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

Dissertation

with the aim of achieving the degree of Doctor rerum naturalium (Dr. rer. nat.)

at the Faculty of Mathematics, Informatics and Natural Sciences

Department of Biology

of the Universität Hamburg

submitted by

Ebrahim Mama Abda

from Bale, Ethiopia

Hamburg 2016

The following evaluators recommend the admission of the dissertation:

1. Prof. Wolfgang R. Streit
2. PD Dr. Andreas Pommerening-Röser

Date of oral defense: 27.05.2016

United States Department of Agriculture | Agricultural Research Service | Midwest Area Plant Genetics Research Unit | 108W Curtis Hall University of Missouri Columbia, MO 65211

voice: (573) 882-8151
fax (573) 884-7850
e-mail: KrishnanH@missouri.edu
Hari.Krishnan@ARS.USDA.GOV

January 27, 2016

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_{L1} and bla_{L2} 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:

ABDA, E. M., KRYSCIAK, D., KROHN-MOLT, I., MAMAT, U., SCHMEISSER, C., FÖRSTNER, K. U., SCHAIBLE, U. E., KOHL, T. A., NIEMAN, S. & STREIT, W. R. 2015. Phenotypic heterogeneity affects *Stenotrophomonas maltophilia* K279a colony morphotypes and β-lactamase expression. *Front Microbiol*, 6, 1373.

KOHL, T. A, MAMAT, U, YUN, Y, SCHMIDT, F, **ABDA, E. M**, KRYSCIAK, D, STREIT, W. R, STEINMANN, J, ROLETOR, I, MARWITZ, S, GOLDMANN, T, NIEMAN, S and SCHAIBLE, U. E. Genotypic and functional characterization of *Stenotrophomonas maltophilia* isolates of diverse habitat. (In prepartion).

Table of contents

List of Tables

List of Figures

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 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. 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_{L2} 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_{L2} 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_{L2} expressing cells. Overexpression of *comE* homologue in *S. maltophilia* K279a reduced the level of cells that were in a bla_{L2}-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_{L2} 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_{L1} and bla_{L2} 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_{L1}, bla_{L2} 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

Das Gram-negative Bakterium *Stenotrophomonas maltophilia* ist ein immer häufiger auftretender Erreger von Krankheiten. Es tritt häufig bei Patienten mit zystischer Fibrose auf, wird aber auch in jeder Umgebung wie im Boden, auf Pflanzen, im Wasser und auch in gesunden menschlichen Individuen gefunden. Der Mikroorganismus trägt viele Gene, die für mehrere Antibiotika-Resistenz-Mechanismen kodieren. Hierzu zählen die beiden β-Lactamase-Gene *bla*$_{L1}$ und *bla*$_{L2}$, die hauptsächlich für den Abbau von β-Lactam-Antibiotika zuständig sind. Im Rahmen dieser Arbeit war vor allem die Identifizierung von nicht genetischen Mechanismen, die die heterogene Expression der beiden β-Lactamase-Gene *bla*$_{L1}$ und *bla*$_{L2}$ beeinflussen können, von Interesse. Dabei lag besondere Aufmerksamkeit auf dem Phänomen der phänotypischen heterogenen Expression der β-Lactamase-Gene. Phänotypische Heterogenität ist eine häufig beschriebene Zell-zu-Zell-Variation in Bakterien, die es isogenen Population erlaubt, sich an wechselnde Konditionen, einschließlich der Anwesenheit von Antibiotika, anzupassen. Die aktuellen Behandlungsstrategien mit Antibiotika, basierend auf dem alten Modell der minimalen Hemmkonzentration, ermöglichen es Subpopulationen der Bakterien der Behandlung mit Antibiotika zu entkommen und ermöglichen somit wiederkehrende Infektionen. Der zugrundeliegende Mechanismus, welcher die phänotypische Heterogenität reguliert, ist vielfältig und muss besser verstanden werden, um Behandlungsstrategien mit Antibiotika zu optimieren. Diesem Ansatz folgend, wurden die phänotypischen Veränderungen von *S. maltophilia* untersucht, die durch die Behandlung der Bakterienzellen mit unterschiedlichen Konzentrationen des Antibiotikums Ampicillin hervorgerufen wurden. Unter dem Einfluss von Ampicillin bilden *S. maltophilia* Populationen zelluläre Subpopulationen mit unterschiedlichen aber reversiblen Morphotypen von großen und kleinen Kolonien aus. Um sicher zu gehen, dass die unterschiedlichen Morphotypen der Kolonien nicht durch stabile Mutationen wie SNPs verursacht wurden, wurden die Genotypen der 24 Kolonievarianten sequenziert. Es konnte eine bedeutende Anzahl an SNPs wie Insertionen und Deletionen identifiziert werden. Interessanterweise sind diese Mutationen nicht hauptsächlich mit dem bakteriellen Resistom assoziiert. Des Weiteren konnte keine der Mutationen in den für das Wachstum essentiellen Genen nachgewiesen werden. Im Anschluss an die Behandlung mit β-Laktam-Antibiotika wurden Transkriptome der resultierenden großen und kleinen Kolonie-Varianten, welche reversible Phänotypen zeigten, erstellt. Es konnten 12 Gene identifiziert werden, die in den großen und kleinen Kolonien unterschiedlich exprimiert wurden. Unter den unterschiedlich exprimierten Genen waren, im Vergleich zu den kleine Kolonien, die Gene *bla*$_{L1}$ und *bla*$_{L2}$ in

großen Kolonievarianten 15.3- beziehungsweise 6.9-fach transkriptionell hochreguliert. In weiterführenden Untersuchungen zu der Expression von β -Laktamase Genen auf Einzel-Zell-Ebene mit Hilfe von Promoterfusionen der Gene *bla*$_{L1}$ und *bla*$_{L2}$, konnte eine hohe phänotypische Heterogenität in Batch-Kulturen nachgewiesen werden. Es ist Bemerkenswert, dass sich einzelne Zellen innerhalb von Filamenten (oder Aggregaten) von exponentiell wachsenden Kulturen in einem „ON“ Modus befanden, während benachbarte Zellen im „OFF“ Modus verblieben. Eine detaillierte statistische Analyse von mehreren hundert Zellen zu jedem Zeitpunkt einer Batch-Kultur ergab, dass sich die Mehrheit der Zellen (95 %) während einer Periode von 24 Stunden im *bla*$_{L2}$ „OFF“ Modus befand. Nach 32 Stunden jedoch exprimierten die meisten Zellen das Rot-fluoreszierende Protein und wechseltenin den *bla* „ON“ Modus. Diese Ergebnisse waren unabhängig von der Anwesenheit des Antibiotikums Ampicillin. Darüber hinaus konnte gezeigt werden, dass sich durch die Zugabe von steril filtriertem Überstand von *S. maltophilia* K279a die Ausprägung der phänotypischen Heterogenität der Expression von *bla*$_{L2}$ in exponentiellen Kulturen stark veränderte. Die Reaktion war hochgradig reproduzierbar und konnte durch eine Wärmebehandlung des zellfreien Überstandes vor Hinzugabe geblockt werden. Dies deutet auf eine Beteiligung eines hitzesensitiven bislang unbekannten Faktors hin, welcher bei der Modulation der heterogenen Expression des Genes *bla*$_{L2}$ auf Einzell-Zell-Ebene beteiligt ist. Um weitere mögliche molekulare Schalter bezüglich der Heterogenität zu entdecken, wurden genomweite Transkriptionsprofile von Zellen nach 27 und 32 Stunden Wachstum mittels RNA-seq analysiert. Hierbei konnte beobachtet werden, dass neben dem *comE*-homologen Gen *smlt134* auch zwei mögliche transmembrane Efflux-Gene (*smlt2851* und *smlt2852*) unterschiedlich exprimiert werden in Zellen, die *bla*$_{L2}$ homogen im Vergleich zu heterogen exprimieren.. Überexpression von *comE* homologen Genen in *S. maltophilia* K279a reduziert die Anzahl an Zellen, welche sich im *bla*$_{L2}$ „ON“ Modus befinden, auf ein Prozent und weniger. Im Gegensatz dazu konnte bei einer Überexpression von Effluxpumpen kein Einfluss auf die phänotypische Heterogenität festgestellt werden. Die Ergebnisse deuten darauf hin, dass das *comE* Homolog einen Einfluss auf die heterogene Expression von *bla*$_{L2}$ hat, dieser Effekt aber durch bislang nicht identifizierte Signalmoleküle, die ins Medium hinzugegeben werden, aufgehoben werden kann. Zellen der konstruierten Mutante SMK279aΔ*smlt3723* (*ampR*), welche keine Regulatoren für die Gene *bla*$_{L1}$ und *bla*$_{L2}$ kodiert, wiesen in mit Ampicillin angereichertem Medium kein Wachstum auf. Dies deutet darauf hin, dass die natürliche sowie die induzierte Expression der Gene *bla*$_{L1}$ und *bla*$_{L2}$ von aktivierenden Liganden in *S. maltophilia* abhängen. Obwohl die natürliche Expression von β-Laktamase zu einer Verringerung des Koloniewachstums führte, war es dennoch notwendig den Antibiotikastress in den kleinen Kolonievarianten zu überwinden. Im Zusammenspiel mit den Sequenzanalysen der kompletten

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

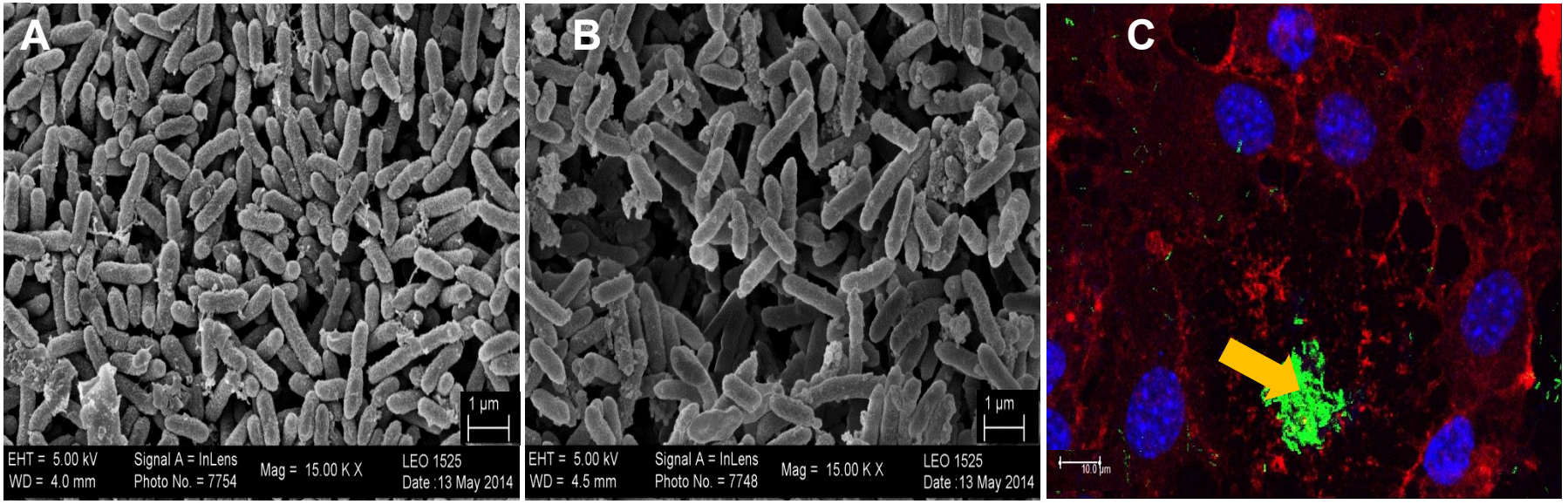


Figure 1: Microscopic images of *S. maltophilia* isolates. Scanning electron microscope images of a patient isolate *S. maltophilia* K279a (A) and an environmental isolate CL5 (B). *S. maltophilia* K279a was isolated from the blood of a cancer patient while CL5 from soil samples taken in the Botanical 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).

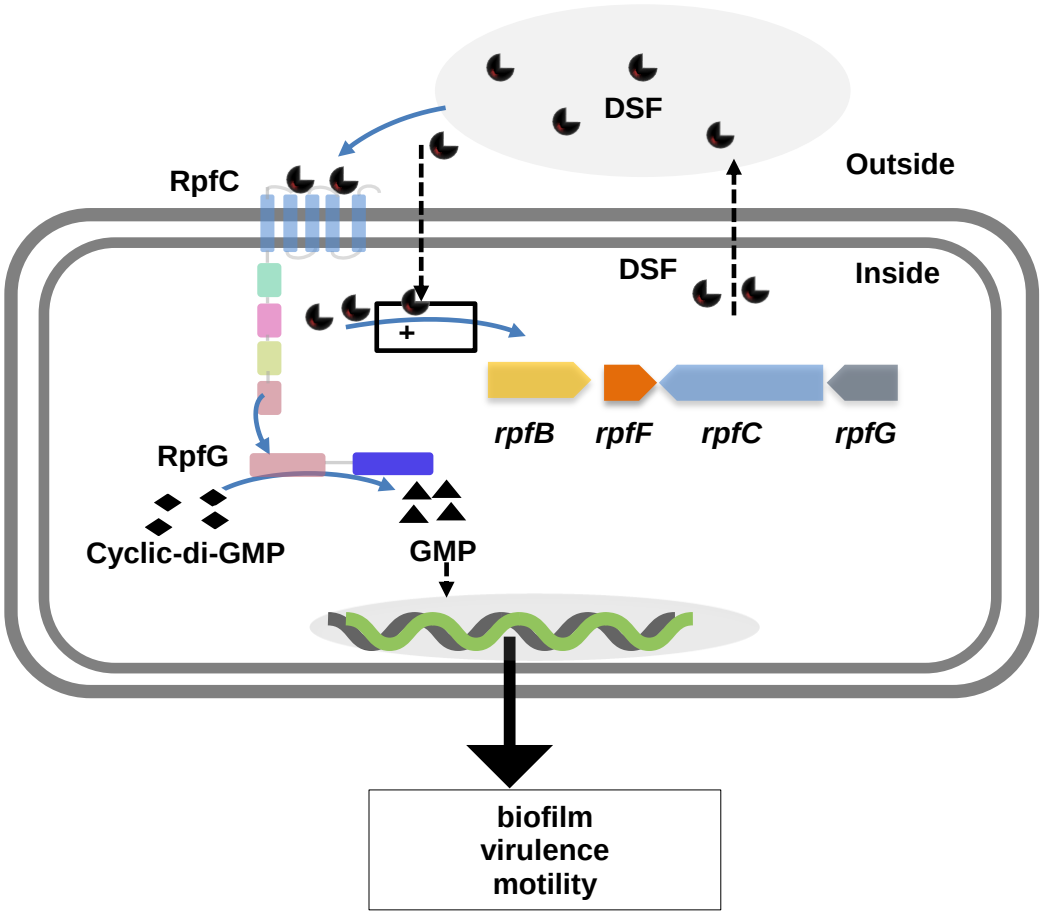


Figure 2: *S. maltophilia* QS system based on diffusible signal factor. The QS genes are arrayed as *rpf* clusters consisting of *rpfB* (fatty acyl-CoA ligase), *rpfF* (enoyl coenzyme A hydratase), *rpfC* (histidine kinase) and *rpfG* (response regulator). The product of *rpfF* gene is mainly responsible 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

(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.

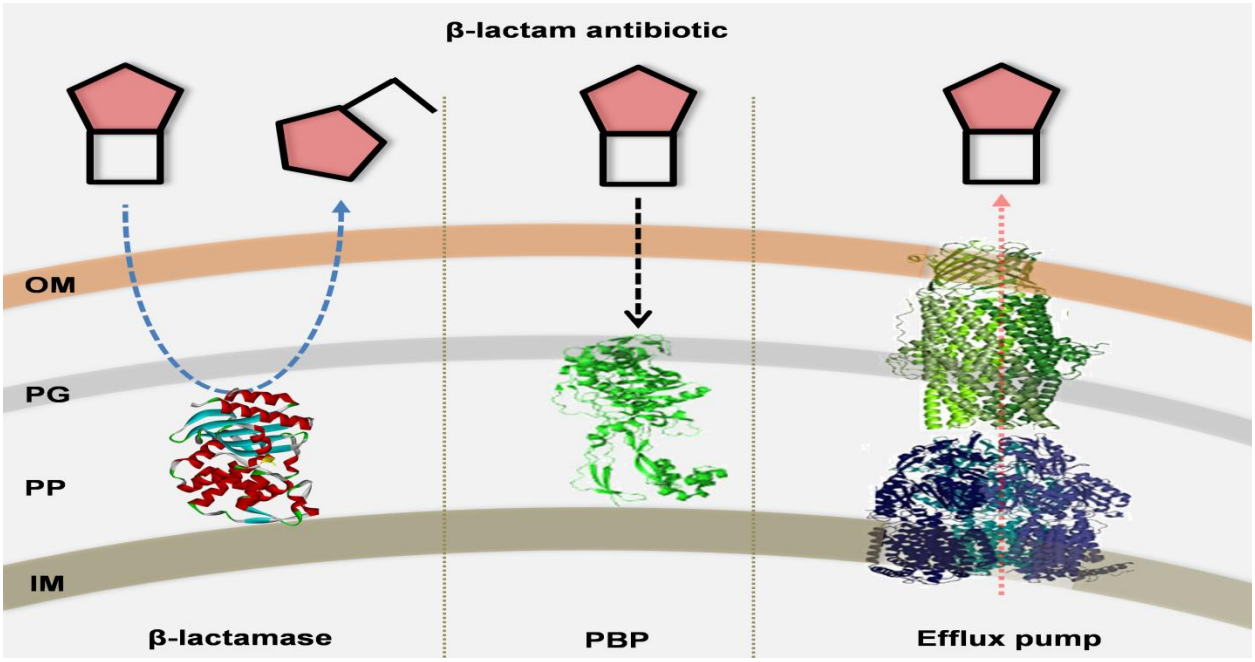


Figure 3: Mechanisms of β-lactam resistance in Gram-negative bacteria. They involve the proteins β-lactamase, PBPs and efflux pumps. Shown above are the ribbon representation of the atomic structure of a *Klebsiella pneumoniae* β-lactamase and a *Pseudomonas aeruginosa* penicillin binding protein 3 and 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). Source of protein structures (Drawz and Bonomo, 2010, Sainsbury *et al*., 2011, Venter *et al*., 2015). Abbreviations: IM, inner membrane; PP, periplasm; PG, peptidoglycan; OM, outer membrane.

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).

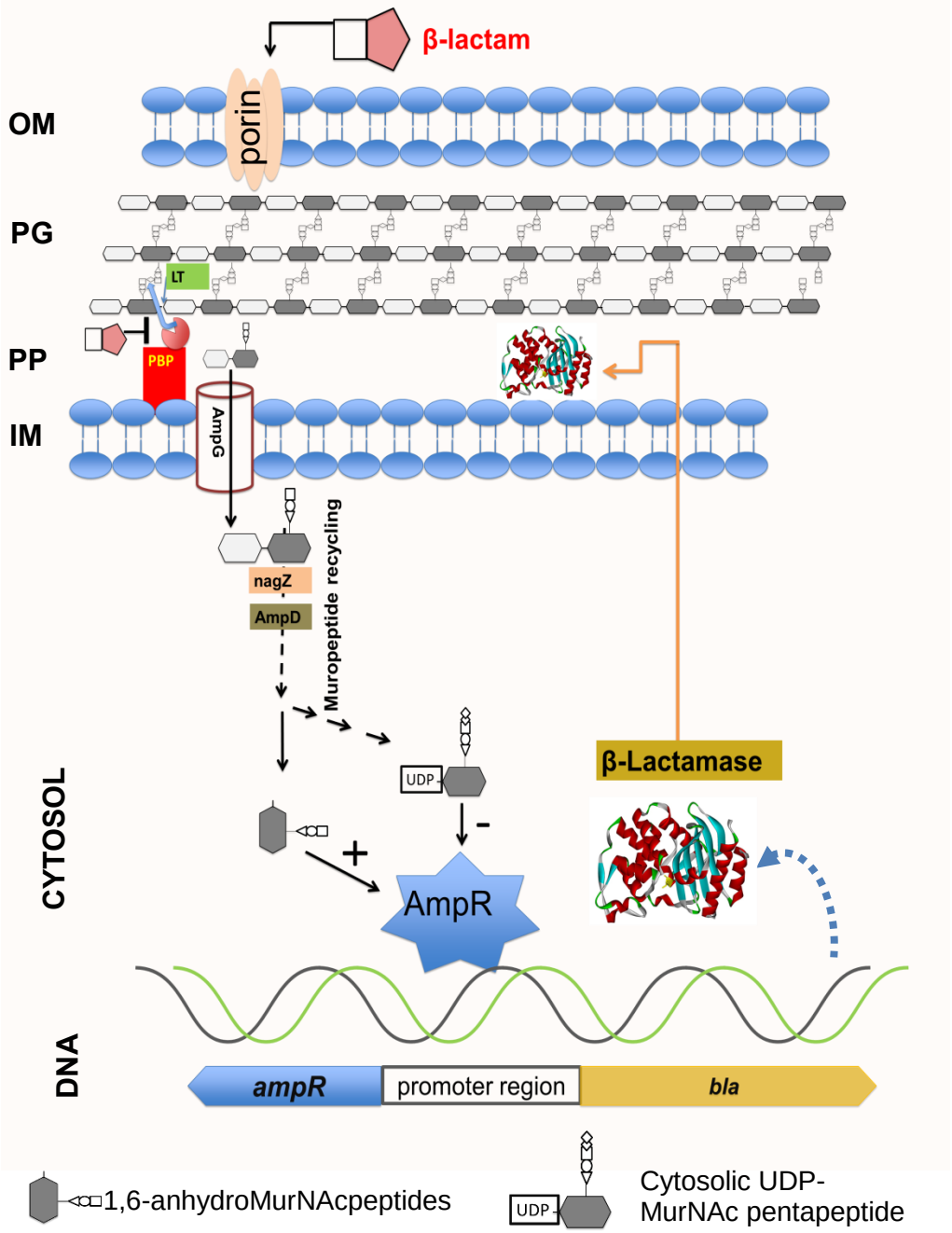


Figure 4: The AmpR–AmpC pathway of β-lactamase induction in Gram-negative bacteria. Expression is controlled by the activities of several proteins including AmpG, NagZ, AmpD and the transcriptional regulator AmpR among others. Upon β-lactam challenge the actions of transpeptidases are blocked or slowed 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 (bla_{L1}) and L2 (bla_{L2}). The product of bla_{L1} is an Ambler class B Zn^{2+}-dependent metalloenzyme that hydrolyses all classes of β-lactams except the monobactams. The bla_{L2} 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*-bla_{L2} 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 bla_{L2} and it is one of the few cases that its product AmpR controls unlinked bla_{L1} expression. Further, it was suggested that the bla_{L2} and bla_{L1} 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 bla_{L1} and bla_{L2} expression in *S. maltophilia,* yet bla_{L1} induction requires more activation of AmpR than bla_{L2} 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,

Grote *et al*., 2014, Mulder and Coombes, 2013, Wang *et al*., 2014).

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 form 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. Heteroresisters 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 a 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 mechanism 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.

Strain or construct	Description	Reference /source
***E. coli* strains**		
HB101	F^-, *hsd*S20($r_B^-m_B^-$), *rec*A13, *ara*14, *pro*A2, *lac*Y1, *gal*K 2,*rps*L20 (Sm^R) *xyl*-5, *mtl*-1, *sup*E44, λ^-,	(Figurski and Helinski, 1979)
SY327	Δ(*lac pro*) *argE*(*Am*) *recA56 rif* ^{R} *nalA* λ *pir*	(Miller and Mekalanos, 1988)
DH5α	F^- Φ80*lacZ*ΔM15 Δ(*lacZYA-argF*) U169 *recA1 endA1 hsdR17*($r_K^-m_K^+$) *phoA supE44 thi-1 gyrA96 relA1* λ^-	(Hanahan, 1983)
***S. maltophilia* strains**		
SMK279a	Clinical isolate from the blood of a cancer patient	(Avison *et al*., 2000)
SMK279aΔ*smlt1134*	SMK279a lacking the *smlt1134* gene, coding for a putative DNA transport competence protein	This study
SMK279aΔ*smlt2851*Δ*smlt2852*	SMK279a lacking the *smlt2851* and *smlt2852*, genes coding for putative transmembrane efflux proteins	This study
SMK279aΔ*smlt3723*	SMK279a lacking the *ampR* (*smlt3723*) gene; a putative HTH and LysR family transcriptional regulator	This study
SMK279aΔ*smlt0387*	SMK279a carrying a deletion in ORF Smlt0387	This study
SMK279a EM2	SMK279a carrying pBBR1MCS-5::P*bla*$_{L2}$::*rfp*	This study
SMK279a EM3	SMK279a carrying pBBR1MCS-5::P*bla*$_{L1}$::*rfp*	This study
SMK279a EM4	SMK279a carrying pBBR1MCS-5::Pno::*rfp*	This study
SMK279a EM5	SMK279a carrying pBBR1MCS-5::P*bla*$_{L2}$::*cfp*	This study
SMK279a EM6	SMK279a carrying pBBR1MCS-5::P*bla*$_{L2}$::*yfp*	This study
SMK279a EM7	SMK279a carrying pBBR1MCS-5::P*bla*$_{L2I}$::*rfp*	This study
SMK279a EM8	SMK279a carrying pBBR1MCS-5::P*bla*$_{L2}$::*rfp::smlt1134*	This study
SMK279a EM9	SMK279a carrying pBBR1MCS-5::P*bla*$_{L2}$::*rfp:: smlt2851*::*smlt2852*	This study
SM-GFP	GFP chromosomal insert	Research Center Borstel
CF148	Clinical isolate from the respiratory tract of a cystic fibrosis patient	Jörg Steinmann (Universitätsklinikum Essen)
DSM-50170	Reference strain from the oropharyngeal region of a patient with cancer	Leibniz institute DSMZ
***Sinorhizobium fredii* NGR234**		
R. fredii NGR234	Wild-type strain, Rf^r	(Trinick, 1980)
R. fredii NGR234	NGR234 P*rpoD::rfp* promoter fusion in pBBR1MCS-5	(Grote *et al*., 2014)
X. campestris		
X. campestris 8004	Wild type, Rf^r	(Turner *et al*., 1984)

Table 2: Plasmids and constructs used in this study.

