textsubscript{L2} gene expression profile in the presence of ComE overexpression (Figure 28B). Interestingly, the expression of extra copies of the ComE homologue in SMK279a abolished bla\textsubscript{L2} heterogeneity on single cell level after 33 h of growth (Figure B 5). While this was certainly an additional piece of evidence that supported the involvement of the ComE protein, phenotypic heterogeneity was unaffected by overexpression of multidrug transporter proteins (Figure 28C). Therefore, in addition to stress (3.2.4), the overexpression of the ComE homologue almost completely reduced
the percentage of cells in bla-ON mode from > 90 % to 1 % or less. Interestingly, this effect could be reverted by addition of cell-free supernatant (3.2.4).

**Figure 28: Expression of ComE homologue under its native promoter.** (A) Physical map and orientation of comE and putative transporter genes in pBBR1MCS-5. (B) Expression of ComE in SMK279a affects blaL2 heterogeneous expression. Cells were cultured in LB medium supplemented with 100 µg/ml ampicillin for 7 h. Left and right panels are a bright-field and fluorescence microscopic image, respectively. (C) Expression of putative transporter genes in SMK279a did not alter phenotypic heterogeneous expression of blaL2 gene. Cells were cultured in same condition as indicated in (B). Left and right panels are a bright-field and fluorescence microscopic image, respectively.

Furthermore, about 150 ml of diluted bacterial cultures were pipetted into a black microplate with transparent bottom and incubated at ambient temperature in a Synergy HT microplate reader (2.9.3.5). The intensity of fluorescence, as measured by relative fluorescence unit (RFU), was collected each h and when required analyzed with fluorescence microscopy. Analysis of the fluorescence and single cell expression data indicated that additional overexpression of multidrug transporter allowed over 90 % of cells to enter bla-ON mode at least 2 h earlier than SMK279a.
only carrying $P_{bla_{2}::rfp}$ reporter fusion (Figure 29). In contrast, cells with ComE overexpression remained in $bla$-OFF mode (Figure 29). Therefore, the data implied that ComE affected heterogeneous $bla_{2}$ expression but its effect could be reverted by unidentified signal molecules (see section 3.2.4).

![Figure 29: Time course of the intensity of fluorescence as measured by relative fluorescence unit (RFU).](image)

**Figure 29:** Time course of the intensity of fluorescence as measured by relative fluorescence unit (RFU). For the measurement, 150 ml diluted bacterial cultures were pipetted into black microplate with transparent bottom and incubated at ambient temperature in Synergy HT microplate reader. Overexpression of multidrug transporter allowed over 90 % of cells to enter $bla$-ON mode at least 2h earlier than SMK279a only carrying $P_{bla_{2}::rfp}$ reporter fusion. Cells with ComE overexpression remained in $bla$-OFF mode.

### 3.4 Heterogeneity and relation to virulence

Phenotypic heterogeneity as demonstrated by colony morphotypes formation resulted from a difference in the transcriptional profile of genes, mainly involving $bla_{1}$ and $bla_{2}$ genes, when the
bacterial cells were challenged with β-lactam. Additionally, gene products with diverse cellular functions were altered transcriptionally in cells of small colonies, where the products of some genes were known as virulence determinants in *S. maltophilia*. The level of altered expression of these genes ranged from 2.0- to 3.6-fold, including down-regulation and up-regulation of eight and six of the 14 genes, respectively (Figure 30). Up-regulated genes in these cells were mainly involved in virulence (Figure 30). Furthermore, as shown in Figure 30, the product of the gene *smlt2601* encoding for putative poly (β-D-mannuronate) lyase was significantly upregulated in small colonies. Compared to uniform colonies, it was 2-fold upregulated in small colonies.

**Figure 30: Differentially regulated genes in small colonies in comparison to big and uniform colonies.** Down-regulation of *bla* subunits affected genes involved in metabolism, transport, regulation, virulence and genetic information processing.

Further analysis of the up-regulated genes in small colonies led to the identification of an operon encoding for fimbriae adhesin proteins SMF-1 (Figure 31). The Smf-1 fimbrial operon includes Smlt0706-Smlt0709 (Figure 31). Among these genes, *smlt0706* (major type 1 subunit fimbrin) and *smlt0709* (fimbria adhesin protein precursor) were upregulated 2.3- and 2-fold respectively when comparing small to big colonies. In particular, the major type 1 subunit fimbrin protein was previously shown to be expressed at 37°C, but not below 18°C and is involved in haemagglutination, biofilm formation and adherence to cultured mammalian cells (de Oliveira-Garcia *et al.*, 2003).
Results

Figure 31: Transcriptome profiles of the Smf-1 fimbrial operon (red arrow) among the colony morphotypes. The smlt0706 (major type 1 subunit fimbrin) and smlt0709 were 2.3-fold and 2.0-fold up-regulated in cells forming small to big colonies, respectively.

Comparative analyses of RNA-seq data further unveiled that the cells of small colonies differentially regulated genes of unique structure—rhs (rearrangement hotspot), a family of enigmatic composite genes that are widespread among Gram-negative bacteria. This family includes rhsA encoded by smlt0044. The product of Smlt0044 has a length of 678 amino acids and was 2-fold upregulated in small colonies. Further, tblastn analysis indicated that RHS families have extended repeat regions. Though an evidence for gene expression and their functions are still elusive in the rhs family, it was shown here that small colonies upregulated smlt0044 in response to β-lactam exposures. The differential regulation of this gene further revealed that stress related factors might be triggered its transcription and supported the diverse roles these proteins in bacterial cells. In summary, the differentially regulated genes in small colonies might also be attributed to the slow growth observed on agar plates (Figure 9B-E).

In line with the above RNA-seq data, the colony morphotypes were analyzed for their ability to build-up a biofilm on plastic surfaces. In these test, cell forming small colonies formed thicker biofilm on polystyrene after 24 h of growth (Figure 32). Biofilm formation in SMK279a cells was further affected by the presence of ampicillin in the culture, that cells treated with ampicillin showed increased biofilm formation. Further, big colonies produced comparable biofilm to the parent population.
Figure 32: Biofilm formation of colony morphotypes. Cells were grown for 24 h and biofilm formation was estimated following crystal violet staining. Data indicate results from triplicate analyses. Sister progeny cells taken from the small colonies formed thicker biofilms compared to big colonies.

3.5 Genotypic and phenotypic characterization of S. maltophilia isolates

A phylogenetic tree of the S. maltophilia isolates was generated utilizing the 394 SNP positions identified in the 106 isolates. The SNPs were identified in a set of 106 S. maltophilia isolates compared to the genome sequence of strain SMK279a. The tree visualizes that the isolates tend to form cluster based on isolation source (clinical, CF, intensive care unit-ICP, environment) (Figure 33). Indeed, a number of isolates from the same sources were present on the same branches of the tree (Figure 33). Furthermore, the S. maltophilia isolates were strewed in the tree that probably indicating the core genome is small (for experimental details see 2.10.2). Therefore, the isolates exhibited more genetic diversity that set to exploit diverse niches.