Plasmids or construct	Description	Reference /source
pTZ19R	High copy cloning vector, Cm^r	Fermentas (St. Leon-Rot, Germany)
pTZ19R:: *bla*$_{L1}$	*bla*$_{L1}$ encoding for Zn^{2}+-dependent β-lactamase cloned in pTZ19R under T7 promoter	This study
pTZ19R:: *bla*$_{L2}$	*bla*$_{L2}$ encoding for serine β-lactamase cloned in pTZ19R under T7 promoter	This study
pRK2013	Kan^R; RK2-derived helper plasmid carrying the *tra* and *mob* genes for mobilization of plasmids containing *oriT*	(Figurski and Helinski, 1979)
pBBR1MCS-5	Broad host range vector, low copy, Gm^r	(Kovach *et al*., 1995)
pBBR1MCS-5::*rfp*	pBBR1MCS-5 carrying the *rfp* gene in the MCS	This study
pBBR1MCS-5::P*bla*$_{L1}$::*rfp*	P*bla*$_{L2}$::*rfp* reporter fusion in pBBR1MCS-5	This study
pBBR1MCS-5::P*bla*$_{L2}$::*rfp*	P*bla*$_{L1}$::*rfp* reporter fusion in pBBR1MCS-5	This study
pBBR1MCS-5::P*bla*$_{L2}$::*yfp*	P*bla*$_{L2}$::*yfp* reporter fusion in pBBR1MCS-5	This study
pBBR1MCS-5::P*bla*$_{L2}$::*cfp*	P*bla*$_{L2}$::*cfp* reporter fusion in pBBR1MCS-5	This study
pBBR1MCS-5::Pno::*rfp*	Promoterless *rfp* reporter fusion in pBBR1MCS-5	This study
pBBR1MCS-5::P*bla*$_{L2I}$::*rfp*	P*bla*$_{L2I}$::*rfp* reporter fusion constructed with the *ampR-bla*$_{L2}$ intergenic region in pBBR1MCS-5	This study
pBBR1MCS-5:: P*bla*$_{L2}$::*rfp::smlt1134*	*smlt1134* gene under its native promoter cloned into the pBBR1MCS-5::P*bla*$_{L2}$::*rfp* reporter fusion	This study
pBBR1MCS-5::P*bla*$_{L2}$::*rfp:: smlt2851*::*smlt2852*	Putative operon of *smlt2851* and *smlt2852* under its native promoter cloned into the pBBR1MCS-5::P*bla*$_{L2}$::*rfp* reporter fusion	This study
pEX18Tc	*sacBoriT*, Tc^r, gene replacement vector	(Hoang *et al*., 1998)
pEX18Tc: Δ0387	pEX18Tc vector with a 950-bp DNA insert of SMK279a containing the flanking region upstream of *smlt0387*	This study
pGPI-SceI-XCm	Cm^R, Tp^R; mobilizable suicide vector; carries the R6Kγ origin of replication, the I-SceI recognition site and a *xylE* reporter gene	(Hamad *et al*., 2010)
pDAI-SceI-SacB	Tet^R; mobilizable broad host range plasmid; carries the gene for the I-SceI homing endonuclease and the *sacB* gene	(Hamad *et al*., 2010, Flannagan *et al*., 2008)
pUDK011	pGPI-SceI-XCm with a 705-bp *Sph*I/*Kpn*I insert of SMK279a containing the flanking region upstream of *smlt1134*	This study
pUDK012	pUDK011with a 741-bp *Kpn*I/*Xba*I insert of SMK279a containing the flanking region downstream of *smlt1134*	This study
pUDK014	pGPI-SceI-XCm with a 779-bp *Not*I/*Kpn*I insert of SMK279a containing the flanking region upstream of *smlt2852*	This study
pUDK015	pUDK014 with a 776-bp *Kpn*I/*Xba*I insert of SMK279a containing the flanking region downstream of *smlt2851*	This study
pUDK017	pGPI-SceI-XCm with a 761-bp *Not*I/*Kpn*I insert of SMK279a containing the flanking region upstream of *smlt3723*	This study
pUDK018	pUDK017 with a 660-bp *Kpn*I/*Xba*I insert of SMK279a containing the flanking region downstream of *smlt3723*	This study

Table 3: Primers used in this study.

Primer	Sequence (5' – 3')
Smlt3722For	CTTAGGTACCCGGATCTGGTGGCTCAGT
Smlt3722Rev	CGATGAATTCCGAGCATGCGGGTTCTCCTG
3722rfpFor	CTTAGGTACCCATCGCGCAGTCGTGA
3722rfpRev	CGTTGAATTCATGCGGGTTCTCCTGG
3722rfp_For	CTTAGGTACCCATCGCGCAGTCGTGA
3722rfp_rev	CGTTGAATTCATGCGGGTTCTCCTGG
0387_Up_F	CGGAATTCTTCGAACAGGGTCTGGCGTG
0387_Up_R	GAGGCATCTTCAGCGCGGGGGGTACACCTTTACT
0387_Down_F	AGTAAAGGTGTACCCCCCGCGCTGAAGATGCCTC
0387_Down_R	CCCAAGCTTGACACCCGCGTTGCAGT
Del_Ch_O_for	CGCTGCTGCACACCATAAG
Del_Ch_O_Rev	TGTCGAGGCATCTTCAG
Del_Ch_I_for	AAGACCGACGTCGATGGCAT
Del_Ch_I_Rev	ATGTCGACGCCGTGCTT
Down_Flank_0387	GCGCGTACTCAACCGTGGTGAA
Up_flank_0387	GCCATTGGCAATCGACGGGCACTTC
pex18Tc_for	TCTTGGAGTGGTGAATCCGT
pex18Tc_rev	TTGCTAACGCAGTCAGGCAC

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®-Spritzenfiler (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.

Antibiotic/ supplement	Stock solution (mg/ml)	Final concentration (µg/ml)				Solvent
		E.coli	*S. maltophilia*	*X. campestris*	*R. fredii* NGR234	
Ampicillin	100	100	100			70 % ethanol
Cefuroxime	50		50			H_2O_{bidest}
Meropenem	4		4			H_2O_{bidest}
Gentamicin	10 or 50	10	50			H_2O_{bidest}
Kanamycin	25	25	25			H_2O_{bidest}
Rifampicin	50			50	25	Methanol
Chloramphenicol	25	25				H_2O_{bidest}
X-Gal	50	50				DMF
IPTG	100	100				H_2O_{bidest}
Cis -11-2-dodecenoic acid	(94.2 mM)					Methanol

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_2O_{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 H_2O_{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**
H_2O_{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))**
H_2O_{bidest}	**ad 200 ml**

P3 buffer (sterile filtered)	
K-acetate	**62.73 g (3 M)**
H_2O_{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 H_2O_{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 H_2O_{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 H_2O_{bidest} as a reference. An OD_{260} 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_{260}:OD_{280} 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, Ger-many).

50x TAE buffer		**Loading dye**	
Tris-Ac	**2 M**	**Glycerol (30 %)**	**60 ml**
EDTA (pH 8.0)	**100 mM**	**EDTA (pH 8.0)**	**50 mM**
H_2O_{bidest}	**ad 1000 ml**	**Bromophenol blue (0.25 %)**	**0.5 g**
pH 8.1 (adjusted with acetic acid)		**Xylencyanol (0.25 %)**	**0.5 g**
		H_2O_{bidest}	**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.

Reactions mixes	Analytical reaction	Preparative reaction
DNA (template)	0.25- 1 µg	1-5 µg
Restriction buffer (10 x)	1 µl	5 µl
Restriction enzyme (10 U/µl)	0.5 µl	1 µl
H_2O_{bidest}	ad 10 µl	ad 50 µl

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).

$$\mathbf{T_m\ (°C) = 69.3 + 0.41 * (\%\ GC) – (650 / bp_{Primer})}$$

$$\mathbf{T_{ann}\ (°C) = T_m – 5°C}$$

% 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.

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)**
H_2O_{bidest}	**ad 50 µl**

Table 6: Thermal cycling conditions for PCR amplification using *Pfu* DNA Polymerase or *Taq* DNA Polymerase.

Steps	Temperatures	Time	Number of cycles
Initial denaturation	95°C	5-10 min	One cycle
Denaturation	95°C	1 min	30 to 35 cycles
Annealing	T_m – 5°C	variable	
Extension	72°C	2 min/kb or 1 min/kb*	
Final extension	72°C	5-10 min	One cycle

*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**
H_2O_{bidest}	**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_m 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).

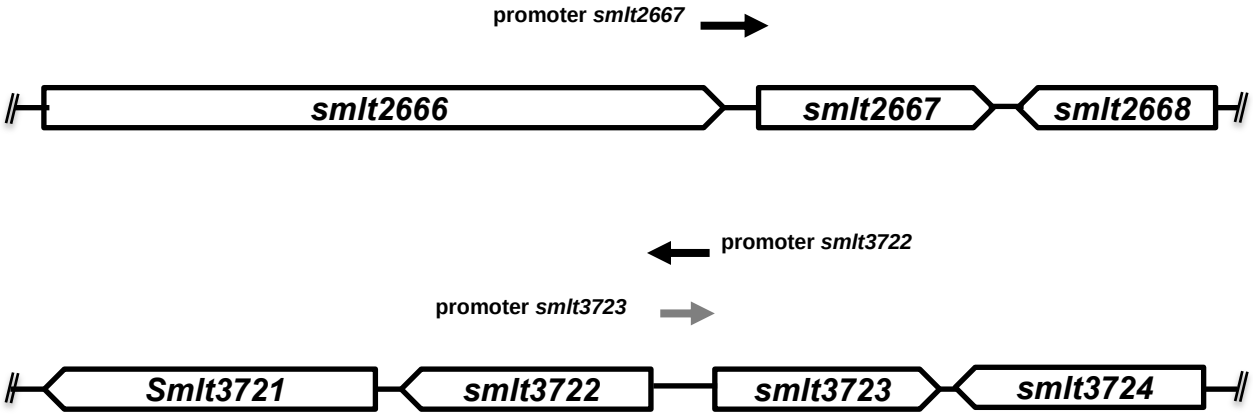
2.9.3 Single cell gene expression studies of β-lactamase

2.9.3.1 Construction of reporter promoter fusions

The activity of the two active β-lactamases, encoded by the *bla*$_{L1}$ and *bla*$_{L2}$ genes, was separately verified by cloning and expressing these genes in *E. 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 *rfp* (DsRed2) (Baird *et al*., 2000), *cfp* (mCerulean) (Rizzo *et al*., 2004) and *yfp* (mVenus) (Shaner *et al*., 2004) were fused to DNA fragments carrying the promoter of the *bla*$_{L1}$ gene or the promoter and intergenic region of the *bla*$_{L2}$.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 *bla*$_{L1}$ or *bla*$_{L2}$ genes (Figure 5). Two different potential promoter regions of *bla*$_{L2}$ were amplified (318 bp and 180 bp). Separately, a 417 bp fragment for *bla*$_{L1}$ were obtained. In subsequent steps, the amplified DNA fragments were fused into the vectors containing the *rfp*, *cfp* and *yfp* genes. The resulting constructs were then used to transform *E.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.



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 (Rangannan and Bansal, 2011) and were located immediately 300 bp upstream of *smlt2667* or *smlt3722* genes (black arrows: *smlt2667* (*bla*$_{L1}$) and *smlt3722* (*bla*$_{L2}$)).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 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 P*bla*$_{L2}$::*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 Pbla_{L2}::*rfp* in the pBBR1MCS-5 vector, resulting in pBBR1MCS-5::Pbla_{L2}::*rfp::smlt1134*; and pBBR1MCS-5::Pbla_{L2}::*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 bla_{L2} 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 bioanylzer. 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 SMK279aΔ*smlt0387* 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 SMK279aΔ*smlt0387* mutant strain. Via sequencing it was further proved that the neighbor’s genes *smlt0386* and *smlt0389* were intact in the SMK279aΔ*smlt0387* 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 SMK279aΔ*smlt1134,* SMK279aΔ*smlt2851*Δ*smlt2852* and SMK279aΔ*smlt3723* 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).

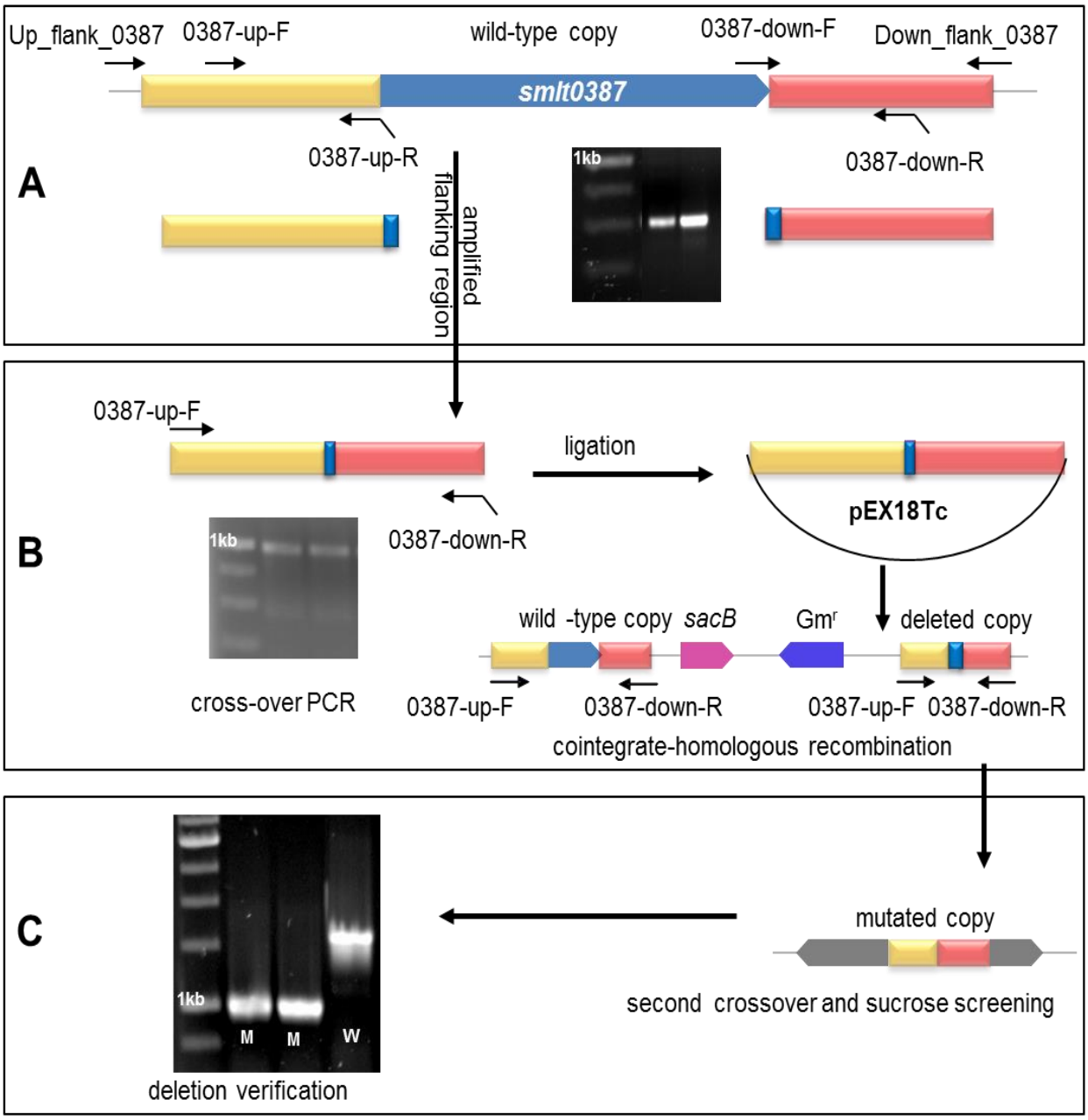


Figure 6: Schematic representation of the cloning procedure and location of PCR fragments used to generate SMK279aΔ*smlt0387*. The flanking region of *smlt0387* was PCR amplified using primer listed in Table 3 and fused together via cross over PCR, ligated into pEX18Tc suicide vector. A SMK279a cell 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.

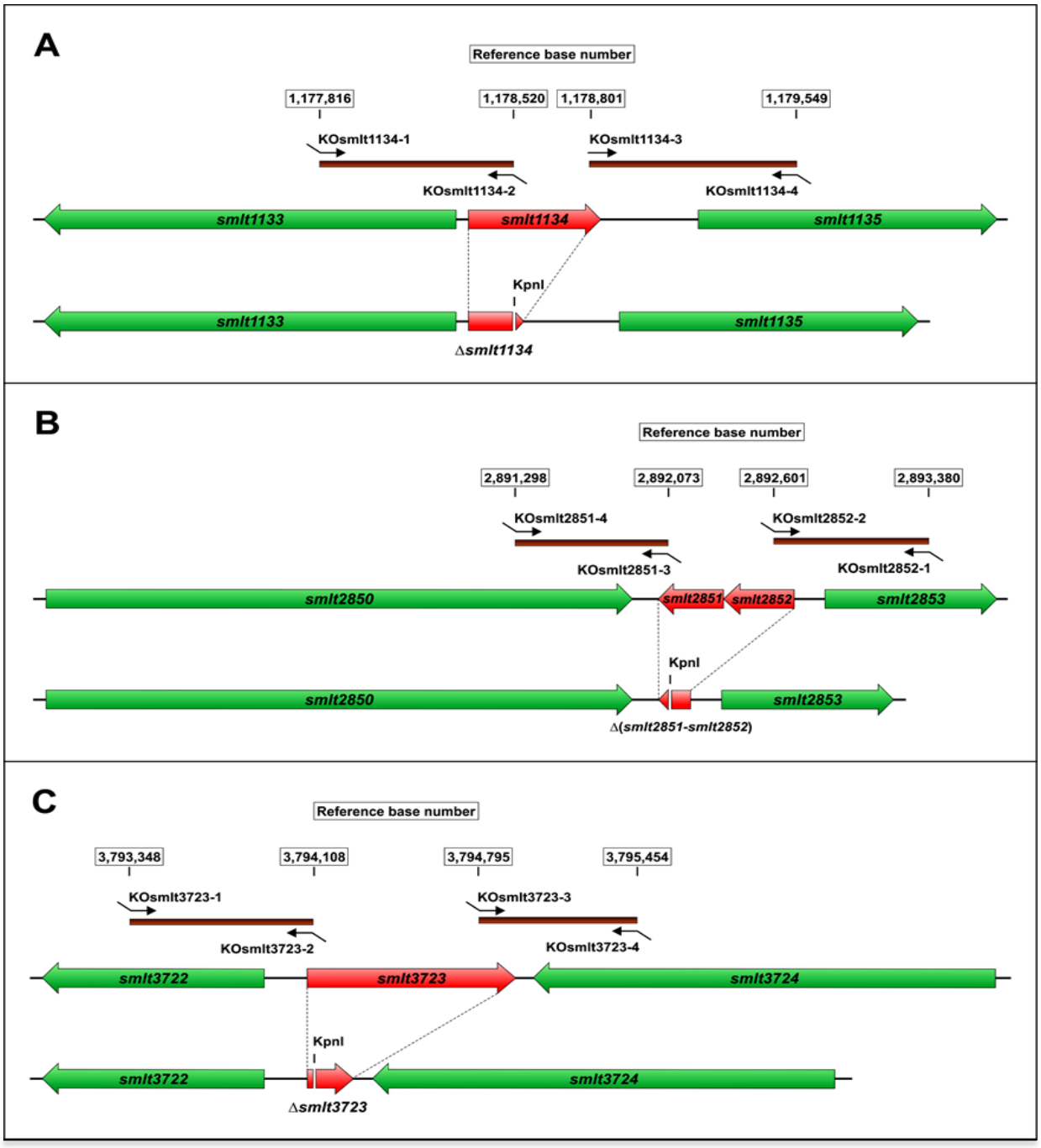


Figure 7: Physical map of genes mutated in SMK279a. Schematic representation of the cloning procedure and location of PCR fragments used to generate markerless *S. maltophilia* K279a mutants of *smlt1134* (A), *smlt2851* & *smlt2852* (B) and *smlt3723* (C). Red arrows indicate the target ORFs and their 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 indicted 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 H_2O.

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.

MOPS buffer (10x)	
MOPS	**200 Mm**
NaAc	**50 Mm**
EDTA	**10 mM**
The pH was adjusted to 7.0, using NaOH	

Formaldehyde gel solution	
Agarose	**1.2 g**
MOPS buffer (10x)	**10 ml**
Formaldehyde	**1.2 ml**
H_2O (RNase free)	**ad 100 ml**

1X MOPS RNA gel electrophoresis buffer	
MOPS buffer (10x)	**100 ml**
Formaldehyde (37 %, v/v)	**20 ml**
H_2O (RNase free)	**880 ml**

DEPC treted RNA free H_2O	
DEPC	**1 ml**
H_2O_{bidest}	**ad 1l H_2O**

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*bla*$_{L2}$::*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

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 ≥ 2.0 and an adjusted *P* value (corrected for multiple testing using the Benjamini-Hochberg procedure) ≤ 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.

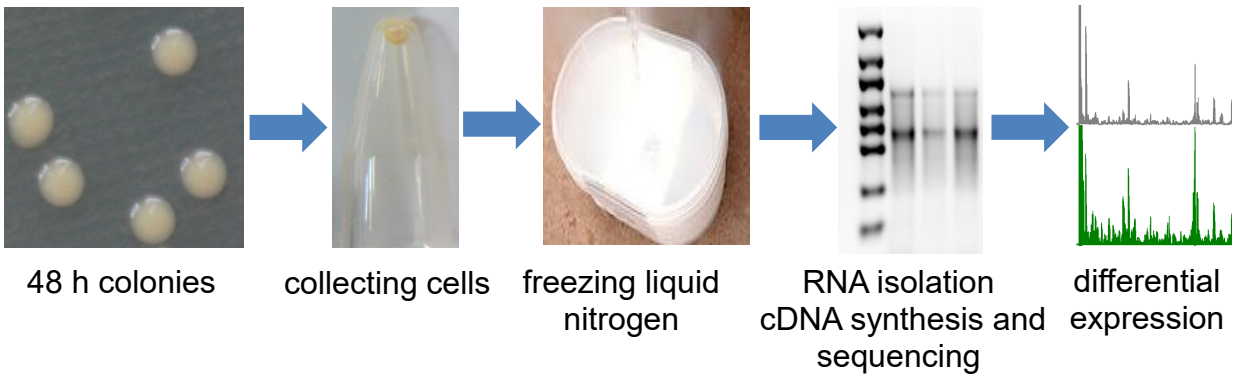


Figure 8: Colonies were scrapped rapidly and collected in E-cups for RNA-seq analysis. The time between scrapping to freezing cells was kept below a minute to avoid possible transcriptional shift. RNA-seq was carried out in the Universität Würzburg in the working group of Prof. Jörg Vogel. Image for liquid nitrogen-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 ($2^{-\Delta\Delta C(t)}$ method).

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

Primer	Sequence [5'-3']	Length of amplified product/gene
qsmlt4165-Bfor	AAGGGCCTGGAACAGGGCTA	145 bp/*rpoD**
qsmlt4165-Brev	CCACATCCGGCGCAACTTCA	
q16S-Afor	AGAGTTTGATCCTGGCTCAG	240 bp/16S rRNA*
q16S-Arev	CTAATCCGACATCGGCTCAT	
qsmlt2667-for	CGGTCACCTGCTGGACAACA	157 bp/bla_{L1}
qsmlt2667-rev	CACCGCCGTTTCTGCATTGG	
qsmlt3722-Afor	CGTCGCCGATTCCTGCAGTT	144 bp/bla_{L2}
qsmlt3722-Arev	TTCCAGCGCGGCGAAATCAC	
qsmlt3721-for	GAGCCATGAACTGATCTACC	196 bp/*smlt3721*
qsmlt3721-rev	GAACAGGAACAGCGAGGAAA	
qsmlt0018-for	CGGAACTGGAGTTCACTCTT	168 bp/*smlt0018*
qsmlt0018-rev	TGATGTCCCAATCCGTTAGC	
qsmlt0019-for	TAATACTAACGGCCCGACCT	205 bp/*smlt0019*
qsmlt0019-rev	TCTTCCTGCACCTGAGTTCT	
qsmlt1007-for	GAAGGCTTCACCAAGCTCAC	126 bp/*smlt1007*
qsmlt1007-rev	GATGCCGGTCCAGATCGTAT	

*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 bla_{L1} and bla_{L2} 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

Locus Tag	Gene product name	Activity in *E.coli*
Smlt0115	putative β-lactamase precursor	-
Smlt0347	metallo-β-lactamase family protein	-
Smlt0580	putative metallo β-lactamase family protein	-
Smlt0581	putative metallo β-lactamase family protein	-
Smlt1470	putative β-lactamase	-
Smlt1815	putative metallo-β-lactamase family protein	-
Smlt2514	putative β-lactamase	-
Smlt2563	putative β-lactamase	-
Smlt2589	putative β-lactamase AmpC protein	-
Smlt2667	putative metallo-β-lactamase, bla_{L1}	**x**
Smlt3114	putative β-lactamase	-
Smlt3132	putative β-lactamase	-
Smlt3495	putative β-lactamase	-
Smlt3652	putative β-lactamase protein	-
Smlt3722	putative β-lactamase protein, bla_{L2}	**x**
Smlt3807	putative metallo-β-lactamase protein	-
Smlt3991	putative metallo-β-lactamase protein	-
Smlt4159	putative β-lactamase protein	-
Smlt4211	putative β-lactamase protein	-

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

morphotypes after 48 h at 37^{o}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.

Treatment	Colony morphotype	% of all colonies	Colony size [diameter, mm]
Amp 100	small	80	1.4 ± 0.26
Amp 100	big	20	3.2 ± 0.27
No Amp	uniform	100	3.0 ± 0.06

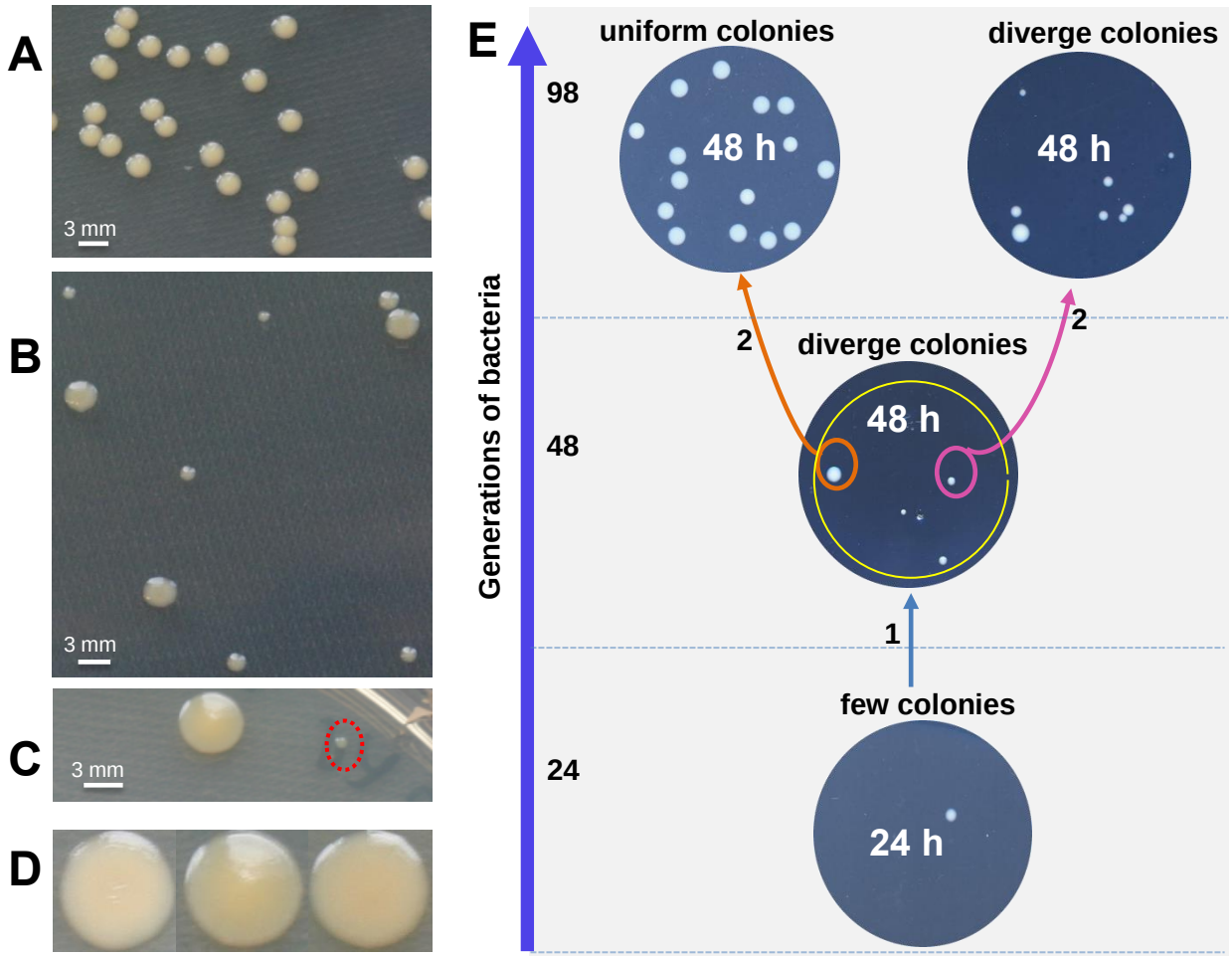


Figure 9: Phenotypic heterogeneity of SMK279a cells during growth on solid media. (A) SMK279a formed round, uniform colonies when grown without ampicillin on LB agar plates for 48 h at 37°C. (B) Colony heterogeneity on LB agar supplemented with ampicillin showing small and big colonies after growth for 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.