S. maltophilia isolates were further assed for their ability to form biofilm on microtiter plates and for extracellular enzymes productions (Figure 34 and Figure 36). The level of biofilm formation by S. maltophilia isolates that were obtained from different isolation sources was determined using the microtiter plate assay and was varied significantly from 3.9 to 0.13 (Figure 34 and Figure 35). Comparing the OD measurements to isolations sources, there was a significant difference between isolates of human origin to that of environmental (Figure 34). However, the biofilm-forming capacity was not varied significantly among isolates of human origin when compared to sources, i.e. cystic fibrosis, intensive care patients, respiratory tract or other parts. The highest
Results

OD\textsubscript{595} value was observed for isolates belonging to LMG10873 (Figure 34).

To further classify the isolate based on biofilm forming capacity a low cut-off (ODc) value was set off as three standard deviations above the mean of control wells. Based on OD, isolates were classified as: no biofilm producer (OD ≤ ODc), weak biofilm producer (ODc < OD ≤ 2 × ODc), moderate biofilm producer (2 × ODc < OD ≤ 4 × ODc), and strong biofilm producer (4 × ODc < OD). The microtiter colorimetric assay results in Figure 35 showed 25.5 % no or little biofilm producers, 49 % weak biofilm producers, 16 % moderate biofilm producers, and 8.5 % strong biofilm producers (Figure 35). It was further noteworthy that a number of isolates of human origin formed strong biofilm after 24 h at 37°C compared to environmental isolates.

In this study, the extracellular enzyme productions by 106 *S. maltophilia* isolates were assessed using two media: TBT and skim milk agar (for experimental details see 2.15.2 and 2.15.3). While all isolate of *S. maltophilia* produced lipase, protease production differed considerably with some isolate of environmental origin demonstrating no clearing zone after 24 h (Figure 36).
Results

Figure 33: Maximum parsimony tree built from 394 single nucleotide polymorphism positions identified in 106 *S. maltophilia* isolates compared to SMK279a. The tree visualizes that the isolates tend to form cluster based on isolation source (clinical, CF, ICP/environment). Numbers on branches indicate the number of distinct SNP positions between isolates. The red arrows indicate the five reference strains with full genome sequence deposited in public data base.
Figure 34: Biofilm formed on microtiter plate by *S. maltophilia* isolates. Biofilm formation capacity was assessed by microtiter colorimetric assay after 24 h incubation at 37°C or 30°C. The level of biofilm formation were varied significantly from 3.9 to 0.13. *S. maltophilia* isolates from human origin varied significantly in their ability to form biofilm in vitro.
Figure 35: Classifications of *S. maltophilia* isolates based on biofilm formation capability. A low cut-off (ODc) value was set off as three standard deviations above the mean of control wells. Based on OD, isolates were classified as: little or no biofilm producer (OD ≤ ODc), weak biofilm producer (ODc < OD ≤ 2 × ODc), moderate biofilm producer (2 × ODc < OD ≤ 4 × ODc), and strong biofilm producer (4 × ODc < OD).
**Results**

**Figure 36: Extracellular protease assay for S. maltophilia isolates.** Protease production differed considerably with some isolate of environmental origin demonstrating no clearing zone after 24 h.

<table>
<thead>
<tr>
<th>KB-KCL</th>
<th>DSM50170</th>
<th>LMG11112</th>
<th>K279a</th>
<th>MP1</th>
<th>MP2</th>
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<th>148CF</th>
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<td>2541CU</td>
<td>3001CU</td>
<td>U1</td>
<td>U2</td>
<td>U3</td>
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<td>SKK27</td>
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<td>LMG10996</td>
<td>LMG10853</td>
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</table>
4 Discussion

Bacterial infection is treated by β-lactam antibiotics and many bacteria secrete active β-lactamases. SMK279a exposed to β-lactams produces two β-lactamases, i.e. L1 and L2 (Saino et al., 1982, Walsh et al., 1994, Walsh et al., 1997, al Naiemi et al., 2006). The molecular mechanisms of β-lactam induction, the structure and the function of β-lactamases were well studied and known (Jacobs et al., 1997, Bush et al., 1995, Bush and Jacoby, 2010, Ambler, 1980, Hall and Barlow, 2005). The expression of β-lactamase is regulated by master regulator AmpR and several other proteins such as AmpG, NagZ and AmpD and others (Hu et al., 2008, Okazaki and Avison, 2008, Cullmann and Dick, 1990, Avison et al., 2002, Gould et al., 2006). Other β-lactam resistance mechanisms are the modification of penicillin binding proteins and efflux pump proteins (Zhang et al., 2000, Li et al., 2002, Sánchez, 2015). SMK279a strain is able to inactivate a range of β-lactam antibiotics and thus making treatment options difficult especially in immuno-compromised host (Chang et al., 2015). Due to this resistance the prevalence of β-lactam antibiotics resistant S. maltophilia infections is raising worldwide (Chang et al., 2015). The resistance is widely observed especially in patients with cystic fibrosis suffered from polymicrobial infections caused by P. aeruginosa, Burkholderia cenocepacia, Acinetobacter baumannii, and S. maltophilia (LiPuma, 2010, Sibley et al., 2006, Tang et al., 2014). These bacteria are a multidrug-resistant (MDR) and opportunistic pathogens. With the exception of S. maltophilia, a heterogeneous response of cells to antibiotic treatment has been previously reported which involved non-genetic mechanisms for escaping antibiotic therapy (Mei et al., 2015, Hermes et al., 2013, Tipton et al., 2015).

4.1 Phenotypic variation in the β-lactam resistant S. maltophilia

Pathogenic bacteria have evolved several mechanisms for the successful colonization and proliferation in their host organisms. The mechanisms are generally involved both genetic and non-genetic changes. The process of infection can be considered as a challenging and stressful for the pathogen, which often requires sudden response that is mostly addressed via set non-genetic mechanisms. In the past several decades, colony morphologies were studied to provide accelerated information on pathogenicity and bacterial stress response. These morphological variants persist during infections often with enhanced ability to form biofilms (Drenkard and Ausubel, 2002, Tipton et al., 2015). In this study, the patient isolate SMK279a developed a colony morphotype in an ampicillin challenged model (Figure 9B-E, Figure 10). Cellular subpopulations with distinct morphotype exhibiting small and big colonies were formed when
bacterial cells were challenged with the β-lactam (Figure 9B and Figure 37A). Although higher antibiotic concentrations are lethal to most cells, a subset bacterial population survived and proliferated in the antibiotic environment exceeding the MIC level (Figure 9C-D). The term ‘heteroresistance’ is often used to describe the subpopulations that tolerate a higher concentration of antibiotic to the rest of the bacterial population. Bacterial colony morphotypes in response to antibiotics treatment have been demonstrated for *Streptococcus pneumoniae* to penicillin (Morand and Mühlemann, 2007). In *Streptococcus pneumoniae*, a subset of populations tolerated high antibiotic concentration as defined by MIC. It further exhibited heterogeneity in colony size when grown on Columbia sheep blood agar plates (CSBA) with or without penicillin (Figure 37B). In spite of previous treatment history, SMK279a cells formed uniform homogenous colonies in the environment free of antibiotic (Figure 37A). However, small colonies of SMK279a and a heteroresistant *Streptococcus pneumoniae*, formed colonies of narrower in size in the presence of the antibiotic than in its absence. Additionally, the reversible variation in colony morphology was also observed for other strains such as *S. maltophilia* CF148, an isolate from of the respiratory tract of a cystic fibrosis patient and *S. maltophilia* DSM-50170 isolated from the human oropharyngeal region (Table 1).
Figure 37: Schematic diagram showing the phenotypic heterogeneity of SMK279a and *Streptococcus pneumoniae* cells during growth on solid media. (A) A colony morphologies observed for SMK279a in response to ampicillin (AMP; 100 µg/ml) treatment on LB agar plates. Up on the removal of the antibiotic, the progeny cells of small and big colonies formed only big colonies. (B) A heteroresistant colony of *Streptococcus pneumonia* (indicated by red rectangular line) was transferred on CSBA plates. This resulted visible heterogeneity of bacterial colony size with or without penicillin (PEN; 0.5 µg/ml) after incubation for 24 h (Morand and Mühlemann, 2007). Arrows indicate the transfer of fresh colonies or liquid culture samples.
4.2 SMK279a cells formed large outer membrane vesicles

The Gram-negative and Gram-positive bacteria secrete OMVs that carry nucleic acids, proteins, toxins and small metabolites which could be used to manipulate host cells (Schwechheimer and Kuehn, 2015, Kieselbach et al., 2015). The SMK279a stress response to β-lactam antibiotic involved a massive secretion of OMVs (Figure 11A and B). In some cells the determined size of the vesicles varied considerably, ranging from 100 nm to 677 nm in diameter (Figure 12A and B), which was twice the size of other Gram-negative bacteria (Beveridge, 1999). When cells were exposed to β-lactams pressure, the vesicles of S. maltophilia were packed with β-lactamase and several other proteins, especially with the two Ax21 homologous Smlt0387 and Smlt0184 that influences biofilm formation (McCarthy et al., 2011, Devos et al., 2015). This feature could enable cells to degrade antibiotics extracellularly and reduces the fitness cost associated with gene expression induced to deal with antibiotics. In addition, other sensitive bacterial populations co-inhabiting with SMK279a might benefit from the degradation of the antibiotic by the virtue of having OMVs in this strain. In this aspect, the β-lactamases packed in the OMVs of Bacteroides species and Haemophilus influenza have been suggested to protect commensal bacteria and other pathogens, such as Salmonella Typhimurium and Group A streptococci, against β-lactam antibiotics (Stentz et al., 2015, Schaar et al., 2014).

Vesiculation in SMK279a could probably be in part due to the disturbed cell wall structure or alteration in peptidoglycan dynamics (Haurat et al., 2015); however, cells were also heavily shelled with vesicles when an aminoglycoside antibiotic is applied that instead targets proteins synthesis (Figure B 1). This emphasis that the application of combination therapy for infection treatments using β-lactams and kanamycin might induces the production of the OMVs in SMK279a. Additionally, several internal and external factors might be responsible for OMVs formation. The envelope stress via the σE pathway and diverse environmental stress, such as oxidative stress are widely described to stimulate the process of vesiculation in bacteria (McBroom and Kuehn, 2007).

4.3 Bistable regulation of blaL1 and blaL2 at the single cell level

Different reporter fusions were constructed in the broad host range vector pBBR1MCS-5 (Kovach et al., 1995), using the promoter regions of blaL1 or blaL2 fused to the red (RFP), yellow (YFP) and cyan (CFP) fluorescent proteins to investigate the β-lactamase expression at a single cell level (Table 10). Analysis of single cells by fluorescence microscopy indicated that blaL1 and blaL2 are heterogeneously expressed between individual bacterial cells (Figure 15A-F). In an isogenic culture, the majority of the cells (95 %) were in the bla-OFF mode during the 24 h of
growth and were subsequently in the bla-ON mode (Figure 18A and B). Fluorescent protein based assays employing the promoter fusions of the two SMK279a β-lactamase resistance genes resulted in two major findings. Firstly, the populations of SMK279a cells differed in their expression profiles of the β-lactamase resistance genes bla\textsubscript{L1} and bla\textsubscript{L2} at the single cell level. The heterogeneous expression of virulence trait was indeed in response to the antibiotic treatment in the exponential and during the early stationary phases. Although, the overall number of cells that were in an ON mode varied between the different promoter fusions, they all shared the common feature of heterogeneous bla\textsubscript{L1} and bla\textsubscript{L2} expression (Figure 17A-F). Furthermore, the level of β-lactamase induction in bacteria was partly affected by the variety of β-lactams used (Hu \textit{et al.}, 2008). In the actively growing cells, however, the bla-OFF mode was the widely observed phenotype with the use of other class of β-lactams that varied in the induction potential (Figure 21A). Secondly, the switch from heterogeneous to homogenous bla\textsubscript{L1} and bla\textsubscript{L2} expression was ampicillin independent but rather growth-phase dependent that was most likely triggered environmental stimuli. Sturm \textit{et al} (2011) reported a similar gene expression pattern for \textit{Salmonella enterica} subspecies 1 serovar Typhimurium at the single cell level (Sturm \textit{et al.}, 2011). In their study, Type Three Secretion System 1 (TTSS-1) expression was demonstrated to be growth-phase dependent in the stationary phase and they further suggested that a signal from the environment plays a role for switching of the population from OFF to ON mode (Sturm \textit{et al.}, 2011).