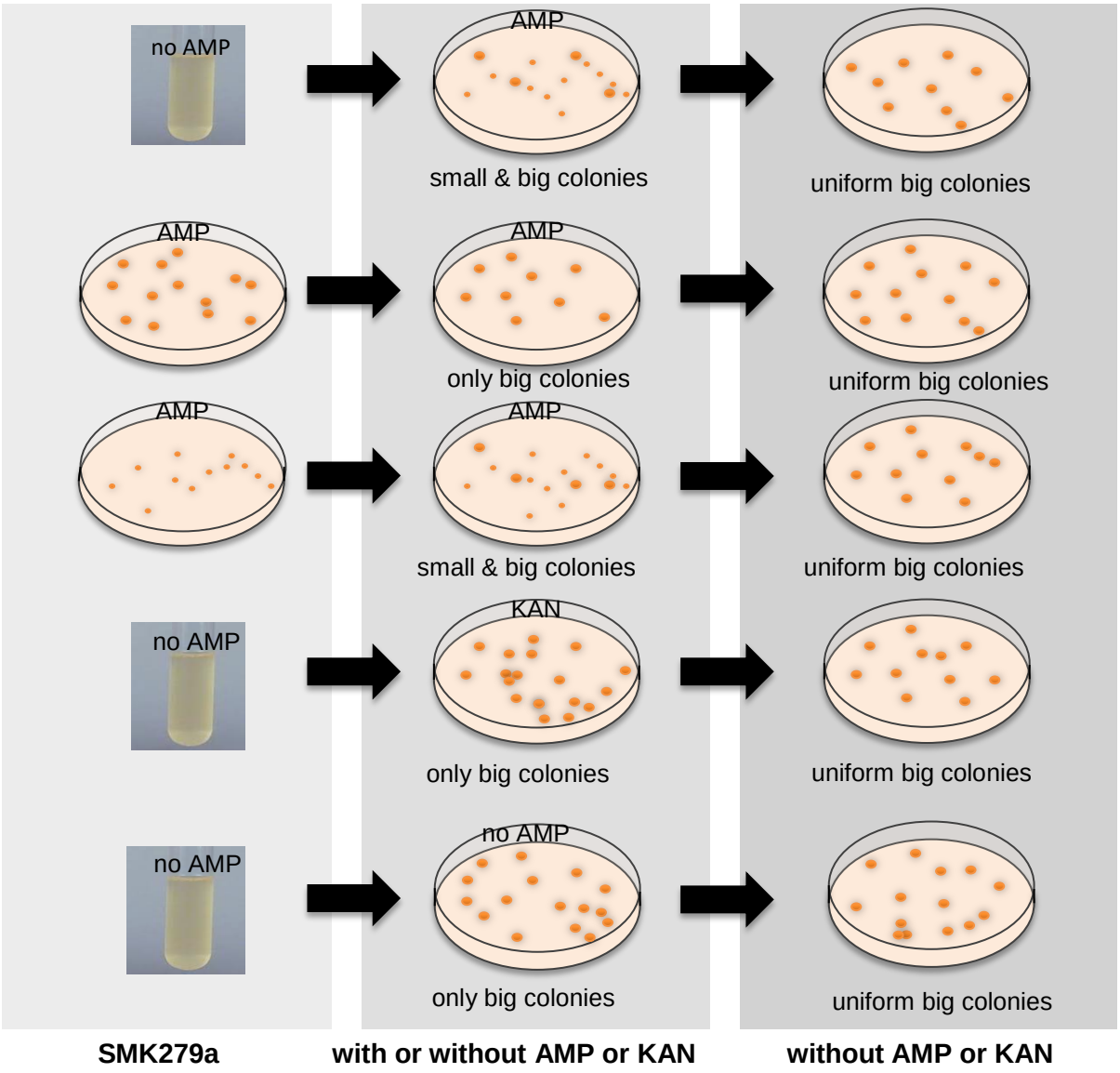


Figure 10: Schematic diagram showing colony morphologies observed for SMK279a in response to ampicillin or kanamycin treatment on LB agar plates. Arrows indicate the transfer of fresh colonies or liquid culture samples. Small colonies formed both, big and small colonies when recultivated in the presence 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.

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

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).

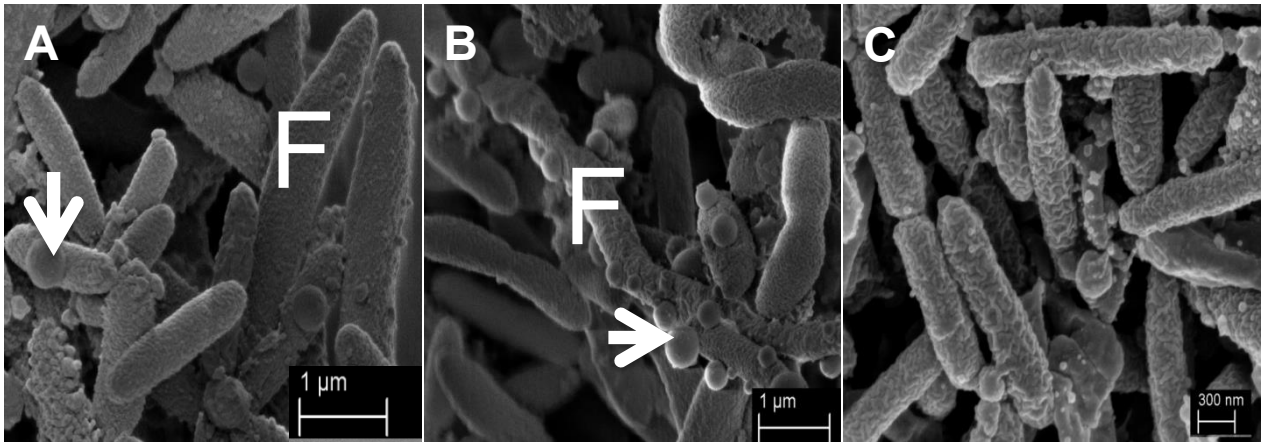


Figure 11: Representative scanning electron micrographs of SMK279a cells grown in the presence or absence of ampicillin at 37°C for 48 h on LB agar plates. SEM images of SMK279a cells were recorded as previously published (Krohn-Molt *et al*., 2013). Cells were pelleted, fixed, and dried progressively 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).

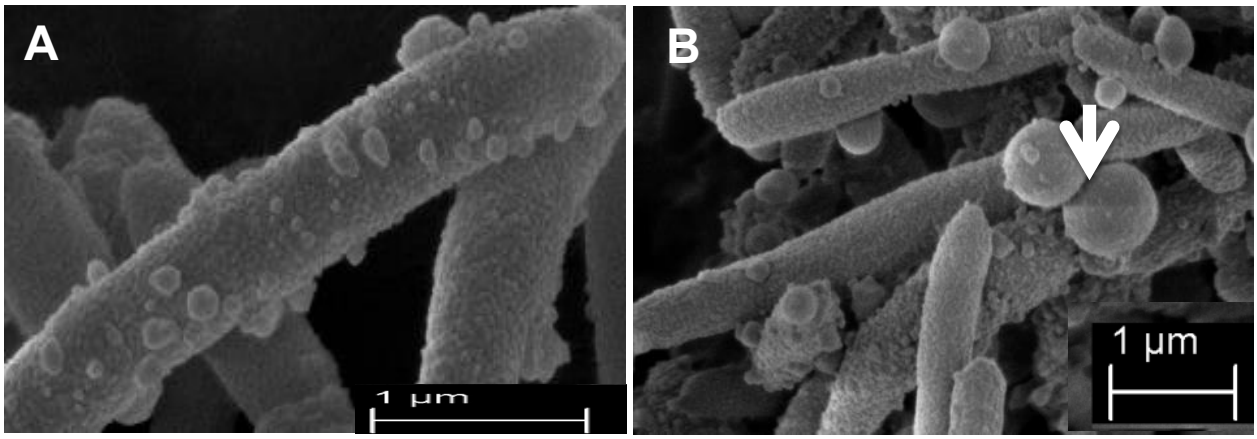


Figure 12: SMK279a cells formed large OMVs in the presence of ampicillin. The size of the OMVs varied considerably, ranging from less than 100 nm (A) up to 677 nm (as shown with an arrow) (B) in diameter in some SMK279a cells.

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).

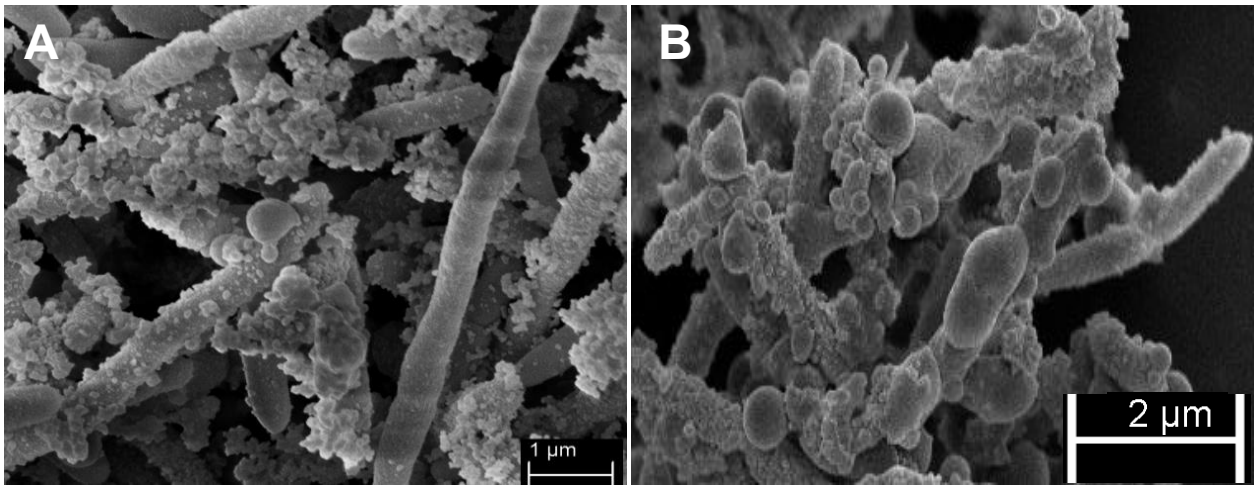


Figure 13: SEM image of cells obtained from big colonies cultured in the presence of 300 µg/ml (A) and 500 µg/ml (B) ampicillin. The cells showed increased extracellular polymeric substances (EPS) and biofilm formation in comparison to cells treated with 100 µg/ml ampicillin (Figure 11A and B).

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_{L1} and bla_{L2} was further investigated at the single cell level using fluorescent reporter proteins that were fused to the promoters regions of bla_{L1} or bla_{L2} 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_{L1} and bla_{L2} were predicted using PromBase (Rangannan and Bansal, 2011). Accordingly, the amplified PCR fragments were 417 bp and 318 bp for the bla_{L1} and $bla_{L2,}$ respectively (Figure 5 and Figure 14). Since the *ampR* is the transcriptional regulator for both genes sharing a divergent overlapping promoter region with bla_{L2}, 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 *bla*$_{L1}$ and *bla*$_{L2}$ were expressed in most reporter strains, but they were not in cells carrying P*bla*$_{L2I}$::*rfp* or Pno::*rfp* (Figure 15A-F). Nevertheless, bacterial cells carrying P*bla*$_{L2}$::*rfp* reporter fusion produced the strongest fluorescent signal with RFP (Figure 15A and B).

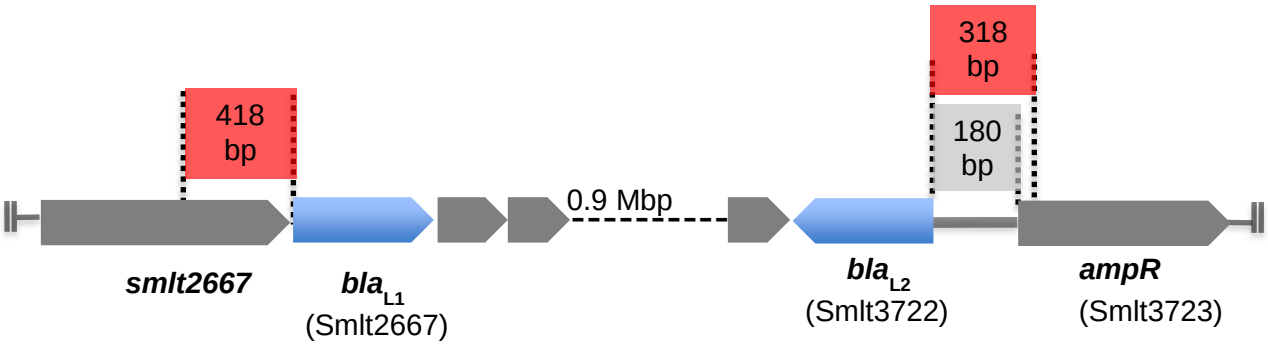


Figure 14: A partial physical map of the SMK279a genome region encoding for β-lactamase resistance genes and the transcriptional regulator *ampR*. Shown above with dashed vertical lines and red boxes are the approximate promoter DNA regions of *bla*$_{L1}$ and *bla*$_{L2}$ genes. Numbers indicate the size of the respective promoters. The *ampR and bla*$_{L2}$ share an overlapping promoter region (indicated with bold grey line).

Table 10: The size and purposes of promoter regions of *bla*$_{L1}$ or *bla*$_{L2}$ fused to the red (RFP), yellow (YFP) and cyan (CFP) fluorescent proteins.

Name	Size(bp)	Partial physical map in pBBRMCS-5	Purpose
P*bla*$_{L1}$::*rfp*	418		For monitoring *bla*$_{L1}$ gene expression on single cell level
P*bla*$_{L2}$::*rfp*	318		For monitoring *bla*$_{L2}$ gene expression on single cell level
P*bla*$_{L2I}$::*rfp*	180		A control promoter fusion constructed only from *ampR-bla*$_{L2}$ intergenic region.
P*bla*$_{L2}$::*cfp*	318		For verification *bla*$_{L2}$ gene expression on single cell level
P*bla*$_{L2}$::*yfp*	318		For verification *bla*$_{L2}$ gene expression on single cell level
Pno::*rfp*	-		A promterless *rfp* construct for monitoring background uninduced fluorescence

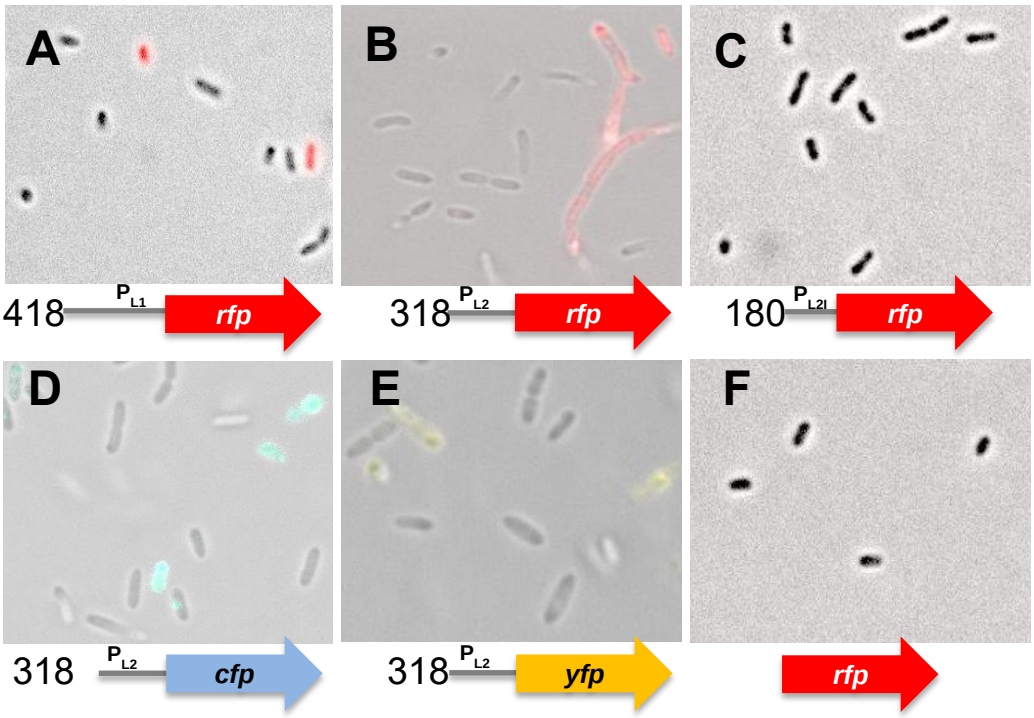


Figure 15: Analysis of single cell fluorescence of P*bla*$_{L1}$ and P*bla*$_{L2}$ promoter gene fusions. (A) Expression of the *bla*$_{L1}$ promoter fused to *rfp* in SMK279a. Cells were grown at 30°C for 17 h under aerobic conditions (200 rpm) in LB medium containing 100 µg/ml ampicillin. (B) Expression of the *bla*$_{L2}$ promoter fused to *rfp* in SMK279a grown under the same conditions as indicated in (A). (C) Cells carrying the P*bla*$_{L2I}$::*rfp* construct were in a *bla*$_{L2}$-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 P*bla*$_{L2}$::*cfp*. (E) and the P*bla*$_{L2}$::*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 P*bla*$_{L2}$::*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 P*bla*$_{L2}$::*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 P*bla*$_{L2}$::*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 β-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 β-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.

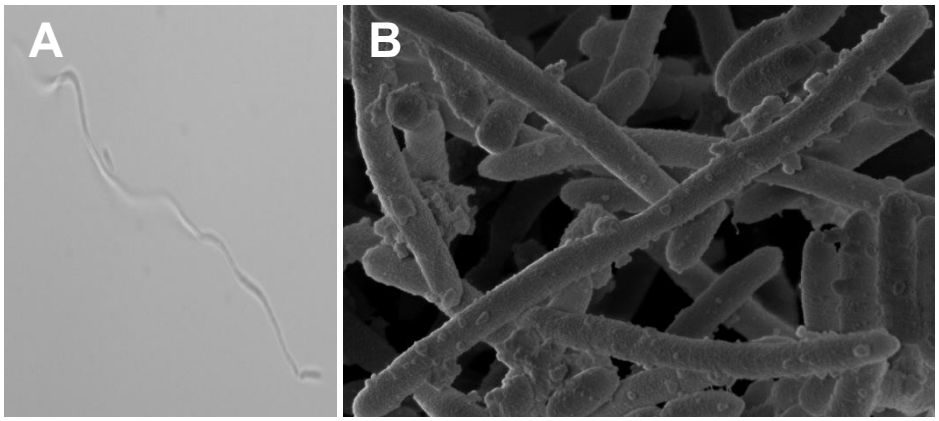


Figure 16: Treatment of cells with ampicillin resulted in formation of long cells and filaments. (A) Very long filament SMK279a observed after 4 h of ampicillin challenge in liquid cultures. (B) SEM images of overnight ampicillin-challenged bacterial cells.

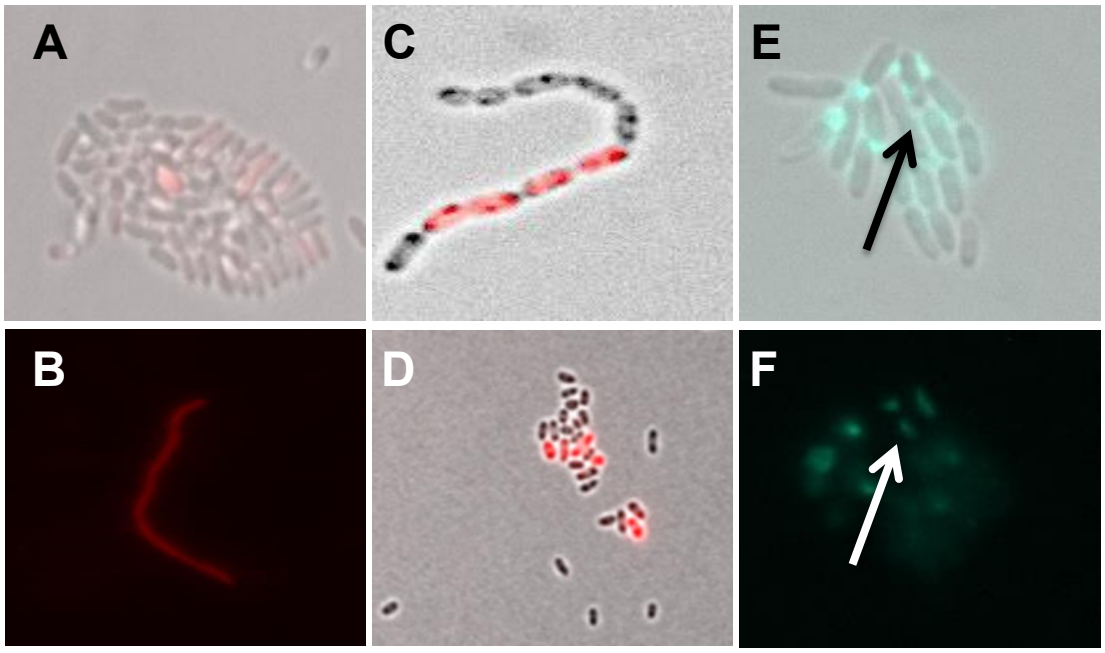


Figure 17: Analysis of single cell fluorescence of P*bla*$_{L2}$ reporter gene fusions in exponentially growing SMK279a. (A) Expression of the *bla*$_{L2}$ gene fused to *rfp* in SMK279a. Cells were grown at 30°C for 7 h under aerobic conditions (200 rpm) in LB medium containing 100 µg/ml ampicillin. (B) Homogenous *bla*$_{L2}$ expression in cells forming filament using the same fusion. (C) Phenotypic heterogeneity observed in cells (carrying the P*bla*$_{L2}$::*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-dependent metallo-β-lactamase with a broad β-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.

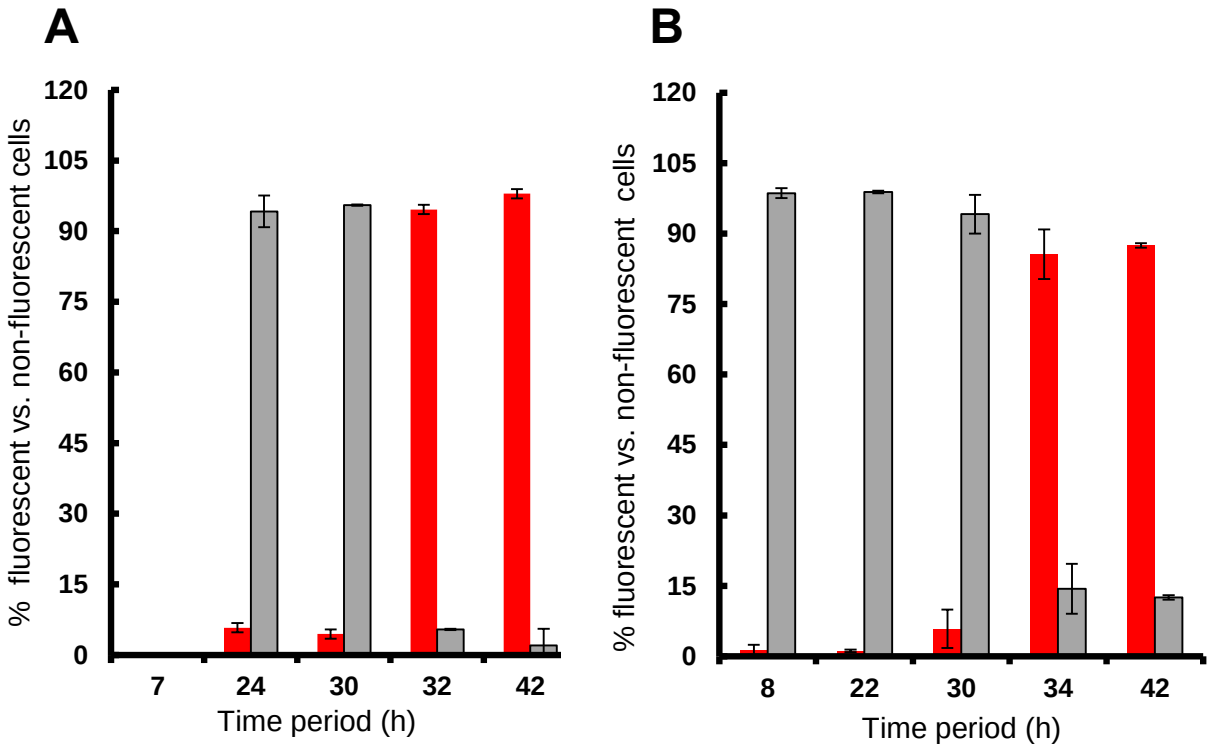


Figure 18: Analysis of *bla*$_{L2}$ and *bla*$_{L1}$ gene expression at single cell level using the RFP. (A) Percentages of fluorescent and non-fluorescent P*bla*$_{L2}$::*rfp* reporter strain cells grown for 24, 30, 32, and 42 h in LB medium containing 50 µg/ml gentamicin and ampicillin (100 µg/ml) at 30°C. (B) Percentages of fluorescent and non-fluorescent P*bla*$_{L1}$::*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*$_{L2}$ 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 P*bla*$_{L2}$::*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 P*bla*$_{L2}$::*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 P*bla*$_{L2}$::*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 was/were produced and released into the medium that were involved in triggering the switch from heterogeneous to homogenous *bla*$_{L2}$ expression.

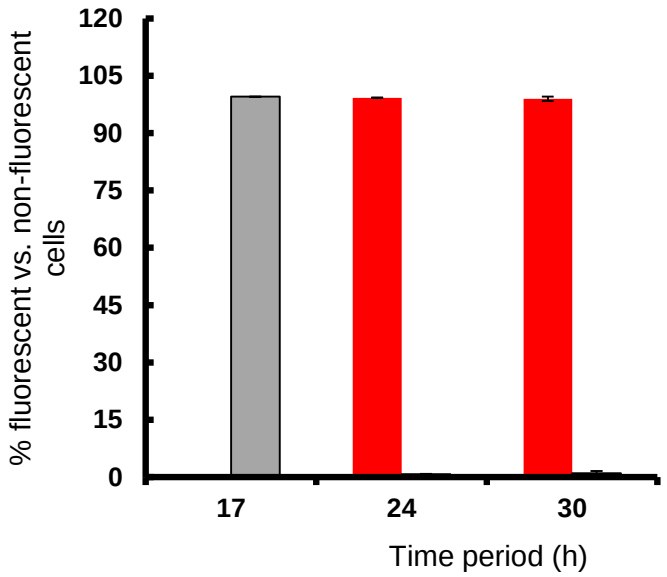


Figure 19: Homogenous expression of *bla*$_{L2}$ triggered by an environmental signal. Percentages of fluorescent and non-fluorescent P*bla*$_{L2}$::*rfp* reporter strain cells grown under aerobic conditions (200 rpm) in 5 ml reaction tubes at 30°C for 17, 24 and 30 h in LB medium, containing 50 µg/ml 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*$_{L2}$ 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 P*bla*$_{L2}$::*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 P*bla*$_{L2}$::*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*$_{L2}$ 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 P*bla*$_{L2}$::*rfp* reporter strain. On the contrary, the use of sterile culture supernatant from *R. fredii* NGR234 or Xcc did not alter the *bla*$_{L2}$ 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*$_{L2}$ 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).