In this study, conversion of bacterial cells from "OFF" into "ON" was achieved using culture supernatants from stationary-phase P\textsubscript{bla\textsubscript{L2}}::\textsubscript{rfp} reporter SMK279a strain or growing overnight cultures of P\textsubscript{bla\textsubscript{L2}}::\textsubscript{rfp} reporter strain in fresh LB medium supplemented with NaCl (1 M) or KCl (0.5 M) (Table 11). This reversible switch between the two states was on "ON" and "OFF" mode at 24 h and at 32 h, respectively, indicating a genetic or epigenetic mechanism that modulates it. Here, the potential effect of plasmid copy-number variation on single cell β-lactamase expression cannot be ruled out. Additionally, promoter fusions of P\textsubscript{bla\textsubscript{L2}}::\textsubscript{cfp} and P\textsubscript{bla\textsubscript{L2}}::\textsubscript{yfp} did not show the switch from a heterogeneous to a homogenous bla expression. Nevertheless, a stress signal obtained from cells carrying the P\textsubscript{bla\textsubscript{L2}}::\textsubscript{rfp} reporter could still switch the cells carrying P\textsubscript{bla\textsubscript{L2}}::\textsubscript{cfp} or P\textsubscript{bla\textsubscript{L2}}::\textsubscript{yfp} from ON to OFF mode. Altogether, these data suggested that a signaling molecule is involved in switching the population from the bla-OFF to the bla-ON mode (Table 11, Figure 20A and B). The postulated signaling molecule most likely accumulates during growth and then mediates the switching from heterogeneous into a homogeneous gene expression. As a putative signaling molecule, the DSF, a primary autoinducer identified in \textit{S. maltophilia} (McCarthy \textit{et al.}, 2011) was tested. However, DSF failed to alter bla\textsubscript{L2} gene expression at the single cell level independent of the source and structure of the DSF used, i.e.
extracted DSF (cis-2-11-methyl-dodecenoic acid and seven structurally related derivatives thereof that were produced by S. maltophilia WR-C (Huang and Wong, 2007)) as well as a commercially available DSF (i.e cis-2-11-methyl-dodecenoic acid).

In general, heterogeneous gene expression in isogenic bacterial populations may occur constitutively or arise in a growth-phase dependent manner in a homogeneous environment as represented by laboratory culture conditions, showing the existence of two equilibrium states (Veening et al., 2008, Dubnau and Losick, 2006). The term ‘bistability’ describes the bifurcation of isogenic bacterial population into two distinct subpopulations showing a different level of gene expression. Therefore, expression profiles of the β-lactamase genes bla\textsubscript{L1} and bla\textsubscript{L2} indicated high levels of bistability at single cell level. This stress response might be prompting bet-hedging and division of labor between two phenotypes for better adaptation and survival in challenging environment. The term ‘bet-hedging’ describes the evolutionary strategy that involves a stochastic switching between phenotypic states (Beaumont et al., 2009).

4.4 SMK279a is hypermutable but not within the resistome

Many colony morphotypes such as the mucoid morphotypes, the rugose small colony variants (RSCVs) and small colony variants (SCVs) are derived from genetic mutations (Qiu et al., 2007, D’Argenio et al., 2002). A mutation in muc\textsubscript{A} was solely responsible for the overproduction of the exopolysaccharide alginate in P. aeruginosa that resulted in phenotypic switching from non-mucoid to mucoid form (Qiu et al., 2007). Additionally, a single nucleotide substitution might be enough to affect resistance to antibiotics when especially located in the essential gene (Carter et al., 2000). These studies demonstrated that mutations are responsible for switching phenotypes in numerous medically important bacteria. Consistent with this studies, the investigated genotypes of 24 colony variants identified a significant number of SNPs and in/dels in the non-coding and coding region of SMK279a genome (Table 12). Within sequenced samples, WGS identified per colony two to 15 SNPs, up to seven deletions and four insertions. About half of variants matched exactly to reference genome and the remaining were matched to reference genome with slight difference of one to three node distances (Figure 23). Interestingly, none of the mutations were identified in previously known resistome of SMK279a. A resistome is a collection of all of the antibiotic resistance genes in bacteria. Therefore, polymorphisms in this strain did not affect the colony morphotypes and indeed the phenotypes were reversible but not based on β-lactam induced mutagenesis, since mutagenesis in bacteria has been shown to be only caused by sub-inhibitory concentration of β-lactams (Gutierrez et al., 2013). Thereby, the
colony morphotypes were a result of heterogeneous behavior within a syngeneic bacterial population mainly involved genetic switches during alternations from one form to another.

The other bacterial variant types were the small colony variants (SCVs) and have been isolated from many pathogens, including *S. maltophilia* (Proctor *et al.*, 2006, Anderson *et al.*, 2007). The SCVs made a subpopulation of slow-growing bacteria that their growth characteristics and morphotypes were distinct from those of the wild type counterparts. They can potentially resist antibiotics and remain persistent eventually causing recurrent infections in the infected host (Proctor *et al.*, 2014). The SCVs contain permanent genetic alternations in their genome for one or more of the essential genes. Several genetic mutations were responsible to the most commonly isolated SCVs that were either deficient in electron transport or deficient in thymidine biosynthesis (Bates *et al.*, 2003 , Cano *et al.*, 2003 , Kahl *et al.*, 2003). However, additional variants which were CO₂ auxotrophs have been described (Proctor *et al.*, 2006). With the current study, in SMK279a, the genes encoding for hypothetical protein (Smlt1844B) and two-component regulatory system (Smlt3944) are identified as hotspots for mutations. The histidine kinase gene (Smlt3944) may be transcribed within an operon with the *smlt3943* gene coding for the phosphate-selective porin OprP (Figure 22). OprP is mainly expressed under conditions of phosphate starvation (Siehnel *et al.*, 1992), and with the flanking gene *smlt3942* (encoding a C4-dicarboxylate transport protein), it is presumably under the regulatory effect of this sensor histidine kinase (Yurgel and Kahn, 2004). The phenotypic heterogeneity described herein was unaffected by the current mutations and none of the mutations occurred in the essential genes. Nevertheless, the nonsynonymous mutations that occurred in the receiver domain of histidine kinase might influence signal processing in response to environmental stimuli.

### 4.5 Cellular variations allows phenotypic variation

Phenotypic variation within isogenic bacterial populations reveals altered level of cellular components that can be depicted at the level of transcription, translation and protein synthesis (Wang *et al.*, 2015, Elowitz *et al.*, 2002). Cellular variations when bacterial cells undergo a phenotypic switching from planktonic to sessile form of growth are well covered for several Gram-positive and Gram-negative bacteria (Ymele-Leki and Ross, 2007, Nadell *et al.*, 2015, Oggioni *et al.*, 2006). With respect to colony morphotypes; however, the transcriptome profiles remain to be uncovered. This study uncovers that the small and big colony variants differed in the transcriptional activation of 12 genes, including the β-lactamase genes *bla*₃₁ and *bla*₃₂. The *bla*₃₁ and *bla*₃₂ were among the strongest differentially regulated genes between the two different colony morphotypes (Table 15, Figure 24). When compared to uniform colonies, 34 genes were
differentially regulated in the small and big colonies. Therefore, the distinct expression patterns were stable enough to allow differentiation into different colony morphotypes.

In a clonal bacterial population, the colony morphotypes of SMK279a and other opportunist pathogen indicate that isogenic population diverges into subpopulation that temporarily modify cellular functions. Bacterial cells have to adapt different phenotypes by expressing different trait and this switch can be triggered by diverse factors including antibiotics. The evidence for the effect on cellular functions aroused from the RNA-seq data that the small colonies altered transcriptionally in diverse genes whose products are known to be involved in bacterial virulence, motility, as well as adhesion and biofilm formation (Figure 30). The diverse role of genes and their products that were associated with the formation of the colony morphotypes are discussed below.

In this study, among fimbriae adhesin proteins (Figure 31) two of them were differentially regulated in the different colony morphotypes. These are the smf-1 and smlt0709 and were previously shown to be involved in haemagglutination, biofilm formation and adherence to cultured mammalian cells (de Oliveira-Garcia et al., 2003). Therefore, phenotypic and functional outcomes modulated by the fimbriae adhesion proteins were affected in the colony variants. Indeed, the sister progeny cells from smaller colonies formed thicker biofilm on plastic surfaces than big colony variant. Bacterial adherence to host cells is also involves the function of the Type IV pili (TFP) (Persat et al., 2015). Type IV pili are surface-exposed fibers with diverse roles in cells including social gliding motility, DNA uptake (competence) and protein secretion (Shi and Sun, 2002, Craig et al., 2004). Their expression is regulated by the response regulator of the two-component system AlgR. Therefore, the differential regulation this key transcriptional regulator AlgR in small colonies influenced the expression of multiple virulence factors including alginate. Although, alginate is not widely produced among clinical strains, it is produced among bacteria causing cystitis fibrosis (Cobb et al., 2004, Massengale et al., 2000). Its' synthesis is increased upon attachment of the bacteria to a cell surface; resulting in stronger bacterial adhesion to the surface (Boyd and Chakrabarty, 1994). The overexpression of the lyase gene in cells forming small colonies might be therefore important in facilitating the dissemination of the bacteria. Additionally, a poly (β-D-mannuronate) lyase (SmLT2601) is presumably involved in the degradation of polysaccharide alginate. Furthermore, the RhsA plays a role in polysaccharide synthesis/export and is also a hotspot for the formation of chromosomal duplication. Moreover, the methyl-accepting chemotaxis protein was transcriptionally affected in small colonies which potentially used to detect concentrations of molecules in the extracellular matrix.
Discussion

Figure 38: Diverse cellular functions that were altered in cells of small colonies based on RNA-seq data and biofilm assay. These functions include virulence determinants in S. maltophilia such as motility, adhesion and biofilm formation. The altered function might promote division of labor and bet-hedging in isogenic population and this can be important during the infection of human epithelia to respond against antimicrobial effectors.

4.6 Potential links of S. maltophilia β-lactamase expression and growth

In this study it was shown that the colony morphotypes of SMK279a cells varied with respect to the level of bla$_{L1}$ and bla$_{L2}$ genes transcription. Indeed, subpopulations comprised of the big colonies up-regulated 15.3- and 6.9-fold of the bla$_{L1}$ and the bla$_{L2}$ genes, respectively; they were among the strongest differentially regulated genes between the two different colony morphotypes. This different expression values could possibly allow heterogeneity as demonstrated with the varying colony size and time of appearance as visible phenotypes when grown on LB agar plates with ampicillin. It was usually after 16-18 h of growth for big colonies and between 24-33 h of growth for small colonies under the same cultivation conditions.

Furthermore, the bla$_{L1}$ and bla$_{L2}$ expression values in big colonies were stable enough to generate homogeneity when cells were transferred to new environments with ampicillin. Particularly, this phenomenon indicated that all cells in the suspension expressed the β-lactamases resistant genes based of the previously achieved cellular state (Figure 9E and
Figure 10, Table 15). This cellular state was partly predefined by genes and by the activities of their products listed in Table 15. In contrast, a subpopulation with the low level of β-lactamase expression could be bifurcated into distinct phenotypes of small and big colonies (Figure 9E and Figure 37A). Thereby, in an isogenic population, a subset of bacterial populations grew slowly even though cells secreted enzymes that provide a means for survival (Figure 39). However, the level of transcriptional activation of bla$_{L1}$ and bla$_{L2}$ genes was only slightly different to the untreated cells that were grown in an environment free of stressful antibiotic challenges. Here, it demonstrated that bacterial cells can maintain low level of the expression of β-lactam resistant genes bla$_{L1}$ and bla$_{L2}$ in the presence and absence of antibiotics. Chromosomally encoded β-lactamase is periplasmic in nature that the feature can be useful to inactivate β-lactams up on sudden exposure. In addition, it protects early damage of cells before enough signals for induction accumulates.

Therefore, the expression values bla$_{L1}$ and bla$_{L2}$ among colony morphotypes suggested that growth and β-lactamase expression are synergistically involved to produce diverse phenotypic and functional outcomes (Figure 39). Indeed, many examples of phenotypic heterogeneity are accompanied by strict control of bacterial growth rate (Wang et al., 2014, Robert et al., 2010, van Dijk et al., 2015). The expression of an accessory antibiotic resistant gene in a model organism such as E. coli was associated with growth penalty (Wang et al., 2014). Additional expression studies using a microfluidic device showed an increased β-lactamase expression in a subset of E. coli cells that slowed growth. This phenomenon of growth control and heterogeneous gene expression is not restricted to antibiotics but also to other stresses and treatment factors. In the same organism, a bistable lactose operon regulation of E. coli involved growth slow-down, which was also associated with higher switching probability (Robert et al., 2010). Additionally, persisters cells can transiently overcome antibiotic stress by stochastically entering a dormant state. The state of dormancy is usually characterized by reduced metabolic activities (Maisonneuve et al., 2013), where there is little requirement for cell wall biogenesis, DNA replication and translation and hence, the antibiotics do not affect these antibiotic sensitive cells. Therefore, growth control might provide a critical regulation for the expression of β-lactamase genes bla$_{L1}$ and bla$_{L2}$ in SMK279a.

The genotypic and phenotypic consequences of slow-growing cells within isogenic population have been investigated for yeast and other eukaryotic organisms (van Dijk et al., 2015). The slow-growing cells upregulated transposons and other genes, consequently leading to more mutations in the genome. Consistent with this, the NGS data presented along with this study supports this hypothesis in bacteria in that more mutations were identified in the morphotypes of
small cells than big (Figure 40). Remarkably, the identified transposase gene \( (smlt0353) \) could perhaps play a similar role as suggested by van Dijk and his co-authors (van Dijk et al., 2015).