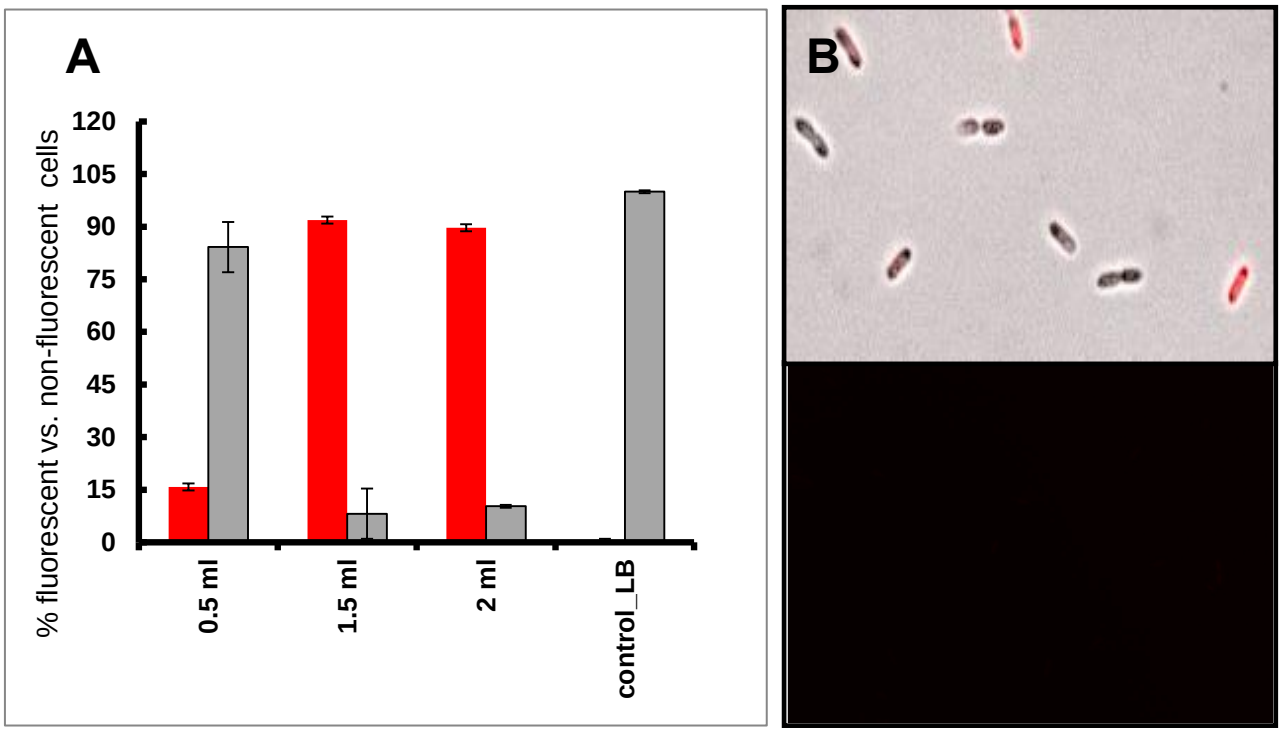


Figure 20: Cell-free culture supernatants trigger phenotypic heterogeneity in SMK279a cells. (A) Phenotypic heterogeneity of cells treated with increasing amounts of cell-free supernatants from stationary-phase P*bla*$_{L2}$::*rfp* reporter strain cultures after 7 h of growth. Data in (A) are mean values 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.

Table 11: Phenotypic heterogeneous *bla*$_{L2}$ gene expression was stimulated by several kinds of stresses.

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

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

3.2.5 Phenotypic heterogeneity with additional β-lactam antibiotics

Ampicillin has been considered as strong inducer of an inducible β-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 β-lactam gene in SMK279a (Table 11, Figure 21A). At single cell level, *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 β- lactam antibiotics used (Table 11 and Figure 21A). The replenishment ampicillin after 7 h of growth did not alter significantly the *bla*-ON and the *bla*-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).

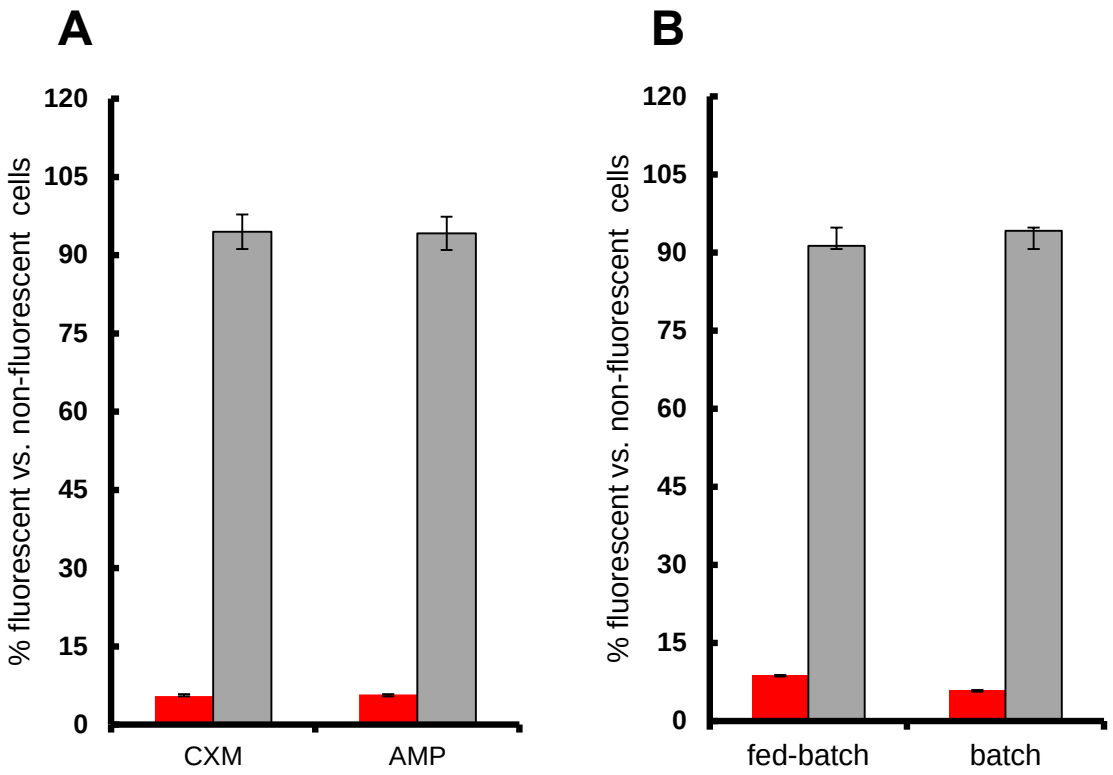


Figure 21: Heterogeneity in *bla*$_{L2}$ expression. (A) Comparison of cefuroxime (CXM) and ampicillin (AMP) on single cell *bla*$_{L2}$ expression in batch culture. (B) Comparison of fed-batch and batch growth conditions on single cell expression of *bla*$_{L2}$ gene in SMK279a. For fed-batch culture 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.

Isolate ID	Colony morphology	Ampicillin [µg/ml]	Coverage after analysis [%]	SNPs	DEL*	INS*
K279a-1	Big	600	98.4	1	0	0
K279a-2	Big	600	104.3	1	1	0
K279a-3	Small	100	52.8	1	0	0
K279a-4	Small	100	56.9	3	0	0
K279a-5	Small	100	56.4	3	0	0
K279a-6	Big	100	51.4	2	0	0
K279a-7	Big	100	62.0	1	0	0
K279a-9	Big	100	71.4	0	0	0
K279a-10	Big	100	77.5	2	0	0
K279a-11	Small	100	75.4	0	6	0
K279a-12	Small	100	73.9	2	0	0
K279a-15	Uniform	0	109.7	2	0	0
K279a-16	Uniform	0	81.6	0	1	0
K279a-17	Big	100	120.2	1	0	0
K279a-18	Big	100	83.6	0	3	0
K279a-19	Big	100	60.2	2	0	0
K279a-20	Small	100	60.2	1	0	0
K279a-21	Small	100	58.4	0	0	0
K279a-22	Small	100	68.9	0	0	0
K279a-23	Small	100	62.4	1	0	0
K279a-24	Small	100	66.6	3	0	0
K279a-25	Small	100	87.4	1	0	0
K279a-26	Small	100	95.3	2	0	0
K279a-27	Small	100	112.3	0	0	0

*DEL, deletion; INS, insertion.

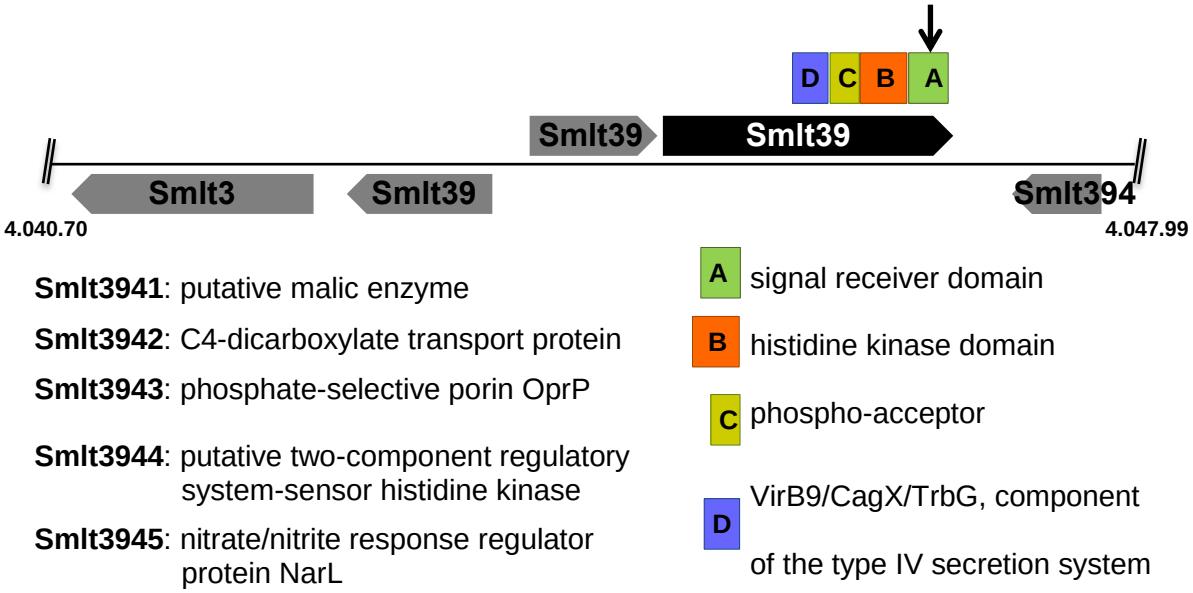


Figure 22: Partial physical arrangement of the two component regulatory system-sensor histidine kinase Smlt3944 and flanking ORFs on the SMK279a genome. The black arrow indicates the histidine kinase Smlt3944, grey-shaded arrows indicate flanking ORFs that are possibly under the regulatory effect of 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.

ORF/gene	Sum of all SNPs in colony samples*			Number of positions affected within ORF
	small	uniform	big	
Smlt0301	0	1	0	1
Smlt0590	0	0	1	1
Smlt0967	1	0	2	1
Smlt1450	1	0	0	1
Smlt1562	6	0	0	6
Smlt1844B	27	3	35	7
Smlt1958	1	0	2	1
Smlt2315 (*flgD*)	0	0	3	3
Smlt2360	1	0	0	1
Smlt3752 (*sucD*)	0	0	1	1
Smlt3885 (*mpl*)	1	2	9	9
Smlt3927 (*pcm*)	1	0	0	1
Smlt3944	22	2	15	9
Smlt4235	4	0	1	1
Smlt4306a2 (*cap*)	3	0	1	1

*Genomes of 13 small, nine big, and two uniform SMK279a colonies were analyzed.

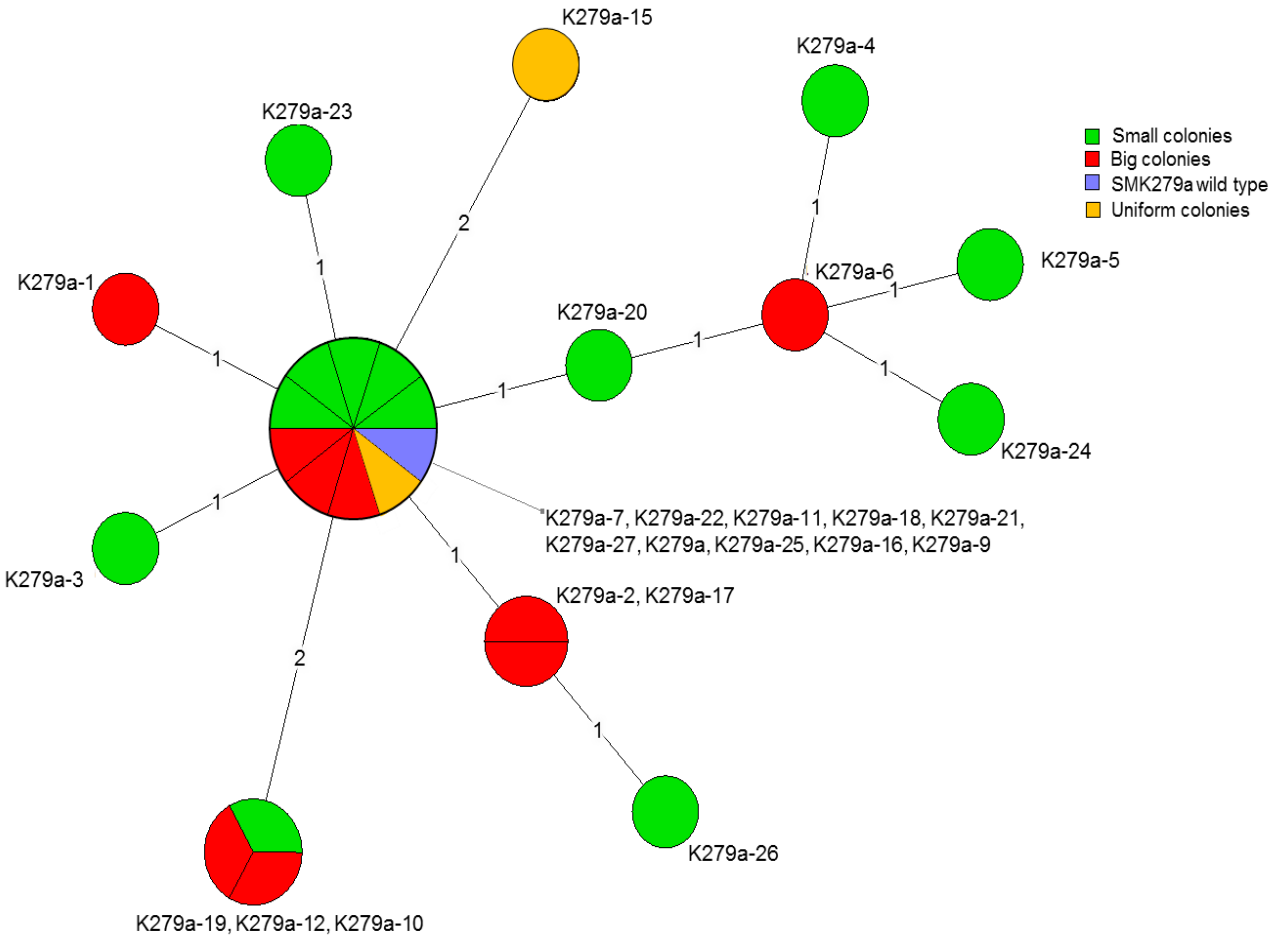


Figure 23: Maximum parsimony tree built from 15 single nucleotide polymorphism positions identified in different colony variants compared to SMK279a. The SNPs were identified in a set of 13 small, nine big and two uniform colony variants compared to the genome sequence of strain SMK279a. Numbers on branches 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

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.

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

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 *bla*$_{L1}$ and *bla*$_{L2}$ (Table 15). Therefore, *bla*$_{L1}$ (*smlt2667*) and *bla*$_{L2}$ (*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 *bla*$_{L1}$ 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 *bla*$_{L1}$ and *bla*$_{L2}$ 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 bla_{L2} flanking gene *ampH* was three-fold up regulated in big colonies. The function of *ampH* remains to be determined.

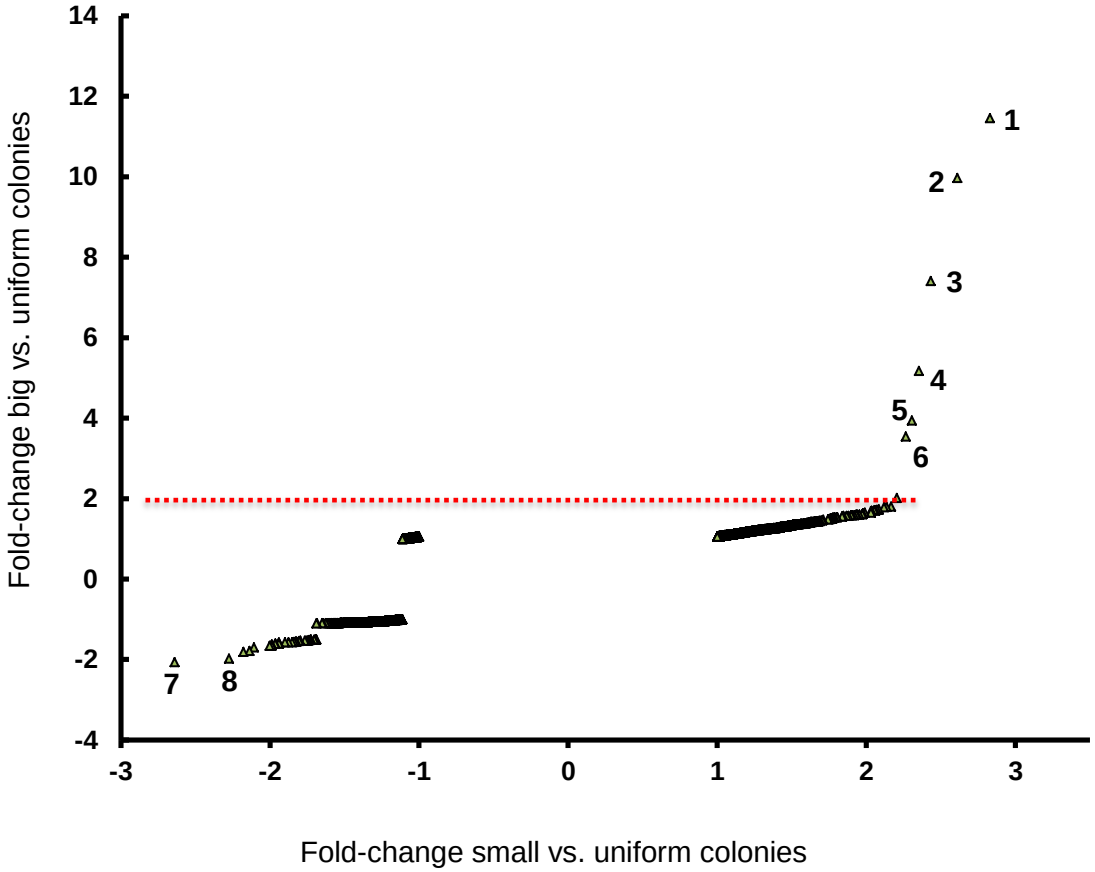


Figure 24: RNA-seq data analysis for different SMK279a colony morphotypes. Scatter plot of fold change in transcription of genes among cells of big (*Y*-axis) and small (*X*-axis) colonies relative to untreated samples of uniform colonies grown in absence of ampicillin on LB agar plates. A two-fold change in the genes transcription with adjusted *P* value of ≤ 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, *smlt0018*; 2, *smlt2667*; 3, *smlt3772*; 4, *smlt2668*; 5, *smlt0019*; 6, *smlt3721*; 7, *smlt1760* and 8, *smlt0596.*

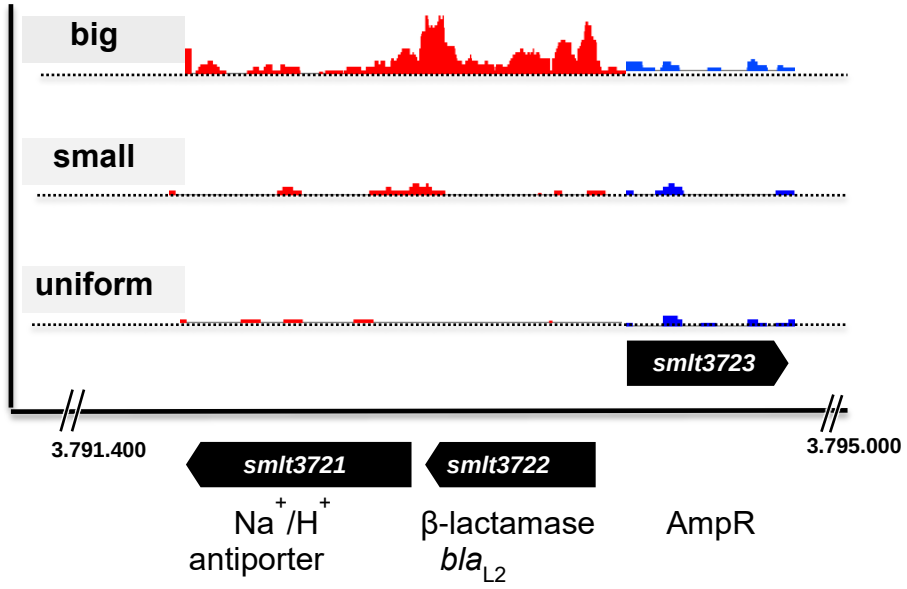


Figure 25: Transcriptome profiles of *bla*$_{L2}$ and the flanking genes *ampR* (*smlt3723*) and *ampH* (*smlt3721*) among the colony morphotypes. The *bla*$_{L2}$ gene was 6.9-fold and 7.4-fold down-regulated in cells forming small colonies in comparison to big and uniform colonies, respectively. Transcriptome 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 *ampR* was shown above for *S. maltophilia* where the level of expression was unaffected by β-lactams pressure, the loss of expression of the *ampR* (*smlt3723*) on colony morphotypes was analyzed by constructing an *ampR*-deletion mutant strain. The SMK279aΔ*smlt3723* deletion was constructed using the pGPI-SceI/pDAI-SceI-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 *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 *bla*$_{L1}$ and *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 *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.

Locus tag	Predicted function	Fold change big vs. small	Fold change big vs. uniform	Fold change small vs. uniform
Smlt0018	hypothetical protein	+7.6	+11.4	-
Smlt0019	hypothetical protein	+2.2	+3.9	-
Smlt0706	fimbria adhesin protein	-2.3	-2.0	-
Smlt0709	fimbria adhesin protein	-2.0	-	-
Smlt1735	delta(2) isopentenylpyrophosphate tRNA	+2.4	-	-
Smlt1766	hypothetical protein	+2.1	-	-
Smlt2667	β-lactamase	+15.3	+9.9	-
Smlt2668	hypothetical protein	+5.9	+5.1	-
Smlt3721	predicted antiporter	+3.6	+3.5	-
Smlt3722	β-lactamase	+6.9	+7.4	-
Smlt1844	modification methylase	-2.0	-	-
Smlt2601	poly (β-D-mannuronate) lyase	-2.1	-	-
Smlt0003	hypothetical protein	-	-	+2.1
Smlt0028	DNA helicase II	-	-	+2.0
Smlt0044	RHS-repeat-containing protein	-	-	+2.0
Smlt0201	hypothetical protein	-	-	+2.2
Smlt0265	acyl CoA dehydrogenase	-	-	-2.1
Smlt0336	hypothetical protein	-	-	+2.3
Smlt0353	transposase	-		+2.0
Smlt0596	sensor histidine kinase RstB	-	-	-2.2
Smlt0641	undecaprenyl-phosphate 4-deoxy-4-formamido-l-arabinose transferase	-	-	+2.4
Smlt1064	hypothetical protein	-	-	+2.8
Smlt1391	Pseudogene	-	-	+2.1
Smlt1402	methyl-accepting chemotaxis protein	-	-	+2.0
Smlt1664	hypothetical protein	-	-	+2.6
Smlt1760	Major facilitator superfamily transmembrane protein	-	-	-2.6
Smlt2041	50S ribosomal protein L36	-	-	+2.0
Smlt3041	hypothetical protein	-	-	+2.3
Smlt3056	transmembrane protein	-	-	+2.1
Smlt3554	anti-sigma factor RseA	-	-	-2.1
Smlt3597	Dehydrogenase	-		-2.1
Smlt4130	two-component system response regulator, AlgR	-	-	-2.0
Smlt4447A	Pseudogene	-	-	+2.2
Smlt4604	endonuclease L-PSP family protein	-	-	+2.0

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.

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

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 bla_{L1} and bla_{L2} 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).

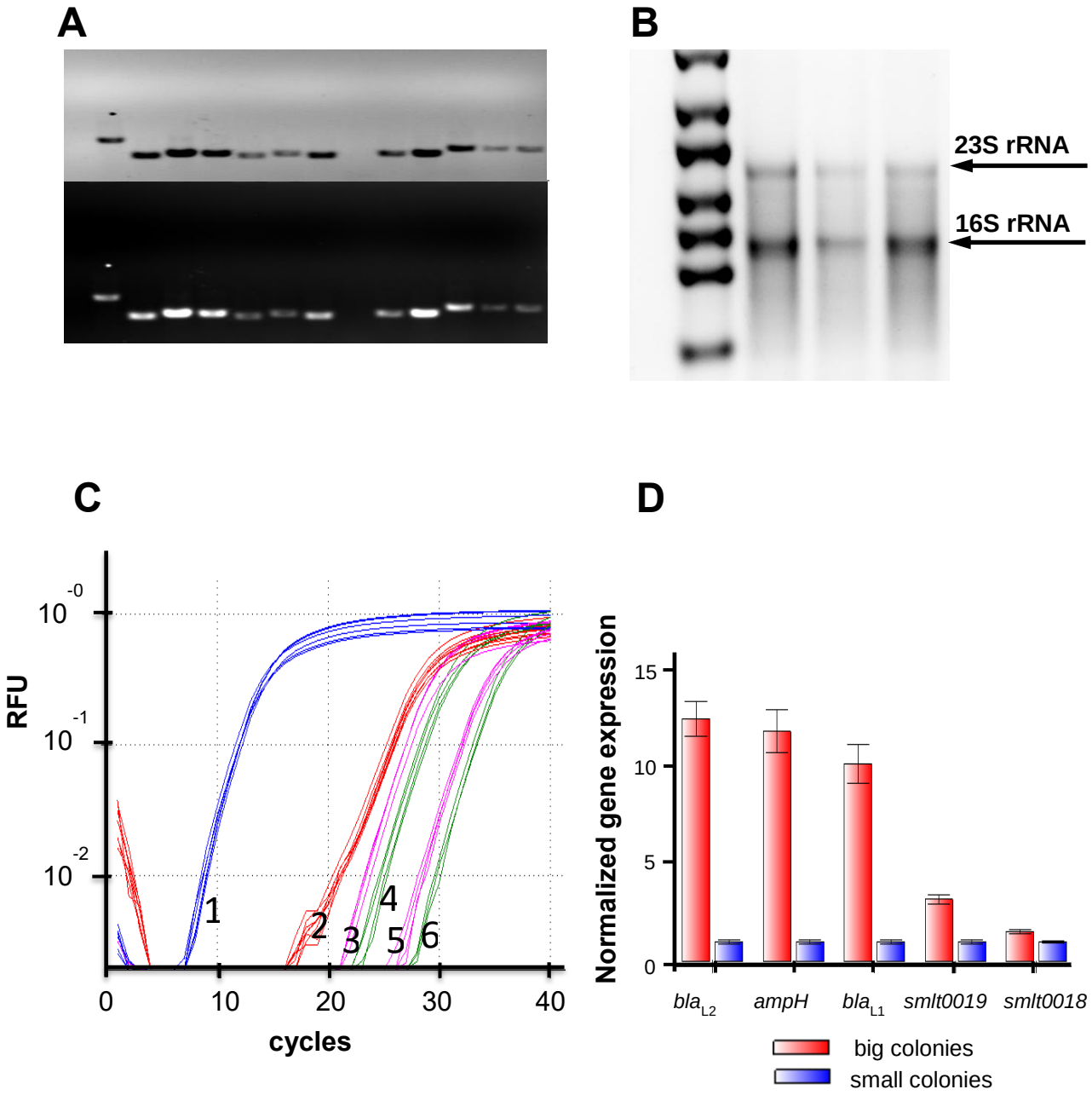


Figure 26: RT-qPCR data analysis for different SMK279a colony morphotypes. (A) The internal DNA fragments of the PCR amplified candidate genes indicating the specificity of the primer used. (B) Total RNA was isolated from 10-15 mg of wet biomass of the colony morphotypes and displayed on a 1.25 % formaldehyde/MOPS 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_q values for *bla*$_{L1}$ and *bla*$_{L2}$ in big vs. small colonies. The C_q 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*$_{L1}$ (big); 4, *bla*$_{L2}$ (big); 5, *bla*$_{L1}$ (small) and 6, *bla*$_{L2}$ (small). (D) RT-qPCR of genes identified in RNA-seq. The SuperScript® VILO™ cDNA synthesis kit (Invitrogen™, life technologies, TX, USA) was used to generate cDNA using 1.2 µg RNA. Both β-lactamase and the Na^+/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 *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 *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 *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 *bla*$_{L2}$-ON and *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 *bla*$_{L2}$-ON vs. *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 *comE,* and two putative transmembrane efflux proteins (Smlt2851 and Smlt2852) encoded in an operon. The *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 *smlt2851* and *smlt2852* genes were 29- and 7.2-fold up-regulated, respectively, while *smlt1134* was 5.4-fold down-regulated. Additional RT-qPCR data supported the observation made for the *comE* homologue. 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 vs. *bla*$_{L2}$-OFF mode cells (Figure B 6). For the *smlt2851* and *smlt2852* loci, RT-qPCR data did not confirm the RNA-seq data.

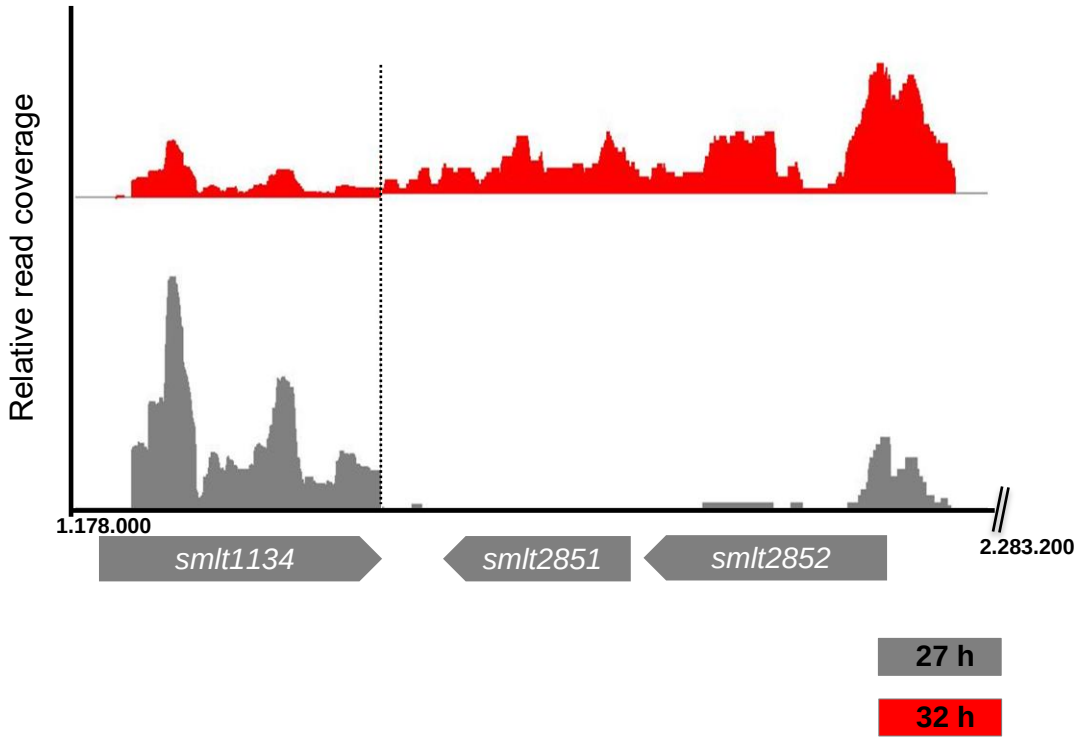


Figure 27: Partial physical arrangement of genes whose fold expression level was significantly altered in "ON" and "OFF" mode cells (32 h vs. 27 h) along with transcription profiles. Between "ON" and "OFF" mode cells, the *smlt2851* and *smlt2852* genes were 29- and 7.2-fold up-regulated, respectively, while *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 *bla*$_{L2}$ expression was also analyzed by knocking out the functional gene *smlt0387*. Therefore, except for *smlt0387*, markerless SMK279a mutants were constructed using the pGPI-SceI/pDAI-SceI-SacB system originally developed for bacteria of the genus *Burkholderia* (Figure 6 and Figure 7). Via triparental mating, the P*bla*$_{L2}$::*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 SMK279aΔ*smlt0387* revealed that the majority of the cells were in *bla*-OFF mode in exponential state, while later in *bla*-ON mode in the cultures after 32 h growth. Therefore, it was noteworthy that the growth phase dependent *bla*$_{L2}$

expression remained unaffected by the loss of the activities of the *smlt0387* gene. Likewise, the mutants SMK279aΔ*smlt1134* and SMK279aΔ*smlt2851*Δ*smlt2852* were analyzed for their effect on single cell *bla*$_{L2}$ expression. However, the loss of neither ComE homologue nor putative multidrug transporter affected the growth phase dependent switch of *bla*$_{L2}$ 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*$_{L2}$.

Table 17: Effects of SMK279a P*bla*$_{L2}$::*rfp* expression on a single cell level.

Assayed SMK279a strain	Gene copy added	Predicted function	% of cells showing the *bla*$_{L2}$-ON mode after 32 h of growth
SMK279a EM2	-	-	>90 %
SMK279a EM8	*smlt1134*	competence protein	< 1 %
SMK279a EM9	*smlt2851-smlt2852*	multidrug transporter	>90 %
SMK279aΔ*smlt1134*	-	-	>90 %
SMK279aΔ*smlt2851*Δ *smlt2852*	-	-	>90 %
SMK279aΔ*smlt0387*	-	-	>90 %

3.3.5 Overexpression proteins in SMK279a

As it is shown above the presence of the SMK279aΔ*smlt1134* or SMK279aΔ*smlt2851*Δ*smlt2852* mutation did not alter *bla*$_{L2}$ gene expression at the single cell level when compared to the parent SMK279a carrying P*bla*$_{L2}$::*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*$_{L2}$ 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::P*bla*$_{L2}$::*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*$_{L2}$ 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*$_{L2}$ 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).

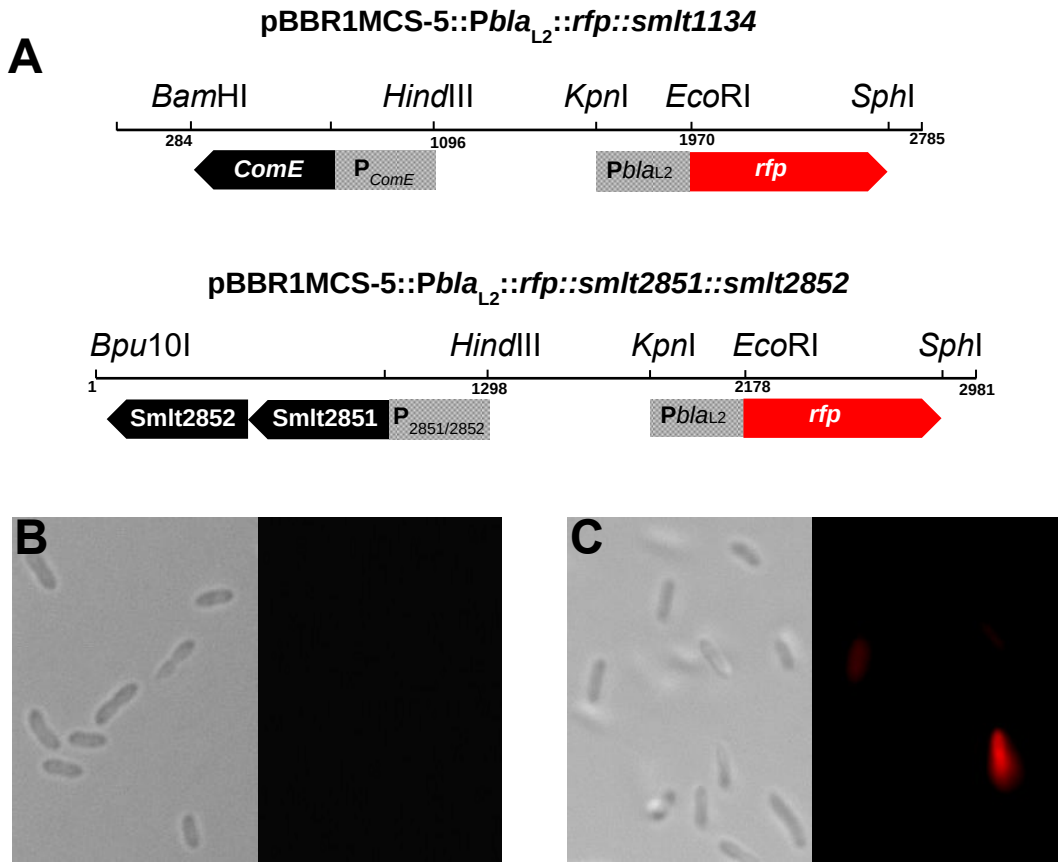


Figure 28: Expression of ComE homologue under its native promoter. (A) Physical map and orientation of *comE* and putative transporter genes in pBBR1MCS-5. (B) Expression of ComE in SMK279a affects *bla*$_{L2}$ heterogeneous expression. Cells were cultured in LB medium supplemented with 100 µg/ml ampicillin 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 *bla*$_{L2}$ 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*$_{L2}$::*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*$_{L2}$ expression but its effect could be reverted by unidentified signal molecules (see section 3.2.4).

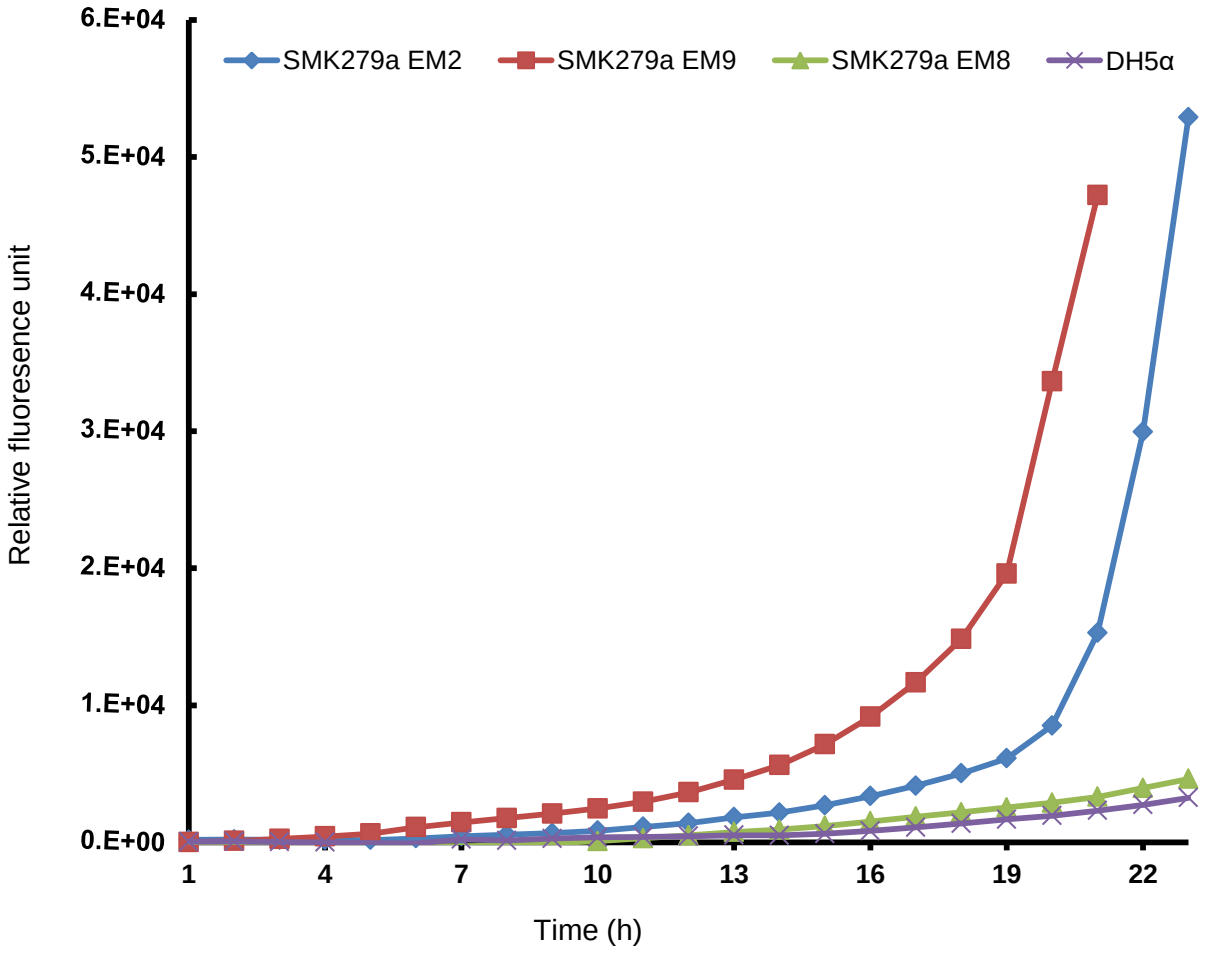


Figure 29: Time course of the intensity of fluorescence as measured by relative fluorescence unit (RFU). For the measurement, 150 ml diluted bacterial cultures were pipetted into black microplate with transparent bottom and incubated at ambient temperature in Synergy HT microplate reader. Overexpression of multidrug transporter allowed over 90 % of cells to enter *bla*-ON mode at least 2h earlier than SMK279a only carrying P*bla*$_{L2}$::*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*$_{L1}$ and *bla*$_{L2}$ 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.

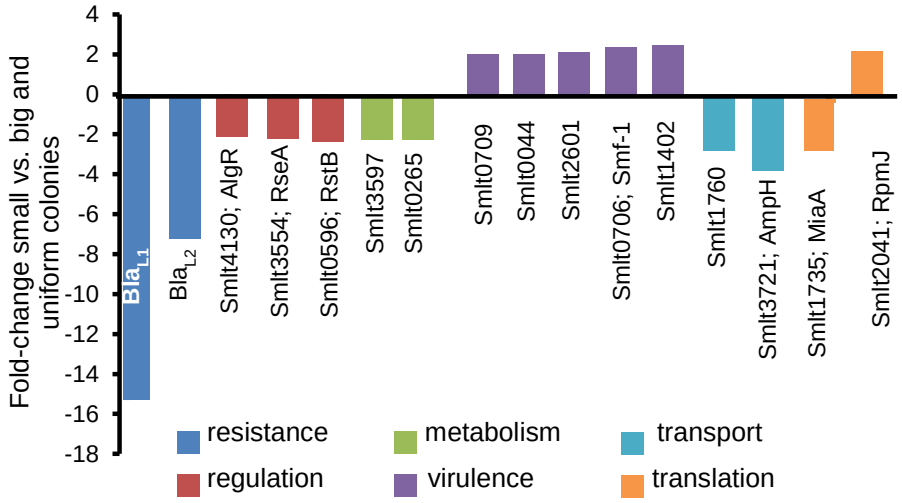


Figure 30: Differentially regulated genes in small colonies in comparison to big and uniform colonies. Down-regulation of *bla*$_{L1}$ and *bla*$_{L2}$ genes affected genes involved in metabolism, transport, regulation, virulence and genetic information processing.

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).

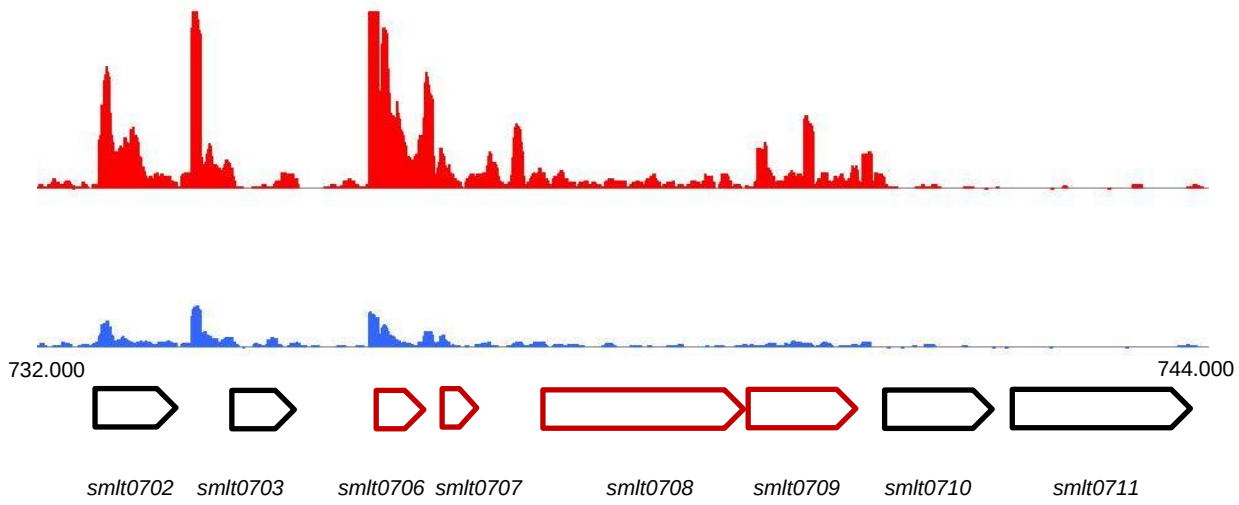


Figure 31: Transcriptome profiles of the Smf-1 fimbrial operon (red arrow) among the colony morphotypes. The *smlt0706* (major type 1 subunit fimbrin) and *smlt0709* were 2.3-fold and 2.0-fold up-regulated in cells forming small to big colonies, respectively.

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.

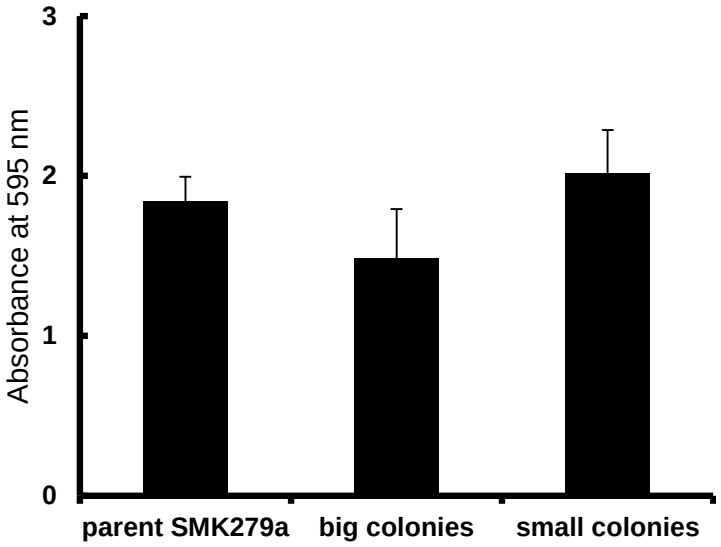


Figure 32: Biofilm formation of colony morphotypes. Cells were grown for 24 h and biofilm formation was estimated following crystal violet staining. Data indicate results from triplicate analyses. Sister progeny cells taken from the small colonies formed thicker biofilms compared to big colonies.

3.5 Genotypic and phenotypic characterization of *S. maltophilia* isolates

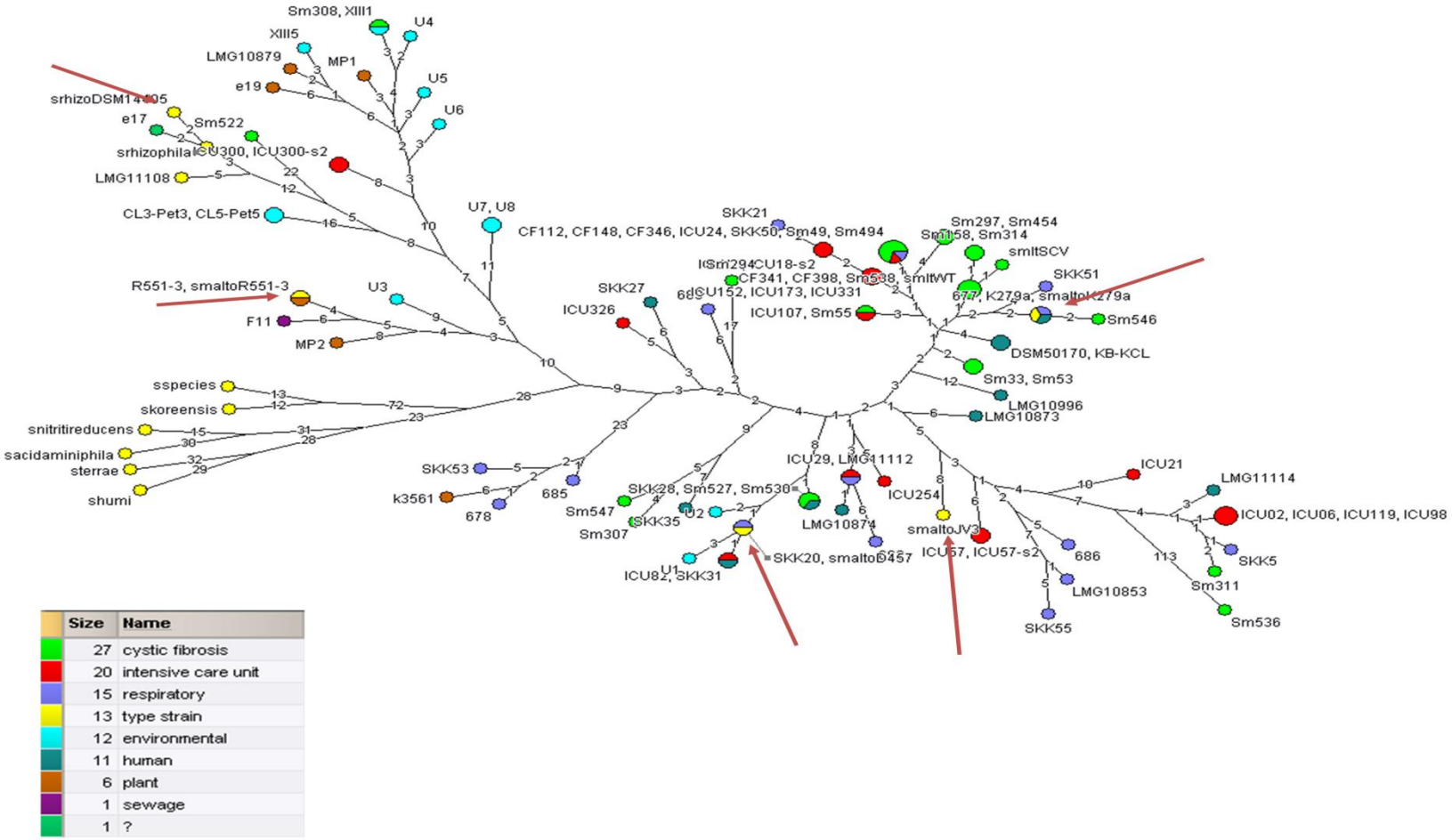
A phylogenetic tree of the *S. maltophilia* isolates was generated utilizing the 394 SNP positions identified in the 106 isolates. The SNPs were identified in a set of 106 *S. maltophilia* isolates compared to the genome sequence of strain SMK279a.The tree visualizes that the isolates tend to form cluster 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

OD_{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).



Size	Name
27	cystic fibrosis
20	intensive care unit
15	respiratory
13	type strain
12	environmental
11	human
6	plant
1	sewage
1	?

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.

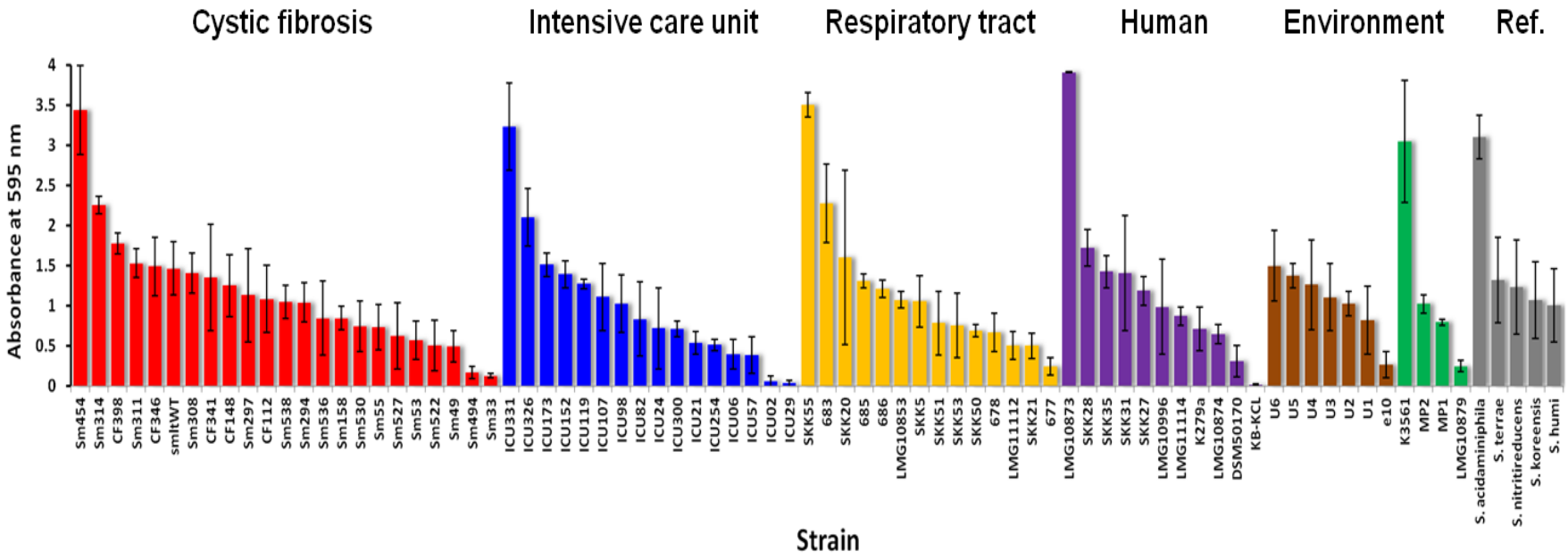


Figure 34: Biofilm formed on microtritre plate by *S. maltophilia* isolates. Biofilm formation capacity was assessed by microtiter colorimetric assay after 24 h incubation at 37°C or 30°C. The level of biofilm formation were varied significantly from 3.9 to 0.13. *S. maltophilia* isolates from human origin varied significantly in their ability to form biofilm in vitro.