Figure 39: Expression of \( \beta \)-lactamase in isogenic SMK279a is controlled in a growth-dependent manner in the presence of \( \beta \)-lactams. The patient isolate SMK279a diverges into cellular subpopulations with distinct but reversible morphotypes of small and big colonies when challenged with ampicillin. A fraction of subpopulations (A) usually grows faster than the average population leading to higher \( \beta \)-lactamase expression and comparatively a less virulent phenotype. A growth penalty results in less enzyme production and consequent hardening of the strain (B). In SMK279a, growth and \( \beta \)-lactamase expression are synergistically involved to produce diverse phenotypic and functional outcomes. The filled circle represents \( \beta \)-lactams.
Figure 40: A section of SMK279a genome that shows cells in small colonies contained more mutations than big colonies. Numbers indicates coordinates on genome map from 2.6 to 3.6 Mbp. The light green lines indicate locations of mutations identified only in the genomic DNA extracted from bacterial cells of small colonies.

4.7 Molecular switches of phenotypic heterogeneity

Phenotypic heterogeneity with respect to the expression of resistant genes might be caused by genetically, non-genetically or epigenetically controlled process and/or acquired after initial exposure to antibiotics (El-Halfawy and Valvano, 2015, Raj and van Oudenaarden, 2008). Genetically determined process leading to variation in population results via a set of mechanisms, including mutations or gene duplications of key resistance genes or regulatory systems (Koch et al., Kao et al., 2014, Fridman et al., 2014). A mutation in gyrA gene was responsible for development a metronidazole-resistant phenotype in subpopulation of Helicobacter pylori (Kao et al., 2014). While mutations or genetic rearrangement is essential in the long term evolution of bacterial resistance, isogenic population can epigenetically switch to temporarily hyper resistant phenotype due to the stochastic nature of gene expression. Differential expression of genes in the regulatory circuit among cells in populations is responsible for heterogeneity and this has been shown by deletion of the regulator/s
(Anetzberger et al., 2009, Deris et al., 2013, Grote et al., 2014, Mulder and Coombes, 2013, Wang et al., 2014). Finally, non-genetically determined heterogeneity may arise from chemical or signal coming from an environment where these cells are exposed, for example, glycine was responsible for the heterogeneous responses of *Staphylococcus aureus* to methicillin (de Jonge et al., 1996).

The well-known mechanisms that lead to different cellular level in the known regulatory proteins of β-lactamase expression are presented in the Figure 41. Cells could show either basal or induced levels of enzyme production based on the cytosolic accumulation level of anhydro-MurNAc-oligopeptides, a cell wall degradation product. Therefore, the phenomenon of differential expression of *bla*<sub>L1</sub> and *bla*<sub>L2</sub> on agar plates in the population of small vs. big colonies could theoretically happen either from the effect of the antibiotic or the regulator or both or other gene products in the regulatory circuit. The effect of the antibiotic will be considered initially as possible molecular switch causing heterogeneity. With the assumption of uniformly distributed antibiotic, the rate of absorption of antibiotic and the physiological state of the individual cell were by far important factors responsible to launch defense machinery targeted towards the antibiotic. Thus, this might create a difference in the initial response, however in the long period the colony forming potential depends on the growth and death rate that partly depends on the capacity of cells forming colonies to overcome antibiotic stress either individually or in a collective manner. Depending on the cellular level the β-lactamase protein, cells cleared the antibiotic in the medium. Furthermore, the colony diameter of small colonies was only less than half wider as of the diameter of big colonies after 48 h (Table 9). This was indeed related to average transcriptional profile within the colony morphotypes. The phenotypic differences on the plates were perhaps caused by significantly differentially regulated genes in the different colony types (Table 15). Additionally, the products of these genes might affect growth that could allow different expression patterns of the *bla*<sub>L1</sub> and *bla*<sub>L2</sub> genes in colony variants.

In SMK279a, the expression of many genes encoding for several proteins involved in the cell wall biosynthesis and β-lactamase production were not differentially regulated among cells of big vs. small colonies, including AmpR. AmpR is the central regulator for the expression of the β-lactamases. It is transcribed in an opposite direction to *bla*<sub>L2</sub> which is located upstream of putative transmembrane Na+/H+ antiporter (Figure 25). The activation of AmpR is responsible for induction of β-lactamase *bla*<sub>L1</sub> and *bla*<sub>L2</sub> (Hu et al., 2008, Okazaki and Avison, 2008). At sub-MIC, isogenic population of SMK279a bifurcated without differentially regulating the *ampR* in response to ampicillin and gene products *bla*<sub>L1</sub> and *bla*<sub>L2</sub>. When untreated cells were compared,
the transcriptional activation of \textit{ampR} was still not differentially regulated. In addition to \textit{ampR}, several of the transcripts of genes involved in the expression of \textit{bla} genes had a fold change of less than two, indicating the absence of differential regulation. Furthermore, the deletion of \textit{ampR} (SMK279aΔsmlt3723) led to complete abolishment of growth on LB agar plates in the presence of ampicillin (100 μg/ml), while the strain showed normal growth on plates without the antibiotic. Accordingly, as a result of the failure to grow in the presence of ampicillin it could not be used to produce colony morphotypes. However, the role of slightly upregulated \textit{ampR} among colony morphotypes cannot under emphasized and needed further studies.

4.7.1 ComE modulated \textit{bla}_{L2} heterogeneous expression in SMK279a

The current study revealed that \textit{bla}_{L1} and \textit{bla}_{L2} genes were subjected to a heterogeneous and bistable expression at the single cell level when it was investigated with the fluorescent proteins based assay. Additionally, RNA-seq indicated a differential regulation of a moderate number of genes, notably including \textit{smlt2851} and \textit{smlt2852}, encoding putative antibiotic resistance transporter, and a putative \textit{comE} homologue (\textit{smlt1134}) between cells that were in the \textit{bla}_{L2}-ON vs. \textit{bla}_{L2}-OFF mode (Figure 27). Furthermore, the impact these genes were investigated by constructing a deletion mutants of both loci, \textit{smlt1134} (\textit{comE} homologue) and \textit{smlt2851}-\textit{smlt2852} (Figure 7). However, heterogeneous gene expression of the \textit{bla}_{L2} reporter fusion was not altered in the background of any of these deletion mutants, suggesting that the lack of expression was not linked to heterogeneous expression (Table 17). Also, the presence of the SMK279aΔsmlt0387 (an Ax21 homologue) mutation did not alter \textit{bla}_{L2} gene expression at the single cell level when compared to the parent strain, implying that Smlt0387 does not play a role in mediating heterogeneous β-lactamase expression (Table 17). The Ax21 homologue peptide was suggested to act as a cell to cell signal to regulate a diverse range of functions, including virulence, in \textit{Stenotrophomonas} (McCarthy \textit{et al.}, 2011).