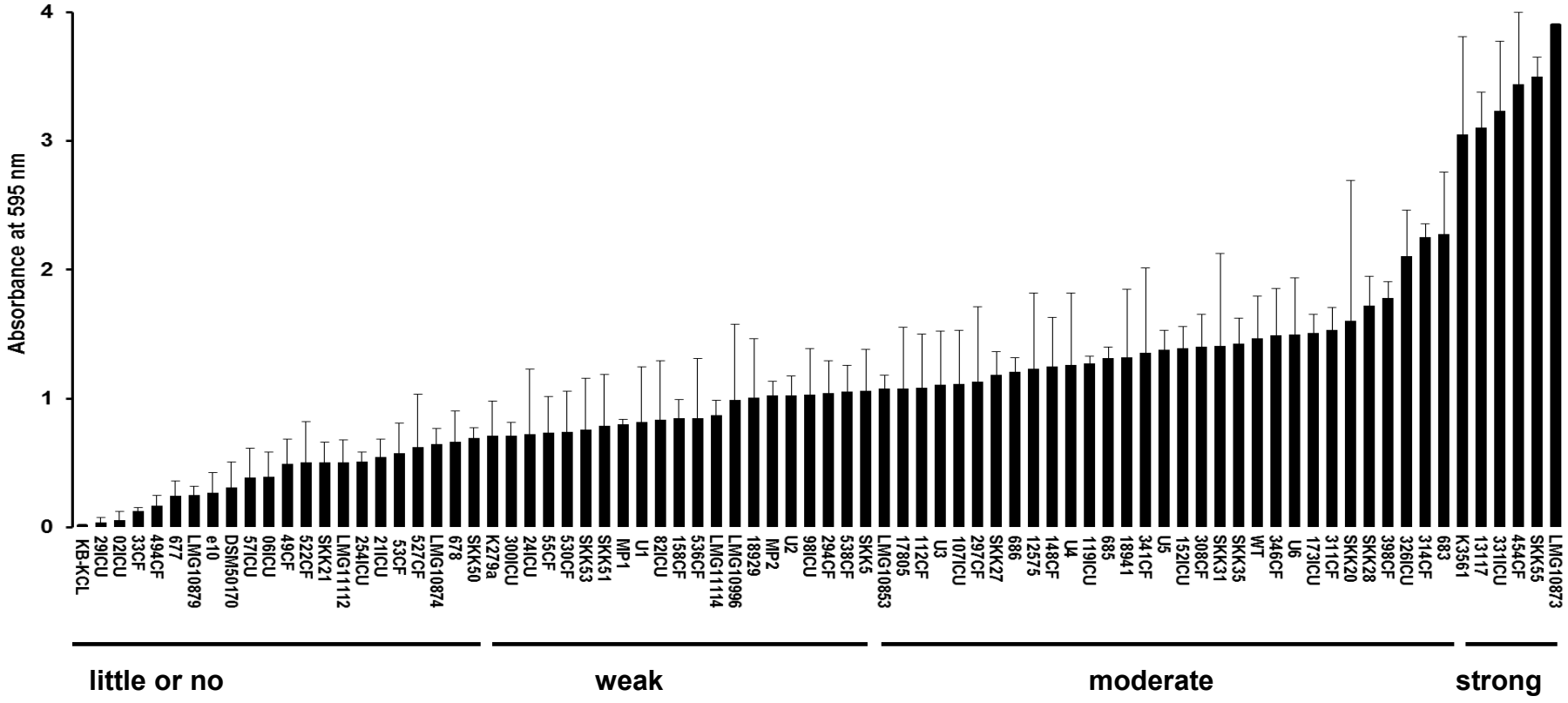


Figure 35: Classifications of *S. maltophilia* isolates based on biofilm formation capability. A low cut-off (ODc) value was set off as three standard deviations above the mean of control wells. Based on OD, isolates were classified as: little or no biofilm producer (OD ≤ ODc), weak biofilm producer (ODc < OD ≤ 2 × ODc), moderate biofilm producer (2 × ODc < OD ≤ 4 × ODc), and strong biofilm producer (4 × ODc < OD).

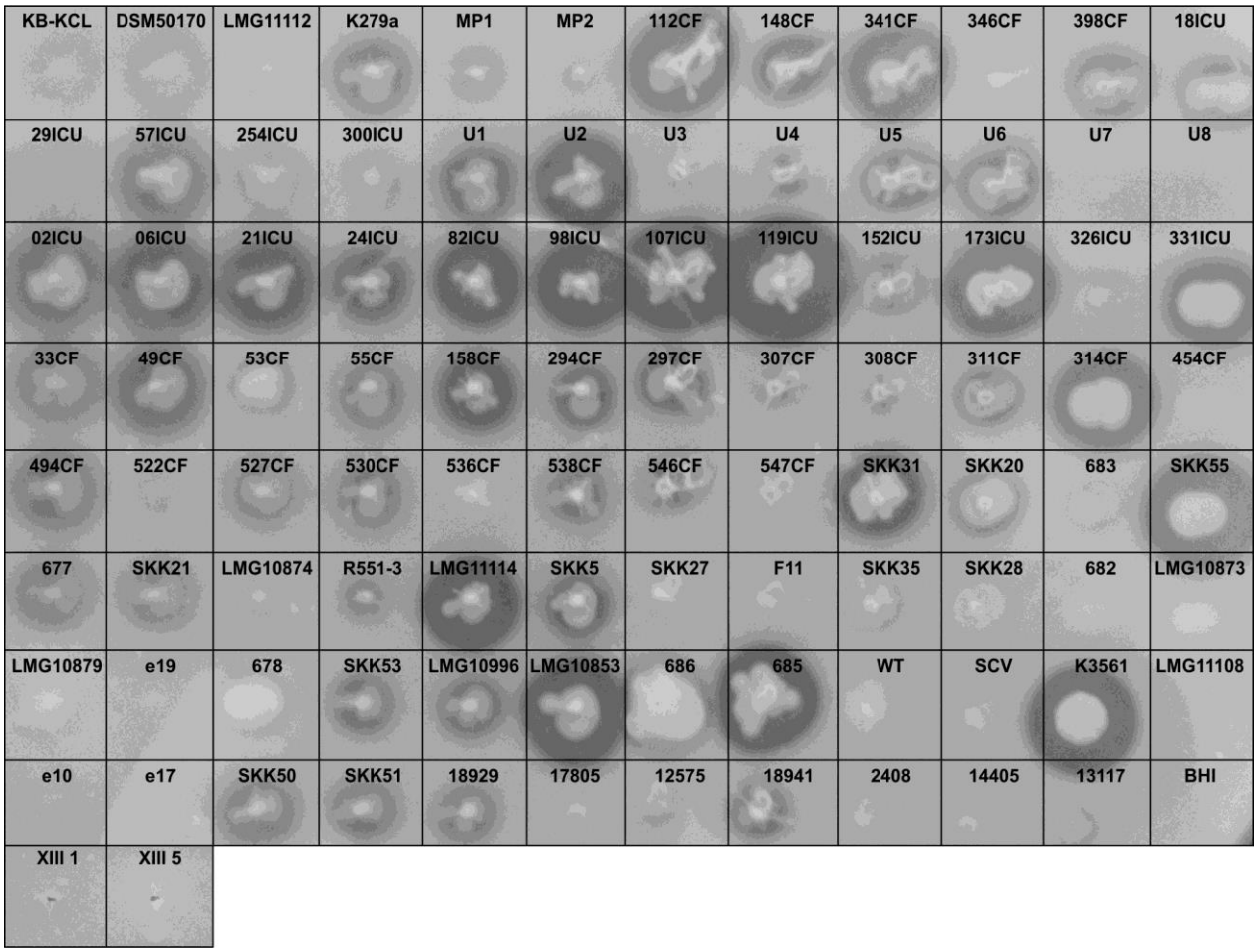


Figure 36: Extracellular protease assay for *S. maltophilia* isolates. Protease production differed considerably with some isolate of environmental origin demonstrating no clearing zone after 24 h.

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. maltophlia* 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. maltophlia* (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. maltophlia,* 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 pneumonia*, 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).

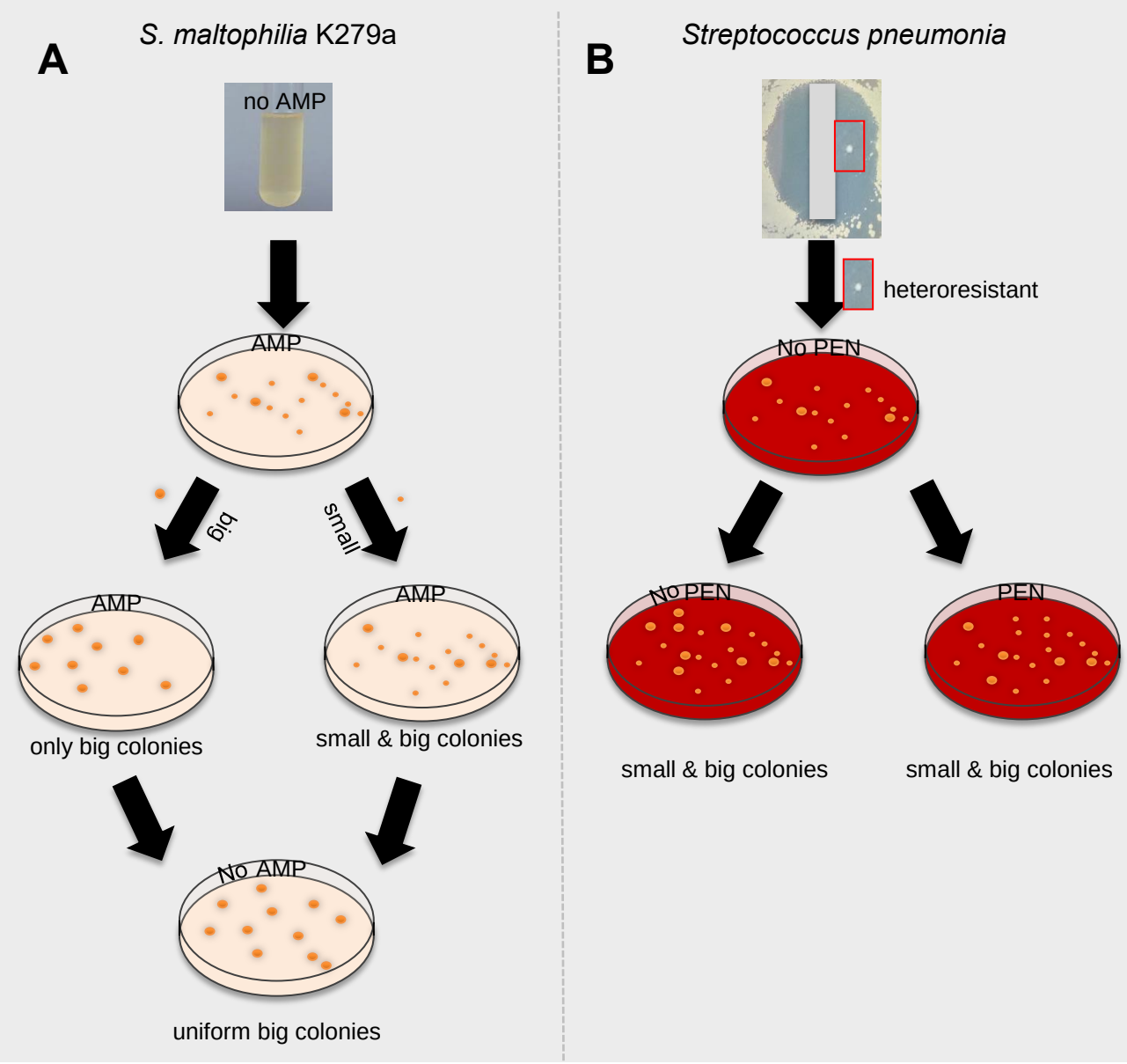


Figure 37: Schematic diagram showing the phenotypic heterogeneity of SMK279a and *Streptococcus pneumoniae* cells during growth on solid media. (A) A colony morphologies observed for SMK279a in response to ampicillin (AMP; 100 µg/ml) treatment on LB agar plates. Up on the removal of the antibiotic, the 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 σ^E 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 *bla*$_{L1}$ and *bla*$_{L2}$ 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 *bla*$_{L1}$ or *bla*$_{L2}$ 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 *bla*$_{L1}$ and *bla*$_{L2}$ 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 SMK279*a* 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_{L1} and bla_{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_{L1} and bla_{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 *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_{L1} and bla_{L2} expression was ampicillin independent but rather growth-phase dependent that was most likely triggered environmental stimuli. Sturm *et al* (2011) reported a similar gene expression pattern for *Salmonella enterica* subspecies 1 *serovar* Typhimurium at the single cell level (Sturm *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 *et al*., 2011).

In this study, conversion of bacterial cells from "OFF" into "ON" was achieved using culture supernatants from stationary-phase Pbla_{L2}::*rfp* reporter SMK279a strain or growing overnight cultures of Pbla_{L2}::*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 Pbla_{L2}::*cfp* and Pbla_{L2}::*yfp* did not show the switch from a heterogeneous to a homogenous *bla* expression. Nevertheless, a stress signal obtained from cells carrying the Pbla_{L2}::*rfp* reporter could still switch the cells carrying Pbla_{L2}::*cfp* or Pbla_{L2}::*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 *S. maltophilia* (McCarthy *et al*., 2011) was tested. However, DSF failed to alter bla_{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_{L1} and bla_{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 *mucA* 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). Addtionally, a single nucleotide susbstitution 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 subpuplation 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 commomly 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 CO_2 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 occured in the reciver domain of histidine kinase might influence signal processing in respose 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_{L1} and bla_{L2}. The bla_{L1} and bla_{L2} 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.

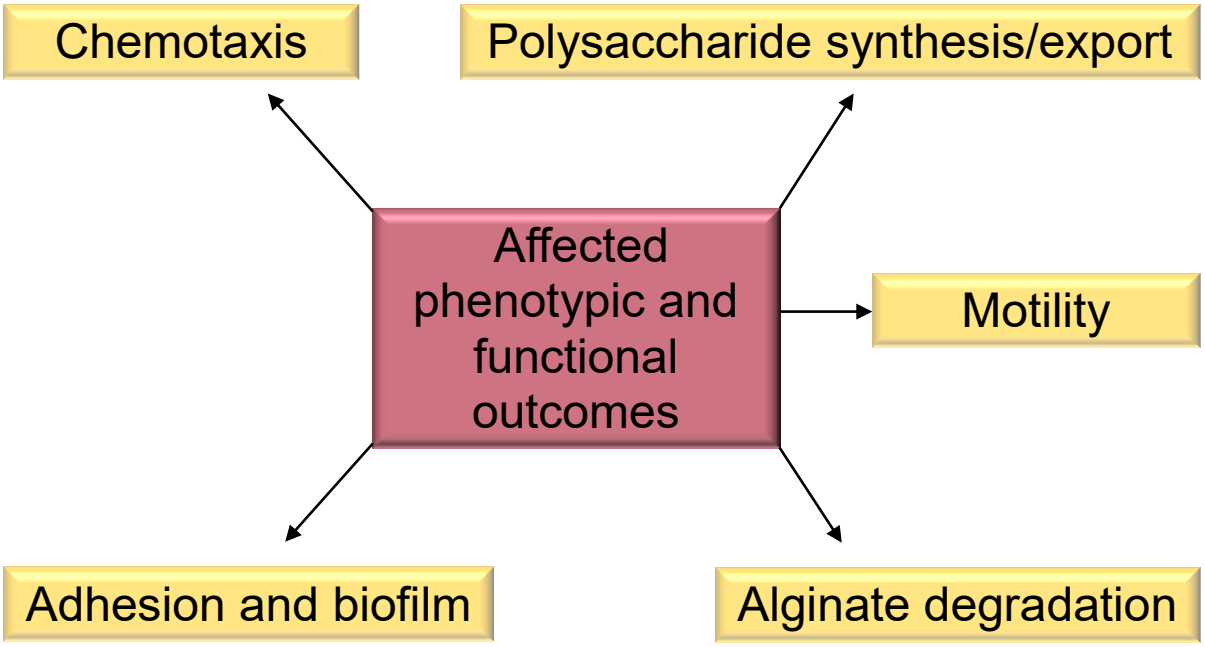


Figure 38: Diverse cellular functions that were altered in cells of small colonies based on RNA-seq data and biofilm assay. These functions include virulence determinants in *S. maltophilia* such as motility, adhesion and biofilm formation. The altered function might promote division of labor and bet-hedging 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).

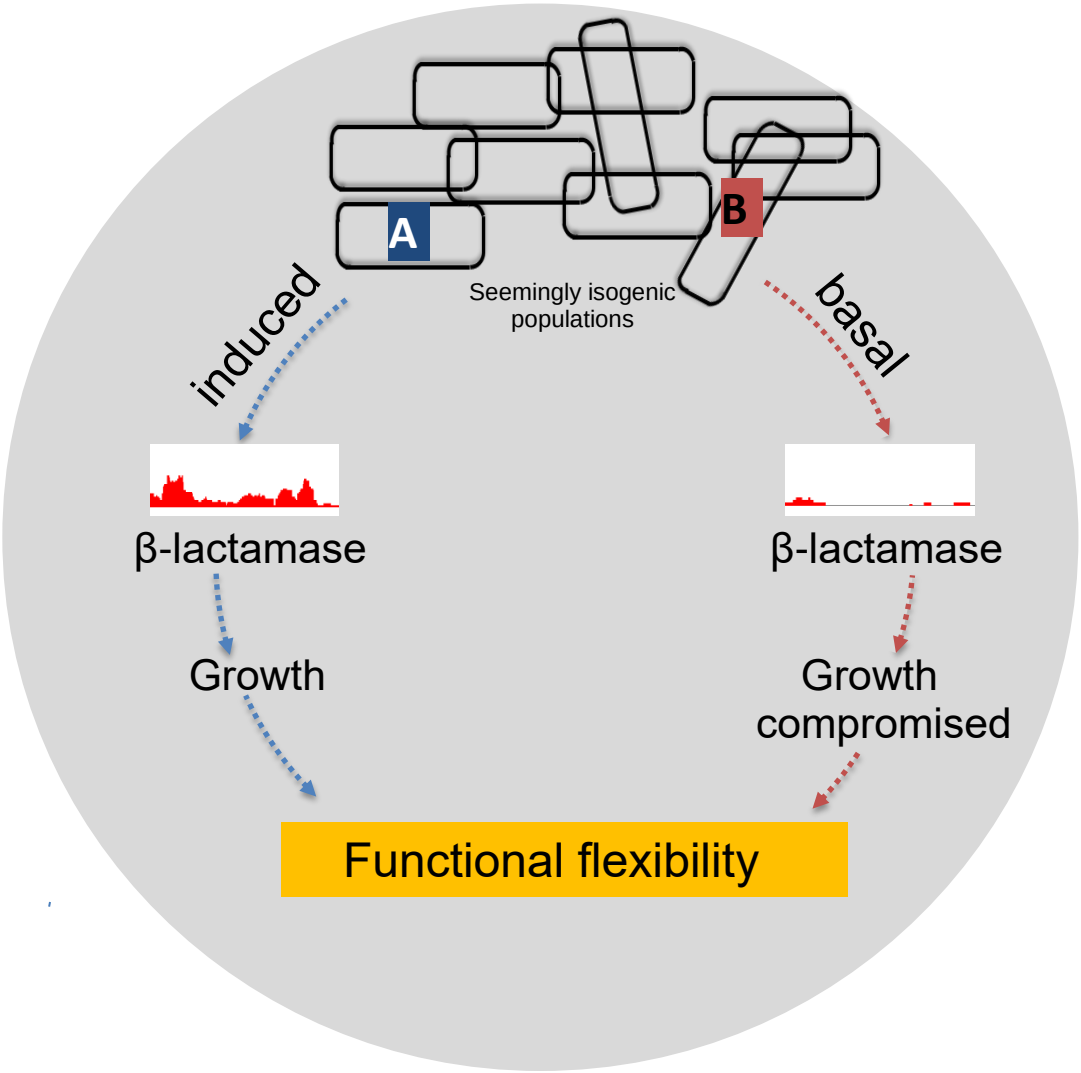


Figure 39: Expression of β-lactamase in isogenic SMK279a is controlled in a growth-dependent manner in the presence of β-lactams. The patient isolate SMK279a diverges into cellular subpopulations with distinct but reversible morphotypes of small and big colonies when challenged with ampicillin. A fraction of subpopulations (A) usually grows faster than the average population leading to higher β-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 β-lactamase expression are synergistically involved to produce diverse phenotypic and functional outcomes. The filled circle represents β-lactams.

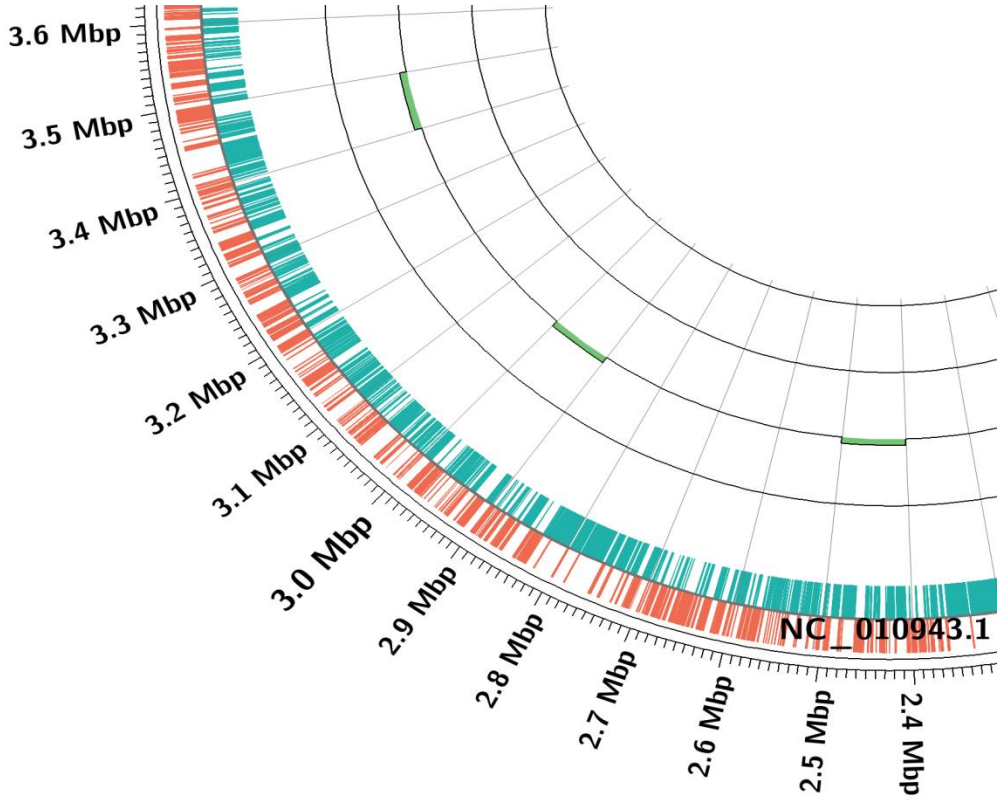


Figure 40: A section of SMK279a genome that shows cells in small colonies contained more mutations than big colonies. Numbers indicates coordinates on genome map from 2.6 to 3.6 Mbp. The light green lines indicate locations of mutations identified only in the genomic DNA extracted from bacterial cells 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_{L1} and bla_{L2} 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_{L1} and bla_{L2} 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_{L2} which is located upstream of putative transmembrane Na+/H+ antiporter (Figure 25). The activation of AmpR is responsible for induction of β-lactamase bla_{L1} and bla_{L2} (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_{L1} and bla_{L2}. When untreated cells were compared,

the transcriptional activation of *ampR* was still not differentially regulated. In addition to *ampR,* several of the transcripts of genes involved in the expression of *bla* genes had a fold change of less than two, indicating the absence of differential regulation. Furthermore, the deletion of *ampR* (SMK279aΔ*smlt3723*) 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 *ampR* among colony morphotypes cannot under emphasized and needed further studies.

4.7.1 ComE modulated *bla*$_{L2}$ heterogeneous expression in SMK279a

The current study revealed that *bla*$_{L1}$ and *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 *smlt2851* and *smlt2852*, encoding putative antibiotic resistance transporter, and a putative *comE* homologue (*smlt1134*) between cells that were in the *bla*$_{L2}$-ON vs. *bla*$_{L2}$-OFF mode (Figure 27). Furthermore, the impact these genes were investigated by constructing a deletion mutants of both loci, *smlt1134* (*comE* homologue) and *smlt2851-smlt2852* (Figure 7). However, heterogeneous gene expression of the *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 SMK279aΔ*smlt0387* (an Ax21 homologue) mutation did not alter *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 *Stenotrophomonas* (McCarthy *et al*., 2011).

Since the *smlt2851* and *smlt2852* genes were 29- and 7.2-fold up-regulated, respectively, in *bla*$_{L2}$-ON mode cells, the genes were overexpressed in SMK279a under their native promoter, which did not alter heterogeneous expression of the *bla*$_{L2}$ gene. The final attempt in this study to uncover what modulates phenotypic heterogeneous expression of *bla*$_{L2}$ was obtained from studies conducted on ComE protein. The overexpression of the ComE homologue resulted in a decreased heterogeneity of *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 *comE* gene involves in part by the RNA-binding protein (Hfq) that regulates a wide variety of cellular responses (including response to

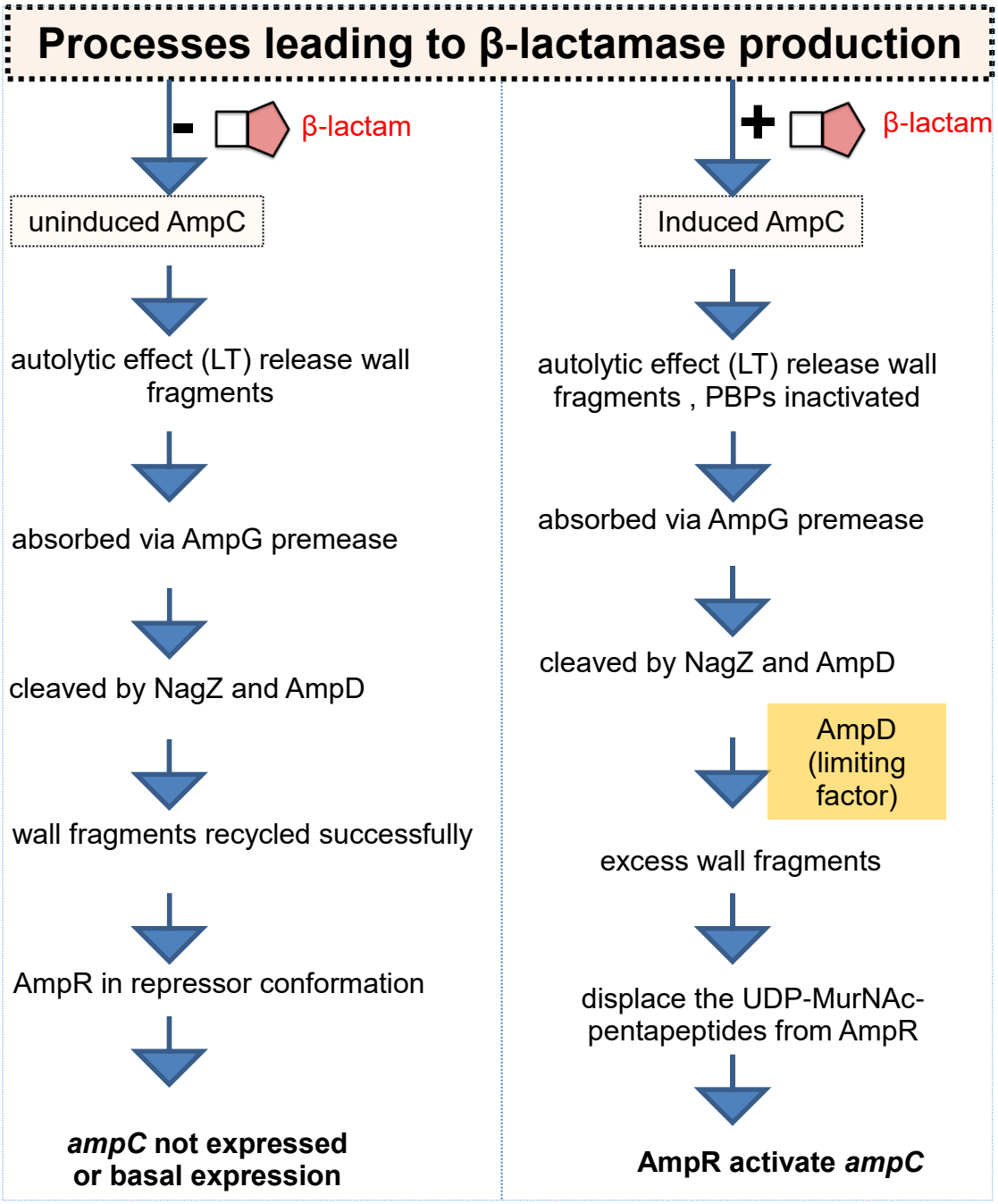


Figure 41: Uninduced and induced β-lactamase production in Gram-negative bacteria. It shows the biological roles of the well-known proteins with or without antibiotic challenges of bacterial cells. Cell wall degradation products are transported to the cytosol via cytoplasmic inner membrane permease AmpG .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 bla_{L1} and 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

ABDA, E. M., KRYSCIAK, D., KROHN-MOLT, I., MAMAT, U., SCHMEISSER, C., FÖRSTNER, K. U., SCHAIBLE, U. E., KOHL, T. A., NIEMAN, S. & STREIT, W. R. 2015. Phenotypic heterogeneity affects *Stenotrophomonas maltophilia* K279a colony morphotypes and β-lactamase expression. *Front Microbiol*, 6, 1373.

AGGER, W. A., COGBILL, T. H., BUSCH, H., JR., LANDERCASPER, J. & CALLISTER, S. M. 1986. Wounds caused by corn-harvesting machines: an unusual source of infection due to gram-negative bacilli. *Rev Infect Dis*, 8**,** 927-31.

AL-HILALI, N., NAMPOORY, M. R., JOHNY, K. V. & CHUGH, T. D. 2000. *Xanthomonas maltophilia* infection in chronic peritoneal dialysis patients. *Scand J Urol Nephrol*, 34**,** 67-9.

AL NAIEMI, N., DUIM, B. & BART, A. 2006. A CTX-M extended-spectrum β-lactamase in *Pseudomonas aeruginosa* and *Stenotrophomonas maltophilia*. *J. Med. Microbiol,* 55**,** 1607-1608.

ALAVI, P., MULLER, H., CARDINALE, M., ZACHOW, C., SANCHEZ, M. B., MARTINEZ, J. L. & BERG, G. 2013. The DSF quorum sensing system controls the positive influence of *Stenotrophomonas maltophilia* on plants. *PLoS ONE,* 8**,** e67103.

ALEKSHUN, M. N. & LEVY, S. B. 2007. Molecular mechanisms of antibacterial multidrug resistance. *Cell,* 128**,** 1037-50.

ALONSO, A. & MARTINEZ, J. L. 2000. Cloning and characterization of SmeDEF, a novel multidrug efflux pump from *Stenotrophomonas maltophilia*. *Antimicrob Agents Chemother,* 44**,** 3079-86.

AMBLER, R. P. 1980. The structure of beta-lactamases. *Philos Trans R Soc Lond B Biol Sci*, 289**,** 321-31.

ANDERSON, S. W., STAPP, J. R., BURNS, J. L. & QIN, X. 2007. Characterization of small-colony-variant *Stenotrophomonas maltophilia* isolated from the sputum specimens of five patients with cystic fibrosis. *J Clin Microbiol*, 45**,** 529-535.

ANETZBERGER, C., PIRCH, T. & JUNG, K. 2009. Heterogeneity in quorum sensing-regulated bioluminescence of *Vibrio harveyi*. *Mol Microbiol*, 73**,** 267-277.

AUBERT, D., HAMAD, M. & VALVANO, M. 2014. A Markerless Deletion Method for Genetic Manipulation of *Burkholderia cenocepacia* and Other Multidrug-Resistant Gram-Negative Bacteria. *In:* VERGUNST, A. C. & O'CALLAGHAN, D. (eds.) *Host-Bacteria Interactions.* Springer New York.

AVISON, M. B., HIGGINS, C. S., FORD, P. J., VON HELDREICH, C. J., WALSH, T. R. & BENNETT, P. M. 2002. Differential regulation of L1 and L2 β-lactamase expression in *Stenotrophomonas maltophilia*. *J Antimicrob Chemother*, 49**,** 387-389.

AVISON, M. B., VON HELDREICH, C. J., HIGGINS, C. S., BENNETT, P. M. & WALSH, T. R. 2000. A TEM-2 beta-lactamase encoded on an active Tn1-like transposon in the genome

of a clinical isolate of *Stenotrophomonas maltophilia*. *J Antimicrob Chemother,* 46**,** 879-84.

BAHAR, O., PRUITT, R., LUU, D. D., SCHWESSINGER, B., DAUDI, A., LIU, F., RUAN, R., FONTAINE-BODIN, L., KOEBNIK, R. & RONALD, P. 2014. The *Xanthomonas* Ax21 protein is processed by the general secretory system and is secreted in association with outer membrane vesicles. *PeerJ*, 2, e242.

BAIRD, G. S., ZACHARIAS, D. A. & TSIEN, R. Y. 2000. Biochemistry, mutagenesis, and oligomerization of DsRed, a red fluorescent protein from coral. *Proc Natl Acad Sci USA*, 97**,** 11984-11989.

BALCEWICH, M. D., REEVE, T. M., ORLIKOW, E. A., DONALD, L. J., VOCADLO, D. J. & MARK, B. L. 2010. Crystal structure of the AmpR effector binding domain provides insight into the molecular regulation of inducible Ampc beta-lactamase. *J Mol Biol,* 400**,** 998-1010.

BATES, D. M., EIFF, C. V., MCNAMARA, P. J., PETERS, G., YEAMAN, M. R., BAYER, A. S. & PROCTOR, R. A. 2003. *Staphylococcus aureus menD* and *hemB* Mutants Are as Infective as the Parent Strains, but the Menadione Biosynthetic Mutant Persists within the Kidney. *J Infect Dis*, 187**,** 1654-1661.

BEAUMONT, H. J. E., GALLIE, J., KOST, C., FERGUSON, G. C. & RAINEY, P. B. 2009. Experimental evolution of bet hedging. *Nature,* 462**,** 90-93.

BERG, G., EGAMBERDIEVA, D., LUGTENBERG, B. & HAGEMANN, M. 2010. Symbiotic Plant–Microbe Interactions: Stress Protection, Plant Growth Promotion, and Biocontrol by *Stenotrophomonas*. *In:* SECKBACH, J. & GRUBE, M. (eds.) *Symbioses and Stress.* Springer Netherlands.

BEVERIDGE, T. J. 1999. Structures of Gram-Negative Cell Walls and Their Derived Membrane Vesicles. *J Bacteriol,* 181**,** 4725-4733.

BJARNSHOLT, T. 2013. The role of bacterial biofilms in chronic infections. *APMIS Suppl*, 1-51.

BLOM, J., JAKOBI, T., DOPPMEIER, D., JAENICKE, S., KALINOWSKI, J., STOYE, J. & GOESMANN, A. 2011. Exact and complete short-read alignment to microbial genomes using Graphics Processing Unit programming. *Bioinformatics*, 27**,** 1351-8.

BLOMBERG, P., WAGNER, E. G. & NORDSTROM, K. 1990. Control of replication of plasmid R1: the duplex between the antisense RNA, CopA, and its target, CopT, is processed specifically in vivo and in vitro by RNase III. *EMBO J,* 9**,** 2331-40.

BOYD, A. & CHAKRABARTY, A. M. 1994. Role of alginate lyase in cell detachment of *Pseudomonas aeruginosa*. *Appl Environ Microbiol*, 60**,** 2355-9.

BROOKE, J. S. 2012. *Stenotrophomonas maltophilia*: an Emerging Global Opportunistic Pathogen. *Clin Microbiol Rev*, 25**,** 2-41.

BUSH, K. & JACOBY, G. A. 2010. Updated Functional Classification of β-Lactamases. *Antimicrob Agents Chemother,* 54**,** 969-976.

BUSH, K., JACOBY, G. A. & MEDEIROS, A. A. 1995. A functional classification scheme for beta-lactamases and its correlation with molecular structure. *Antimicrob Agents Chemother,* 39**,** 1211-1233.

CANO, D. A., PUCCIARELLI, M. G., MARTÍNEZ-MOYA, M., CASADESÚS, J. & GARCÍA-DEL PORTILLO, F. 2003. Selection of Small-Colony Variants of *Salmonella enterica* Serovar Typhimurium in Nonphagocytic Eucaryotic Cells. *Infect Immun*, 71**,** 3690-3698.

CARTER, A. P., CLEMONS, W. M., BRODERSEN, D. E., MORGAN-WARREN, R. J., WIMBERLY, B. T. & RAMAKRISHNAN, V. 2000. Functional insights from the structure of the 30S ribosomal subunit and its interactions with antibiotics. *Nature,* 407**,** 340-8.

CASAGRANDE PROIETTI, P., STEFANETTI, V., HYATT, D. R., MARENZONI, M. L., CAPOMACCIO, S., COLETTI, M., BIETTA, A., FRANCIOSINI, M. P. & PASSAMONTI, F. 2015. Phenotypic and genotypic characterization of canine pyoderma isolates of *Staphylococcus pseudintermedius* for biofilm formation. *J Vet Med Sci*, 77**,** 945-951.

CHANG, Y. T., LIN, C. Y., CHEN, Y. H. & HSUEH, P. R. 2015. Update on infections caused by *Stenotrophomonas maltophilia* with particular attention to resistance mechanisms and therapeutic options. *Front Microbiol*, 6**,** 893.

CHESTER, N. & MARSHAK, D. R. 1993. Dimethyl sulfoxide-mediated primer Tm reduction: a method for analyzing the role of renaturation temperature in the polymerase chain reaction. *Anal Biochem*, 209**,** 284-90.

COBB, L. M., MYCHALECKYJ, J. C., WOZNIAK, D. J. & LOPEZ-BOADO, Y. S. 2004. *Pseudomonas aeruginosa* flagellin and alginate elicit very distinct gene expression patterns in airway epithelial cells: implications for cystic fibrosis disease. *J Immunol*, 173**,** 5659-70.

CRAIG, L., PIQUE, M. E. & TAINER, J. A. 2004. Type IV pilus structure and bacterial pathogenicity. *Nat Rev Microbiol*, 2**,** 363-378.

CROSSMAN, L., GOULD, V., DOW, J., VERNIKOS, G., OKAZAKI, A., SEBAIHIA, M., SAUNDERS, D., ARROWSMITH, C., CARVER, T., PETERS, N., ADLEM, E., KERHORNOU, A., LORD, A., MURPHY, L., SEEGER, K., SQUARES, R., RUTTER, S., QUAIL, M., RAJANDREAM, M., HARRIS, D., CHURCHER, C., BENTLEY, S., PARKHILL, J., THOMSON, N. & AVISON, M. 2008. The complete genome, comparative and functional analysis of *Stenotrophomonas maltophilia* reveals an organism heavily shielded by drug resistance determinants. *Genome Biol,* 9**,** R74.

CULLMANN, W. & DICK, W. 1990. Heterogeneity of beta-lactamase production in *Pseudomonas maltophilia*, a nosocomial pathogen. *Chemotherapy,* 36**,** 117-26.

D'ARGENIO, D. A., CALFEE, M. W., RAINEY, P. B. & PESCI, E. C. 2002. Autolysis and autoaggregation in *Pseudomonas aeruginosa* colony morphology mutants. *J Bacteriol,* 184**,** 6481-9.

DE JONGE, B. L., CHANG, Y. S., XU, N. & GAGE, D. 1996. Effect of exogenous glycine on peptidoglycan composition and resistance in a methicillin-resistant *Staphylococcus aureus* strain. *Antimicrob Agents Chemother,* 40**,** 1498-1503.

DE OLIVEIRA-GARCIA, D., DALL'AGNOL, M., ROSALES, M., AZZUZ, A. C., ALCANTARA, N., MARTINEZ, M. B. & GIRON, J. A. 2003. Fimbriae and adherence of *Stenotrophomonas maltophilia* to epithelial cells and to abiotic surfaces. *Cell Microbiol,* 5**,** 625-36.

DENTON, M. & KERR, K. G. 1998. Microbiological and Clinical Aspects of Infection Associated with *Stenotrophomonas maltophilia*. *Clin Microbiol Rev,* 11**,** 57-80.

DERIS, J. B., KIM, M., ZHANG, Z., OKANO, H., HERMSEN, R., GROISMAN, A. & HWA, T. 2013. The Innate Growth Bistability and Fitness Landscapes of Antibiotic-Resistant Bacteria. *Science,* 342.

DEVOS, S., VAN OUDENHOVE, L., STREMERSCH, S., VAN PUTTE, W., DERYCKE, R., VAN DRIESSCHE, G., VITSE, J., RAEMDONCK, K. & DEVREESE, B. 2015. The effect of imipenem and diffusible signaling factors on the secretion of outer membrane vesicles and associated Ax21 proteins in *Stenotrophomonas maltophilia*. *Front Microbiol*,6, 298.

DIETZ, H., PFEIFLE, D. & WIEDEMANN, B. 1997. The signal molecule for beta-lactamase induction in *Enterobacter cloacae* is the anhydromuramyl-pentapeptide. *Antimicrob Agents Chemother,* 41**,** 2113-20.

DRAWZ, S. M. & BONOMO, R. A. 2010. Three decades of beta-lactamase inhibitors. *Clin Microbiol Rev,* 23**,** 160-201.

DRENKARD, E. & AUSUBEL, F. M. 2002. *Pseudomonas* biofilm formation and antibiotic resistance are linked to phenotypic variation. *Nature,* 416**,** 740-743.

DUBNAU, D. & LOSICK, R. 2006. Bistability in bacteria. *Mol Microbiol,* 61**,** 564-72.

EL-HALFAWY, O. M. & VALVANO, M. A. 2013. Chemical Communication of Antibiotic Resistance by a Highly Resistant Subpopulation of Bacterial Cells. *PLoS ONE,* 8**,** e68874.

EL-HALFAWY, O. M. & VALVANO, M. A. 2015. Antimicrobial heteroresistance: an emerging field in need of clarity. *Clin Microbiol Rev,* 28**,** 191-207.

ELOWITZ, M. B., LEVINE, A. J., SIGGIA, E. D. & SWAIN, P. S. 2002. Stochastic Gene Expression in a Single Cell. *Science,* 297**,** 1183-1186.

ELSNER, H. A., DUHRSEN, U., HOLLWITZ, B., KAULFERS, P. M. & HOSSFELD, D. K. 1997. Fatal pulmonary hemorrhage in patients with acute leukemia and fulminant pneumonia caused by *Stenotrophomonas maltophilia*. *Ann Hematol*, 74**,** 155-61.

ELTING, L. S. & BODEY, G. P. 1990. Septicemia due to *Xanthomonas* species and non-aeruginosa *Pseudomonas* species: increasing incidence of catheter-related infections. *Medicine (Baltimore),* 69**,** 296-306.

ENGEL, H., GUTIERREZ-FERNANDEZ, J., FLUCKIGER, C., MARTINEZ-RIPOLL, M., MUHLEMANN, K., HERMOSO, J. A., HILTY, M. & HATHAWAY, L. J. 2013. Heteroresistance to fosfomycin is predominant in *Streptococcus pneumoniae* and depends on the *murA1* gene. *Antimicrob Agents Chemother,* 57**,** 2801-8.

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.

FERRER-NAVARRO, M., PLANELL, R., YERO, D., MONGIARDINI, E., TORRENT, G., HUEDO, P., MARTÍNEZ, P., ROHER, N., MACKENZIE, S., GIBERT, I. & DAURA, X. 2013. Abundance of the Quorum-Sensing Factor Ax21 in Four Strains of *Stenotrophomonas maltophilia* Correlates with Mortality Rate in a New Zebrafish Model of Infection. *PLoS ONE,* 8**,** e67207.

FIGURSKI, D. H. & HELINSKI, D. R. 1979. Replication of an origin-containing derivative of plasmid RK2 dependent on a plasmid function provided in trans. *Proc Natl Acad Sci USA,* 76**,** 1648-52.

FILIPE, S. R., SEVERINA, E. & TOMASZ, A. 2002. The murMN operon: A functional link between antibiotic resistance and antibiotic tolerance in *Streptococcus pneumoniae*. *Proc Natl Acad Sci USA,* 99**,** 1550-1555.

FLANNAGAN, R. S., LINN, T. & VALVANO, M. A. 2008. A system for the construction of targeted unmarked gene deletions in the genus *Burkholderia*. *Environ Microbiol,* 10**,** 1652-60.

FÖRSTNER, K. U., VOGEL, J. & SHARMA, C. M. 2014. READemption – A tool for the computational analysis of deep-sequencing-based transcriptome data. *Bioinformatics*. 30, 3421–3423.

FRIDMAN, O., GOLDBERG, A., RONIN, I., SHORESH, N. & BALABAN, N. Q. 2014. Optimization of lag time underlies antibiotic tolerance in evolved bacterial populations. *Nature,* 513**,** 418-21.

GOULD, V. C., OKAZAKI, A. & AVISON, M. B. 2006. Beta-lactam resistance and beta-lactamase expression in clinical *Stenotrophomonas maltophilia* isolates having defined phylogenetic relationships. *J Antimicrob Chemother*, 57**,** 199-203.

GREEN, M. R. & SAMBROOK, J. 2012. *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratory Press.

GROTE, J., KRYSCIAK, D., SCHORN, A., DAHLKE, R. I., SOONVALD, L., MULLER, J., HENSE, B. A., SCHWARZFISCHER, M., SAUTER, M., SCHMEISSER, C. & STREIT, W. R. 2014. Evidence of autoinducer-dependent and -independent heterogeneous gene expression in *Sinorhizobium fredii* NGR234. *Appl Environ Microbiol*, 80**,** 5572-82.

GUTIERREZ, A., LAURETI, L., CRUSSARD, S., ABIDA, H., RODRÍGUEZ-ROJAS, A., BLÁZQUEZ, J., BAHAROGLU, Z., MAZEL, D., DARFEUILLE, F., VOGEL, J. & MATIC, I. 2013. β-lactam antibiotics promote bacterial mutagenesis via an RpoS-mediated reduction in replication fidelity. *Nat Commun,* 4**,** 1610.

GUTIERREZ RODERO, F., MASIA, M. M., CORTES, J., ORTIZ DE LA TABLA, V., MAINAR, V. & VILAR, A. 1996. Endocarditis caused by *Stenotrophomonas maltophilia*: case report and review. *Clin Infect Dis,* 23**,** 1261-5.

HALL, B. G. & BARLOW, M. 2005. Revised Ambler classification of β-lactamases. *J Antimicrob Chemother,* 55**,** 1050-1051.

HAMAD, M. A., SKELDON, A. M. & VALVANO, M. A. 2010. Construction of aminoglycoside-sensitive *Burkholderia cenocepacia* strains for use in studies of intracellular bacteria with the gentamicin protection assay. *Appl Environ Microbiol,* 76**,** 3170-6.

HANAHAN, D. 1983. Studies on transformation of *Escherichia* coli with plasmids. *J Mol Biol,* 166**,** 557-80.

HAURAT, M. F., ELHENAWY, W. & FELDMAN, M. F. 2015. Prokaryotic membrane vesicles: new insights on biogenesis and biological roles. *Biol Chem,* 396**,** 95-109.

HERMES, D. M., PORMANN PITT, C., LUTZ, L., TEIXEIRA, A. B., RIBEIRO, V. B., NETTO, B., MARTINS, A. F., ZAVASCKI, A. P. & BARTH, A. L. 2013. Evaluation of heteroresistance to polymyxin B among carbapenem-susceptible and -resistant *Pseudomonas aeruginosa*. *J Med Microbiol,* 62**,** 1184-9.

HOANG, T. T., KARKHOFF-SCHWEIZER, R. R., KUTCHMA, A. J. & SCHWEIZER, H. P. 1998. A broad-host-range Flp-FRT recombination system for site-specific excision of chromosomally-located DNA sequences: application for isolation of unmarked *Pseudomonas aeruginosa* mutants. *Gene,* 212**,** 77-86.

HOFFMANN, S., OTTO, C., KURTZ, S., SHARMA, C. M., KHAITOVICH, P., VOGEL, J., STADLER, P. F. & HACKERMÜLLER, J. 2009. Fast Mapping of Short Sequences with Mismatches, Insertions and Deletions Using Index Structures. *PLoS Comput Biol*, 5**,** e1000502.

HU, R. M., HUANG, K. J., WU, L. T., HSIAO, Y. J. & YANG, T. C. 2008. Induction of L1 and L2 beta-lactamases of *Stenotrophomonas maltophilia*. *Antimicrob Agents Chemother,* 52**,** 1198-200.

HU, R. M., LIAO, S. T., HUANG, C. C., HUANG, Y. W. & YANG, T. C. 2012. An inducible fusaric acid tripartite efflux pump contributes to the fusaric acid resistance in *Stenotrophomonas maltophilia*. *PLoS ONE,* 7**,** e51053.

HUANG, T. P. & WONG, A. C. 2007. A cyclic AMP receptor protein-regulated cell-cell communication system mediates expression of a FecA homologue in *Stenotrophomonas maltophilia*. *Appl Environ Microbiol,* 73**,** 5034-40.

HUANG, Y.-W., HU, R.-M., LIN, C.-W., CHUNG, T.-C. & YANG, T.-C. 2012. NagZ-Dependent and NagZ-Independent Mechanisms for β-Lactamase Expression in *Stenotrophomonas maltophilia*. *Antimicrob Agents Chemother,* 56**,** 1936-1941.

HUANG, Y. W., HU, R. M., CHU, F. Y., LIN, H. R. & YANG, T. C. 2013. Characterization of a major facilitator superfamily (MFS) tripartite efflux pump EmrCABsm from *Stenotrophomonas maltophilia*. *J Antimicrob Chemother,* 68**,** 2498-505.

HUEDO, P., YERO, D., MARTÍNEZ-SERVAT, S., RUYRA, À., ROHER, N., DAURA, X. & GIBERT, I. 2015. Decoding the genetic and functional diversity of the DSF Quorum-Sensing system in *Stenotrophomonas maltophilia*. *Front Microbiol,* 6, 761.

HUGH, R. & LEIFSON, E. 1963. A description of the type strain of *Pseudomonas maltophilia*. *Int J Syst Evol Microbiol,* 13**,** 133-138.

JACOBS, C., FRERE, J. M. & NORMARK, S. 1997. Cytosolic intermediates for cell wall biosynthesis and degradation control inducible beta-lactam resistance in gram-negative bacteria. *Cell,* 88**,** 823-32.

KAHL, B. C., BELLING, G., REICHELT, R., HERRMANN, M., PROCTOR, R. A. & PETERS, G. 2003. Thymidine-Dependent Small-Colony Variants of *Staphylococcus aureus* Exhibit Gross Morphological and Ultrastructural Changes Consistent with Impaired Cell Separation. *J Clin Microbiol*, 41**,** 410-413.

KAO, C.-Y., LEE, A.-Y., HUANG, A.-H., SONG, P.-Y., YANG, Y.-J., SHEU, S.-M., CHANG, W.-L., SHEU, B.-S. & WU, J.-J. 2014. Heteroresistance of *Helicobacter pylori* from the same patient prior to antibiotic treatment. *Infect Genet Evol,* 23**,** 196-202.

KEREN, I., SHAH, D., SPOERING, A., KALDALU, N. & LEWIS, K. 2004. Specialized persister cells and the mechanism of multidrug tolerance in *Escherichia coli*. *J Bacteriol,* 186**,** 8172-80.

KIESELBACH, T., ZIJNGE, V., GRANSTROM, E. & OSCARSSON, J. 2015. Proteomics of *Aggregatibacter actinomycetemcomitans* Outer Membrane Vesicles. *PLoS ONE,* 10**,** e0138591.

KOCH, G., YEPES, A., FÖRSTNER, KONRAD U., WERMSER, C., STENGEL, STEPHANIE T., MODAMIO, J., OHLSEN, K., FOSTER, KEVIN R. & LOPEZ, D. Evolution of Resistance to a Last-Resort Antibiotic in *Staphylococcus aureus* via Bacterial Competition. *Cell,* 158**,** 1060-1071.

KOVACH, M. E., ELZER, P. H., HILL, D. S., ROBERTSON, G. T., FARRIS, M. A., ROOP, R. M., 2ND & PETERSON, K. M. 1995. Four new derivatives of the broad-host-range cloning vector pBBR1MCS, carrying different antibiotic-resistance cassettes. *Gene,* 166**,** 175-6.

KROHN-MOLT, I., WEMHEUER, B., ALAWI, M., POEHLEIN, A., GULLERT, S., SCHMEISSER, C., POMMERENING-ROSER, A., GRUNDHOFF, A., DANIEL, R., HANELT, D. & STREIT, W. R. 2013. Metagenome survey of a multispecies and alga-associated biofilm revealed key elements of bacterial-algal interactions in photobioreactors. *Appl Environ Microbiol,* 79**,** 6196-206.

LENZ, P. & SØGAARD-ANDERSEN, L. 2011. Temporal and spatial oscillations in bacteria. *Nat Rev Microbiol*, 9**,** 565-577.

LEVINE, J. H., LIN, Y. & ELOWITZ, M. B. 2013. Functional Roles of Pulsing in Genetic Circuits. *Science,* 342**,** 1193-1200.