Since the \textit{smlt2851} and \textit{smlt2852} genes were 29- and 7.2-fold up-regulated, respectively, in \textit{bla}_{L2}-ON mode cells, the genes were overexpressed in SMK279a under their native promoter, which did not alter heterogeneous expression of the \textit{bla}_{L2} gene. The final attempt in this study to uncover what modulates phenotypic heterogeneous expression of \textit{bla}_{L2} was obtained from studies conducted on ComE protein. The overexpression of the ComE homologue resulted in a decreased heterogeneity of \textit{bla}_{L2} expression (Figure 28). ComE contains a Helix-hairpin-helix motif, which is mainly involved in non-sequence-specific DNA binding (Shao and Grishin, 2000). Thereby, post-transcription regulation of the \textit{comE} gene involves in part by the RNA-binding protein (Hfq) that regulates a wide variety of cellular responses (including response to
Figure 41: Uninduced and induced β-lactamase production in Gram-negative bacteria. It shows the biological roles of the well-known proteins with or without antibiotic challenges of bacterial cells. Cell wall degradation products are transported to the cytosol via cytoplasmic inner membrane permease AmpG. They are processed by β-N-acetylglucosaminidase (NagZ) and an anhydro-N-acetylmuramyl-L-alanine amidase (AmpD). The AmpC is the first bacterial β-lactamase discovered being able to destroy penicillin. The expression values \( \text{bla}_{L1} \) and \( \text{bla}_{L2} \) genes in big colonies suggested that the system was activated in response to ampicillin.
antibiotics) via interacting with small RNAs (sRNAs) and mRNAs (Roscetto et al., 2012), indicating the complex network for comE regulation and this may in part owning to its diverse role in cells. Consistent with this finding, there is increasing evidence that additional genes are involved in the regulatory circuit of β-lactamase expression in Gram-negative bacteria (Filipe et al., 2002, Zahner et al., 2002, Zamorano et al., 2014). This includes the murMN genes and the ciaH/R two-component system that are involved in the expression of pneumococcal penicillin resistance. The CreBC system of P. aeruginosa also controls the expression of genes of diverse functions including β-lactam resistance, biofilm formation or anaerobic respiration (Zamorano et al., 2014). However, a similar role of the CreBC system in S. maltophilia has not been found (Lin et al., 2011). Finally, it is noteworthy that the mechanism involved in phenotypic heterogeneity is complex and involves several genes (Morand and Mühlemann, 2007).

4.8 S. maltophilia is genotypically diverse

The genus Stenotrophomonas currently comprises at least 13 species (http://www.bacterio.net/stenotrophomonas.html), indicating much more differentiation at species level. Among these species, S. maltophilia is the sp. that most frequently associated with human infections. Surprisingly, a strong association to plant hosts is also restricted to the two species S. maltophilia and S. rhizophila (Berg et al., 2010, Wolf et al., 2002). The genotypes of the 106 S. maltophilia isolates that were obtained from human and environmental isolation sources indicated that the isolates exhibited more genetic diversity (Figure 33). Furthermore, S. maltophilia isolates showed considerable variations with regard to biofilm formation on abiotic surfaces (Figure 34).
5 Conclusion and outlook

The data presented in this work have provided two insights into the regulatory network triggering phenotypic heterogeneity of SMK279a with the following conclusion. First, in an ampicillin challenged model, SMK279a cells showed heterogeneity in colony and cell morphology in line with differential gene expression including the β-lactam resistance ones, \( bla_{L1} \) and \( bla_{L2} \). The genotypes of the different colony morphotypes revealed that SMK279a is hypermutable but not within the resistome. Second, the phenotypic heterogeneity within isogenic populations of SMK279a is a result of non-genetic variations in individual cells. This cellular variation was mainly triggered by the gene products and activities of \( bla_{L1}, \ bla_{L2} \) and \( comE \) homologue and might be affecting bacterial virulence, bacterial motility, as well as adhesion and biofilm formation. The phenotypic heterogeneity in SMK279a cells provides a selective advantage in natural environments and during the infection of human epithelia to respond against antimicrobial effectors. This adaptation is probably also relevant during acute and chronic human infections associated with \( S. \ maltophilia \) and effectiveness of antibiotic treatment. Future work to identify the underlying molecular switches involved in triggering phenotypic heterogeneity in \( S. \ maltophilia \) includes;

- Investigation heterogeneous expression using chromosomal-based reporter fusion
- Single cell transcriptomes and RT-qPCR analysis
- Identifying the signal molecules of SMK279a under stress condition and defining the structure
- Studying the mechanism involved in the heteroresistance SMK279a
- Defining the role of growth control to β-lactamase expression in SMK279a
- Defining the role heterogeneity during infections
References


EUROPEAN COMMITTEE FOR ANTIMICROBIAL SUSCEPTIBILITY TESTING OF THE EUROPEAN SOCIETY OF CLINICAL, M. & INFECTIOUS, D. 2003. Determination of
minimum inhibitory concentrations (MICs) of antibacterial agents by broth dilution. *Clin Microbiol Infect*, 9, ix-xv.


REFERENCES


Appendix

A List of abbreviations

%  Percentage
μg  Micro gram
°C  Degree Celsius
aa(s)  Amino acid(s)
Acc.no  Accession number
AMP  Ampicillin
Amp’  Ampicillin resistance
approx.  Approximately
bidest  Bidistilled water
bla-OFF  Red fluorescent protein non-expressing cells
bla-ON  Red fluorescent protein expressing cells
bp  Base pair(s)
cDNA  Complementary DNA
CF  Cystic fibrosis
cfp  Cyan fluorescent protein
Cm’  Chloramphenicol resistance
CXM  Cefuroxime
DEL  Deletion
DMF  Dimethylene formamide
DMSO  Dimethyl-sulfoxide
DNA  Deoxyribonucleic acid
DNTP(s)  Deoxyribonucleotide triphosphate
DSF  Diffusible signal factor
DTT  Dithiothreitol
E.coli  Escherichia coli
E-cup  Eppendorf cups
EDTA  Ethylene-diamine-tetraacetic acid
EMBL  European Molecular Biology Laboratory
ENA  European Nucleotide Archive
ESBL  Extended spectrum β-lactamase
EtOH  Ethanol
EUCAST  European Committee on Antimicrobial Susceptibility Testing
GFP  Green fluorescent protein
Appendix

Gm’  Gentamicin resistance
h    Hour
ICP  Intensive care patient
IGB  Integrated Genome Browser
INS  Insertion
IPTG Isopropyl β-D-1-thiogalactopyranoside
KAN  Kanamycin
kb   Kilo base pairs
L    Liter
L1   Putative metallo-β-lactamase, bla\textsubscript{L1}
L2   Putative β-lactamase protein, bla\textsubscript{L2}
LA4  Murine lung epithelial cell line
LB   Luria Bertani
LT   Lytic transglycosylases
M    Mole(mol/L)
mA   Milli Ampere
max. Maximum
Mbp  Mega base pairs
MCS  Multiple cloning site
mg   Milli gram
MICs Minimum inhibitory concentrations
min  Minute
ml   Milli liter
mM   Milli mole
mM   Milli mole
mol  Molar
n    Nano
NCBI National Center for Biotechnology Information
NGS  Next generation sequencing
NYGB Nutrient yeast glycerol medium
OD   Optical density
OMVs Outer membrane vesicles
ORFs Open reading frames
PBP  Penicillin binding proteins
PG   Peptidoglycan
PMF  Proton motive force
QS   Quorum sensing
r Resistance
rfp Red fluorescent protein
RFU Relative fluorescence unit
RNA Ribonucleic acid
RNase Ribonuclease
rpf Regulation of pathogenicity factors
rpm Rounds per minute
rRNA Ribosomal ribonucleic acid
RT Room temperature
RT-qPCR Reverse transcription-quantitative PCR
SDS Sodium-dodecylsulphate
Sec Second
SEM Scanning electron micrographs
Smf-1 Fimbrial operon
SMK279a *Stenotrophomonas maltophilia* K279a
SNPs Single nucleotide polymorphism
sp Species
TA Toxin-antitoxin
U Unit
V Volt
v/v Volume per volume
W Wilde type
w/v Weight per volume
WGS Whole genome sequencing
Xcc *Xanthomonas campestris* 8004
X-Gal 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside
yfp Yellow fluorescent protein
B Supplementary Tables and Figures

Figure B 1: Vesicle formation by the SMK279a cells exposed to kanamycin. Bacterial cells were cultured on LB agar plate supplemented with kanamycin (25 µg/ml).

Figure B 2: Salt stress triggers Pbla₂₂ heterogeneity in SMK279a. Overnight cultures of Pbla₂₂::rfp reporter strain were diluted in fresh LB medium supplemented with high concentrations of NaCl (1 M). After 7 h of growth at 30°C, aliquot of withdrawn sample for image analysis revealed that Pbla₂₂::rfp gene expression was heterogeneous among salt stressed cells. Cells were also curved at the poles.
Figure B 3: Phenotypic heterogeneity observed in SMK279a cells grown in batch culture and in the presence of 1.5 ml l cell free supernatant of a 48 hour-old culture added at time ‘0’. Data are mean values of at least three independent cultures and for each time point and culture a minimum of 408 cells were analyzed. The volume of control cultures were adjusted with 1.5 ml fresh LB at time ‘0’.

Figure B 4: Cell-free culture supernatants obtained from Pbla\textsubscript{L2}::rfp reporter strain cultures trigger phenotypic heterogeneity in SMK279a carrying the Pbla\textsubscript{L2}::cfp (A) and the Pbla\textsubscript{L2}::yfp (B) promoter gene fusion. Cells carrying either of the reporter fusion was cultured in the presence of 1.5 ml l cell free supernatant for 7 h and analyzed as before. The volume of control cultures were adjusted with 1.5 ml fresh LB at time ‘0’.
Figure B 5: Overexpression of ComE homologue abolishes heterogeneity. The \textit{smlt1134} gene with the predicted promoter coding region was PCR amplified and inserted upstream of the RFP in the reporter plasmid in \textit{pBBR1MCS-5::Pbla_{L2}::rfp}. The construct \textit{pBBR1MCS-5::Pbla_{L2}::rfp::smlt1134} was transferred into SMK279a and challenged with 100 µg/ml ampicillin. Samples for the fluorescence microscopy analysis were taken at 7, 24, 30, and 48 h and revealed that an altered population-wide \textit{bla_{L2}} gene expression profile in the presence of comE homologue overexpression.

Table B 1: Overall transcriptome metadata for the analyzed SMK279a liquid culture samples

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Figure B 6: RT-qPCR data analysis of bla_{L2} ON vs. OFF cells (A) Amplification curves for rpoD gene generated from a 10-fold serial dilutions of cDNA. It was used to evaluate the primer efficiency and for data analysis. (B) The standard curve generated for rpoD gene using the CFX Manager™ software. The efficiency, slope and correlation coefficient were with the recommend range described for the RT-qPCR. _comE_ homologue. (C) RT-qPCR data analysis for the _comE_ homologue _smlt1134_ and two putative transmembrane efflux genes (_smlt2851_ and _smlt2852_). RT-qPCR indicated a 1.74±0.29 fold down-regulation in the transcription of the _smlt1134_ gene among cells that were in the _bla_{L2}^ON_ mode vs. _bla_{L2}^OFF_ cells.
Table B 2: Summary of genome sequence data of *S. maltophilia* strains K279a, SMK279a∆smlt1134, SMK279a∆smlt2851∆smlt2852 and SMK279a∆smlt3723.

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## Appendix

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*Table shows all variations identified by mapping of the sequence reads to the S. maltophilia K279a genome sequence [GenBank: NC_010943.1]. Uncovered regions corresponding to the deleted smlt1134, smlt2851 & smlt3723 genes are not included.*
Appendix

C Programs and databases

**Programs**

- **BioEdit** (Sequence alignment editor)  [http://www.mbio.ncsu.edu/bioedit/bioedit.html](http://www.mbio.ncsu.edu/bioedit/bioedit.html)
- **Clone Manager Suite 7** (Edition of sequence files)  [http://www.scied.com/in_508.htm](http://www.scied.com/in_508.htm)
- **EndNote** (Literature)  [http://www.endnote.com](http://www.endnote.com)
- **Mega 6** (Phylogenetic analysis)  [http://www.megasoftware.net/](http://www.megasoftware.net/)
- **Quality One** (Gel Documentation software)  [http://www.bio-rad.com/](http://www.bio-rad.com/)
- **GAP4 software** (DNA sequence edit / assembly)  [http://staden.sourceforge.net/](http://staden.sourceforge.net/)
- **IGB** (Integrated Genome Browser)  [http://bioviz.org/igb/index.html](http://bioviz.org/igb/index.html)

**Databases**

- **BRENDA** (Enzyme database)  [http://www.brenda-enzymes.info/](http://www.brenda-enzymes.info/)
- **EMBL-EBI** (InterPro Scan)  [http://www.ebi.ac.uk/embl/](http://www.ebi.ac.uk/embl/)
- **ExPASy** (Proteomics server)  [http://expasy.org/](http://expasy.org/)
- **RAST** (Annotation server)  [http://rast.nmpdr.org/](http://rast.nmpdr.org/)
- **RCSB PDB** (Protein Data Bank)  [http://www.rcsb.org/pdb/explore.do/](http://www.rcsb.org/pdb/explore.do/)
- **Signal IP** (Signal peptide prediction)  [http://www.cbs.dtu.dk/services/SignalP/](http://www.cbs.dtu.dk/services/SignalP/)
Declaration on oath

"I hereby declare on oath, that I have written the present dissertation by my own and have not used other than the acknowledged resources and aids."

Ebrahim Mama

Hamburg, 29 January 2016
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