LEWIS, K. 2007. Persister cells, dormancy and infectious disease. *Nat Rev Microbiol,* 5**,** 48-56.

LI, X. Z., ZHANG, L. & POOLE, K. 2002. SmeC, an outer membrane multidrug efflux protein of *Stenotrophomonas maltophilia*. *Antimicrob Agents Chemother,* 46**,** 333-43.

LIN, C. W., HUANG, Y. W., HU, R. M., CHIANG, K. H. & YANG, T. C. 2009. The role of AmpR in regulation of L1 and L2 beta-lactamases in *Stenotrophomonas maltophilia*. *Res Microbiol,* 160**,** 152-8.

LIN, C. W., LIN, H. C., HUANG, Y. W., CHUNG, T. C. & YANG, T. C. 2011. Inactivation of mrcA gene derepresses the basal-level expression of L1 and L2 beta-lactamases in *Stenotrophomonas maltophilia*. *J Antimicrob Chemother,* 66**,** 2033-7.

LIN, Y. T., HUANG, Y. W., LIOU, R. S., CHANG, Y. C. & YANG, T. C. 2014. MacABCsm, an ABC-type tripartite efflux pump of *Stenotrophomonas maltophilia* involved in drug resistance, oxidative and envelope stress tolerances and biofilm formation. *J Antimicrob Chemother,* 69**,** 3221-6.

LIPUMA, J. J. 2010. The Changing Microbial Epidemiology in Cystic Fibrosis. *Clin Microbiol Rev,* 23**,** 299-323.

LOVE, M. I., HUBER, W. & ANDERS, S. 2014. Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. *Genome Biol,* 15**,** 550.

MAISONNEUVE, E., CASTRO-CAMARGO, M. & GERDES, K. 2013. (p)ppGpp Controls Bacterial Persistence by Stochastic Induction of Toxin-Antitoxin Activity. *Cell,* 154**,** 1140-1150.

MASSENGALE, A. R., QUINN, F. J., WILLIAMS, A., GALLAGHER, S. & ARONOFF, S. C. 2000. The effect of alginate on the invasion of cystic fibrosis respiratory epithelial cells by clinical isolates of *Pseudomonas aeruginosa*. *Exp Lung Res,* 26**,** 163-78.

MCBROOM, A. J. & KUEHN, M. J. 2007. Release of outer membrane vesicles by Gram-negative bacteria is a novel envelope stress response. *Mol Microbiol*, 63**,** 545-558.

MCCARTHY, Y., DOW, J. M. & RYAN, R. P. 2011. The Ax21 Protein Is a Cell-Cell Signal That Regulates Virulence in the Nosocomial Pathogen *Stenotrophomonas maltophilia*. *J Bacteriol*, 193**,** 6375-6378.

MEI, S., GAO, Y., ZHU, C., DONG, C. & CHEN, Y. 2015. Research of the heteroresistance of *Pseudomonas aeruginosa* to imipenem. *Int J Clin Exp Med,* 8**,** 6129-6132.

MILLER, V. L. & MEKALANOS, J. J. 1988. A novel suicide vector and its use in construction of insertion mutations: osmoregulation of outer membrane proteins and virulence determinants in *Vibrio cholerae* requires toxR. *J Bacteriol,* 170**,** 2575-83.

MORAND, B. & MÜHLEMANN, K. 2007. Heteroresistance to penicillin in *Streptococcus pneumonia*e. *Proc Natl Acad Sci USA,* 104**,** 14098-14103.

MOYA, B., DOTSCH, A., JUAN, C., BLAZQUEZ, J., ZAMORANO, L., HAUSSLER, S. & OLIVER, A. 2009. Beta-lactam resistance response triggered by inactivation of a nonessential penicillin-binding protein. *PLoS Pathog*, 5**,** e1000353.

MULDER, D. T. & COOMBES, B. K. 2013. Infection biology: Cheats never prosper. *Nature,* 494**,** 321-322.

NADELL, C. D., DRESCHER, K., WINGREEN, N. S. & BASSLER, B. L. 2015. Extracellular matrix structure governs invasion resistance in bacterial biofilms. *ISME J,* 9**,** 1700-9.

NGUYEN, M. H. & MUDER, R. R. 1994. Meningitis due to *Xanthomonas maltophilia*: case report and review. *Clin Infect Dis,* 19**,** 325-6.

NICOL, J. W., HELT, G. A., BLANCHARD, S. G., RAJA, A. & LORAINE, A. E. 2009. The Integrated Genome Browser: free software for distribution and exploration of genome-scale datasets. *Bioinformatics,* 25**,** 2730-2731.

O'TOOLE, G., KAPLAN, H. B. & KOLTER, R. 2000. Biofilm formation as microbial development. *Annu Rev Microbiol*, 54**,** 49-79.

O'TOOLE, G. A. 2011. Microtiter Dish Biofilm Formation Assay. *J Vis Exp***,** 2437.

OGGIONI, M. R., TRAPPETTI, C., KADIOGLU, A., CASSONE, M., IANNELLI, F., RICCI, S., ANDREW, P. W. & POZZI, G. 2006. Switch from planktonic to sessile life: a major event in pneumococcal pathogenesis. *Mol Microbiol*, 61**,** 1196-1210.

OKAZAKI, A. & AVISON, M. B. 2008. Induction of L1 and L2 β-Lactamase Production in *Stenotrophomonas maltophilia* Is Dependent on an AmpR-Type Regulator. *Antimicrob Agents Chemother,* 52**,** 1525-1528.

PALLERONI, N. J. & BRADBURY, J. F. 1993. *Stenotrophomonas*, a New Bacterial Genus for *Xanthomonas maltophilia* (Hugh 1980) Swings *et al*. 1983. *Int J Syst Bacteriol*, 43**,** 606-609.

PARK, H. J., LEE, S. W. & HAN, S. W. 2014. Proteomic and functional analyses of a novel porin-like protein in *Xanthomonas oryzae* pv. oryzae. *J Microbiol*, 52**,** 1030-5.

PERSAT, A., INCLAN, Y. F., ENGEL, J. N., STONE, H. A. & GITAI, Z. 2015. Type IV pili mechanochemically regulate virulence factors in *Pseudomonas aeruginosa*. *Proc Natl Acad Sci USA*, 112**,** 7563-7568.

PROCTOR, R. A., KRIEGESKORTE, A., KAHL, B. C., BECKER, K., LÖFFLER, B. & PETERS, G. 2014. *Staphylococcus aureus* Small Colony Variants (SCVs): a road map for the metabolic pathways involved in persistent infections. *Front Cell Infect Microbiol,* 4**,** 99.

PROCTOR, R. A., VON EIFF, C., KAHL, B. C., BECKER, K., MCNAMARA, P., HERRMANN, M. & PETERS, G. 2006. Small colony variants: a pathogenic form of bacteria that facilitates persistent and recurrent infections. *Nat Rev Microbiol,* 4**,** 295-305.

PUTMAN, M., VAN VEEN, H. W. & KONINGS, W. N. 2000. Molecular Properties of Bacterial Multidrug Transporters. *Microbiol Mol Biol Rev*, 64**,** 672-693.

QIU, D., EISINGER, V. M., ROWEN, D. W. & YU, H. D. 2007. Regulated proteolysis controls mucoid conversion in *Pseudomonas aeruginosa*. *Proc Natl Acad Sci USA,* 104**,** 8107-8112.

RAJ, A. & VAN OUDENAARDEN, A. 2008. Nature, Nurture, or Chance: Stochastic Gene Expression and Its Consequences. *Cell,* 135**,** 216-226.

RANGANNAN, V. & BANSAL, M. 2011. PromBase: a web resource for various genomic features and predicted promoters in prokaryotic genomes. *BMC Res Notes,* 4**,** 257.

REUVEN, P. & ELDAR, A. 2011. Macromotives and microbehaviors: the social dimension of bacterial phenotypic variability. *Curr Opin Genet Dev,* 21**,** 759-67.

RIZZO, M. A., SPRINGER, G. H., GRANADA, B. & PISTON, D. W. 2004. An improved cyan fluorescent protein variant useful for FRET. *Nat Biotechnol*, 22**,** 445-9.

ROBERT, L., PAUL, G., CHEN, Y., TADDEI, F., BAIGL, D. & LINDNER, A. B. 2010. Pre-dispositions and epigenetic inheritance in the *Escherichia coli* lactose operon bistable switch. *Mol Syst Biol,* 6**,** 357.

ROETZER, A., DIEL, R., KOHL, T. A., RÜCKERT, C., NÜBEL, U., BLOM, J., WIRTH, T., JAENICKE, S., SCHUBACK, S., RÜSCH-GERDES, S., SUPPLY, P., KALINOWSKI, J. & NIEMANN, S. 2013. Whole Genome Sequencing versus Traditional Genotyping for Investigation of a *Mycobacterium tuberculosis* Outbreak: A Longitudinal Molecular Epidemiological Study. *PLoS Med,* 10**,** e1001387.

ROSCETTO, E., ANGRISANO, T., COSTA, V., CASALINO, M., FORSTNER, K. U., SHARMA, C. M., DI NOCERA, P. P. & DE GREGORIO, E. 2012. Functional characterization of the RNA chaperone Hfq in the opportunistic human pathogen *Stenotrophomonas maltophilia*. *J Bacteriol,* 194**,** 5864-74.

RYAN, R. P., AN, S.-Q., ALLAN, J. H., MCCARTHY, Y. & DOW, J. M. 2015. The DSF Family of Cell–Cell Signals: An Expanding Class of Bacterial Virulence Regulators. *PLoS Pathog,* 11**,** e1004986.

RYAN, R. P., MONCHY, S., CARDINALE, M., TAGHAVI, S., CROSSMAN, L., AVISON, M. B., BERG, G., VAN DER LELIE, D. & DOW, J. M. 2009. The versatility and adaptation of bacteria from the genus *Stenotrophomonas*. *Nat Rev Microbiol,* 7**,** 514-25.

SAINO, Y., KOBAYASHI, F., INOUE, M. & MITSUHASHI, S. 1982. Purification and properties of inducible penicillin beta-lactamase isolated from *Pseudomonas maltophilia*. *Antimicrob Agents Chemother,* 22**,** 564-570.

SAINSBURY, S., BIRD, L., RAO, V., SHEPHERD, S. M., STUART, D. I., HUNTER, W. N., OWENS, R. J. & REN, J. 2011. Crystal structures of penicillin-binding protein 3 from *Pseudomonas aeruginosa*: comparison of native and antibiotic-bound forms. *J Mol Biol,* 405**,** 173-84.

SÁNCHEZ, M. B. 2015. Antibiotic resistance in the opportunistic pathogen *Stenotrophomonas maltophilia*. *Front Microbiol,* 6**,** 658.

SCHAAR, V., UDDBÄCK, I., NORDSTRÖM, T. & RIESBECK, K. 2014. Group A streptococci are protected from amoxicillin-mediated killing by vesicles containing β-lactamase derived from *Haemophilus influenzae*. *J Antimicrob Chemother,* 69**,** 117-120.

SCHWECHHEIMER, C. & KUEHN, M. J. 2015. Outer-membrane vesicles from Gram-negative bacteria: biogenesis and functions. *Nat Rev Microbiol,* 13**,** 605-619.

SHAIKH, S., FATIMA, J., SHAKIL, S., RIZVI, S. M. D. & KAMAL, M. A. 2015. Antibiotic resistance and extended spectrum beta-lactamases: Types, epidemiology and treatment. *Saudi J Biol Sci*, 22**,** 90-101.

SHANER, N. C., CAMPBELL, R. E., STEINBACH, P. A., GIEPMANS, B. N., PALMER, A. E. & TSIEN, R. Y. 2004. Improved monomeric red, orange and yellow fluorescent proteins derived from *Discosoma* sp. red fluorescent protein. *Nat Biotechnol,* 22**,** 1567-72.

SHAO, X. & GRISHIN, N. V. 2000. Common fold in helix–hairpin–helix proteins. *Nucleic Acids Res,* 28**,** 2643-2650.

SHARMA, C. M., HOFFMANN, S., DARFEUILLE, F., REIGNIER, J., FINDEISZ, S., SITTKA, A., CHABAS, S., REICHE, K., HACKERMULLER, J., REINHARDT, R., STADLER, P. F. & VOGEL, J. 2010. The primary transcriptome of the major human pathogen *Helicobacter pylori*. *Nature,* 464**,** 250-255.

SHI, W. & SUN, H. 2002. Type IV pilus-dependent motility and its possible role in bacterial pathogenesis. *Infect Immun,* 70**,** 1-4.

SIBLEY, C. D., RABIN, H. & SURETTE, M. G. 2006. Cystic fibrosis: a polymicrobial infectious disease. *Future Microbiol,* 1**,** 53-61.

SIEHNEL, R. J., EGLI, C. & HANCOCK, R. E. 1992. Polyphosphate-selective porin OprO of *Pseudomonas aeruginosa*: expression, purification and sequence. *Mol Microbiol,* 6**,** 2319-26.

STENTZ, R., HORN, N., CROSS, K., SALT, L., BREARLEY, C., LIVERMORE, D. M. & CARDING, S. R. 2015. Cephalosporinases associated with outer membrane vesicles released by *Bacteroides* spp. protect gut pathogens and commensals against β-lactam antibiotics. *J Antimicrob Chemother,* 70**,** 701-709.

STURM, A., HEINEMANN, M., ARNOLDINI, M., BENECKE, A., ACKERMANN, M., BENZ, M., DORMANN, J. & HARDT, W.-D. 2011. The Cost of Virulence: Retarded Growth of *Salmonella Typhimurium* Cells Expressing Type III Secretion System 1. *PLoS Pathog,* 7**,** e1002143.

SWINGS, J., DE VOS, P., VAN DEN MOOTER, M. & DE LEY, J. 1983. Transfer of *Pseudomonas maltophilia* Hugh 1981 to the Genus *Xanthomonas as Xanthomonas maltophilia* (Hugh 1981) comb. nov. *Int J Syst Evol Microbiol*, 33**,** 409-413.

TANG, A. C., TURVEY, S. E., ALVES, M. P., REGAMEY, N., TÜMMLER, B. & HARTL, D. 2014. Current concepts: host–pathogen interactions in cystic fibrosis airways disease. *Eur Respir Rev*, 23**,** 320-332.

THOMAS, R., HAMAT, R. A. & NEELA, V. 2014. Extracellular enzyme profiling of *Stenotrophomonas maltophilia* clinical isolates. *Virulence,* 5**,** 326-330.

TIPTON, K. A., DIMITROVA, D. & RATHER, P. N. 2015. Phase-Variable Control of Multiple Phenotypes in *Acinetobacter baumannii* Strain AB5075. *J Bacteriol,* 197**,** 2593-9.

TRINICK, M. J. 1980. Relationships Amongst the Fast-growing Rhizobia of *Lablab purpureus*, *Leucaena leucocephala*, *Mimosa* spp., *Acacia farnesiana* and *Sesbania grandiflora* and their Affinities with Other Rhizobial Groups. *J Appl Bacteriol,* 49**,** 39-53.

TURNER, P., BARBER, C. & DANIELS, M. 1984. Behaviour of the transposons Tn5 and Tn7 in *Xanthomonas campestris* pv. campestris. *Mol Gen Genet*, 195**,** 101-107.

TYPAS, A., BANZHAF, M., GROSS, C. A. & VOLLMER, W. 2012. From the regulation of peptidoglycan synthesis to bacterial growth and morphology. *Nat Rev Microbiol,* 10**,** 123-36.

VADLAMANI, G., THOMAS, M. D., PATEL, T. R., DONALD, L. J., REEVE, T. M., STETEFELD, J., STANDING, K. G., VOCADLO, D. J. & MARK, B. L. 2015. The beta-lactamase gene regulator AmpR is a tetramer that recognizes and binds the D-Ala-D-Ala motif of its repressor UDP-N-acetylmuramic acid (MurNAc)-pentapeptide. *J Biol Chem,* 290**,** 2630-43.

VAN DIJK, D., DHAR, R., MISSAROVA, A. M., ESPINAR, L., BLEVINS, W. R., LEHNER, B. & CAREY, L. B. 2015. Slow-growing cells within isogenic populations have increased RNA polymerase error rates and DNA damage. *Nat Commun,* 6, 7972.

VEENING, J. W., SMITS, W. K. & KUIPERS, O. P. 2008. Bistability, epigenetics, and bet-hedging in bacteria. *Annu Rev Microbiol,* 62**,** 193-210.

VENTER, H., MOWLA, R., OHENE-AGYEI, T. & MA, S. 2015. RND-type drug e ffl ux pumps from Gram-negative bacteria: molecular mechanism and inhibition. *Front Microbiol,* 6**,** 377.

WALSH, T. R., HALL, L., ASSINDER, S. J., NICHOLS, W. W., CARTWRIGHT, S. J., MACGOWAN, A. P. & BENNETT, P. M. 1994. Sequence analysis of the L1 metallo-beta-lactamase from *Xanthomonas maltophilia*. *Biochim Biophys Acta*, 1218**,** 199-201.

WALSH, T. R., MACGOWAN, A. P. & BENNETT, P. M. 1997. Sequence analysis and enzyme kinetics of the L2 serine beta-lactamase from *Stenotrophomonas maltophilia*. *Antimicrob Agents Chemother,* 41**,** 1460-4.

WANG, J., CHEN, L., CHEN, Z. & ZHANG, W. 2015. RNA-seq based transcriptomic analysis of single bacterial cells. *Integr Biol (Camb),* 7**,** 1466-76.

WANG, X., KANG, Y., LUO, C., ZHAO, T., LIU, L., JIANG, X., FU, R., AN, S., CHEN, J., JIANG, N., REN, L., WANG, Q., BAILLIE, J. K., GAO, Z. & YU, J. 2014. Heteroresistance at the single-cell level: adapting to antibiotic stress through a population-based strategy and growth-controlled interphenotypic coordination. *MBio,* 5**,** e00942-13.

WILKE, M. S., LOVERING, A. L. & STRYNADKA, N. C. J. 2005. β-Lactam antibiotic resistance: a current structural perspective. *Curr Opin Microbiol*, 8, 525-533.

WINDHORST, S., FRANK, E., GEORGIEVA, D. N., GENOV, N., BUCK, F., BOROWSKI, P. & WEBER, W. 2002. The major extracellular protease of the nosocomial pathogen *Stenotrophomonas maltophilia*: characterization of the protein and molecular cloning of the gene. *J Biol Chem,* 277**,** 11042-9.

WOLF, A., FRITZE, A., HAGEMANN, M. & BERG, G. 2002. *Stenotrophomonas rhizophila* sp. nov., a novel plant-associated bacterium with antifungal properties. *Int J Syst Evol Microbiol,* 52**,** 1937-44.

WU, H., MOSER, C., WANG, H.-Z., HOIBY, N. & SONG, Z.-J. 2015a. Strategies for combating bacterial biofilm infections. *In J Oral Sci,* 7**,** 1-7.

WU, N., HE, L., CUI, P., WANG, W., YUAN, Y., LIU, S., XU, T., ZHANG, S., WU, J., ZHANG, W. & ZHANG, Y. 2015b. Ranking of persister genes in the same *Escherichia coli* genetic background demonstrates varying importance of individual persister genes in tolerance to different antibiotics. *Front Microbiol,* 6**,** 1003.

YANG, T. C., HUANG, Y. W., HU, R. M., HUANG, S. C. & LIN, Y. T. 2009. AmpDI is involved in expression of the chromosomal L1 and L2 beta-lactamases of *Stenotrophomonas maltophilia*. *Antimicrob Agents Chemother,* 53**,** 2902-7.

YMELE-LEKI, P. & ROSS, J. M. 2007. Erosion from *Staphylococcus aureus* Biofilms Grown under Physiologically Relevant Fluid Shear Forces Yields Bacterial Cells with Reduced Avidity to Collagen. *Appl Environ Microbiol*, 73**,** 1834-1841.

YURGEL, S. N. & KAHN, M. L. 2004. *Dicarboxylate transport by rhizobia*.

ZAHNER, D., KAMINSKI, K., VAN DER LINDEN, M., MASCHER, T., MERAL, M. & HAKENBECK, R. 2002. The ciaR/ciaH regulatory network of *Streptococcus pneumoniae*. *J Mol Microbiol Biotechnol,* 4**,** 211-6.

ZAMORANO, L., MOYA, B., JUAN, C., MULET, X., BLAZQUEZ, J. & OLIVER, A. 2014. The *Pseudomonas aeruginosa* CreBC two-component system plays a major role in the response to beta-lactams, fitness, biofilm growth, and global regulation. *Antimicrob Agents Chemother,* 58**,** 5084-95.

ZENG, X. & LIN, J. 2013. Beta-lactamase induction and cell wall metabolism in Gram-negative bacteria. *Front Microbiol,* 4, 128.

ZHANG, L., LI, X. Z. & POOLE, K. 2000. Multiple antibiotic resistance in *Stenotrophomonas maltophilia*: involvement of a multidrug efflux system. *Antimicrob Agents Chemother,* 44**,** 287-93.

ZHOU, L., YU, Y., CHEN, X., DIAB, A. A., RUAN, L., HE, J., WANG, H. & HE, Y. W. 2015. The Multiple DSF-family QS Signals are Synthesized from Carbohydrate and Branched-chain Amino Acids via the FAS Elongation Cycle. *Sci Rep,* 5**,** 13294.

Appendix

A List of abbreviations

%	Percentage
µg	Micro gram
°C	Degree Celsius
aa(s)	Amino acid(s)
Acc.no	Accession number
AMP	Ampicillin
Amp^r	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^r	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

Gm^r	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_{L1}
L2	Putative β-lactamase protein, bla_{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

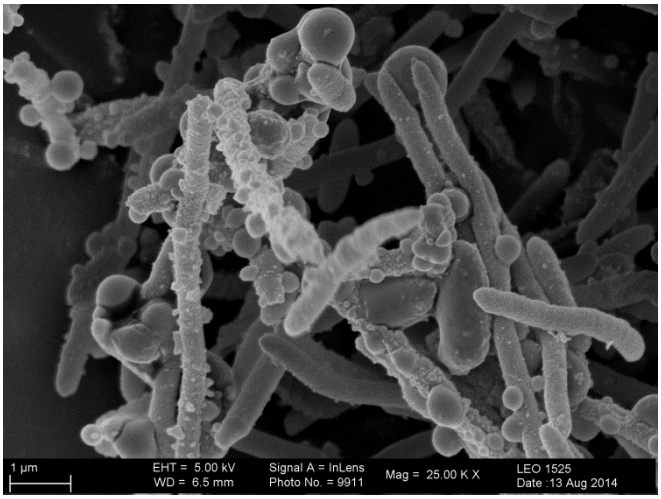


Figure B 1: Vesicle formation by the SMK279a cells exposed to kanamycin. Bacterial cell were cultured on LB agar plate supplemented with kanamycin (25 µg/ml).

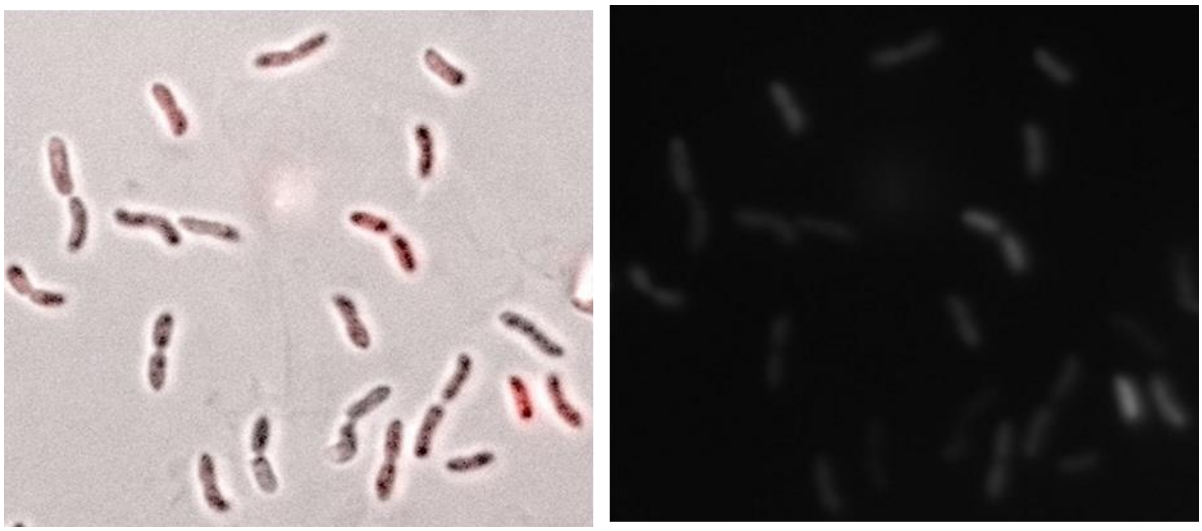


Figure B 2: Salt stress triggers P*bla*$_{L2}$ heterogeneity in SMK279a. Overnight cultures of P*bla*$_{L2}$::*rfp* reporter strain were diluted in fresh LB medium supplemented with high concentrations of NaCl (1 M). After 7 h of growth at 30°C, aliquot of withdrawn sample for image analysis revealed that P*bla*$_{L2}$::*rfp* gene expression was heterogeneous among salt stressed cells. Cells were also curved at the poles.

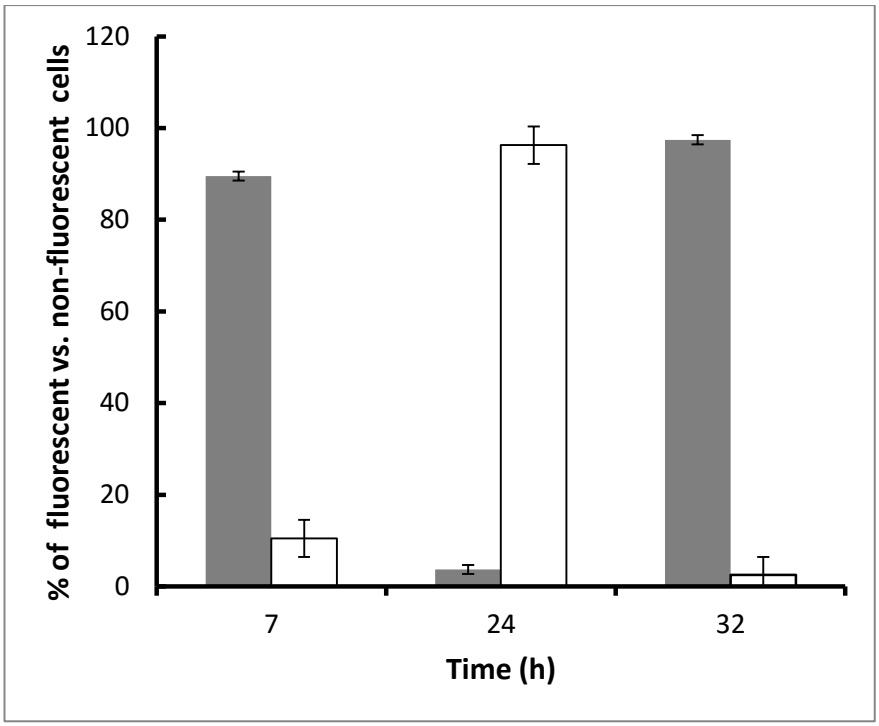


Figure B 3: Phenotypic heterogeneity observed in SMK279a cells grown in batch culture and in the presence of 1.5 ml l cell free supernatant of a 48 hour-old culture added at time '0'. Data are mean values of at least three independent cultures and for each time point and culture a minimum of 408 cells were analyzed. The volume of control cultures were adjusted with 1.5 ml fresh LB at time '0'

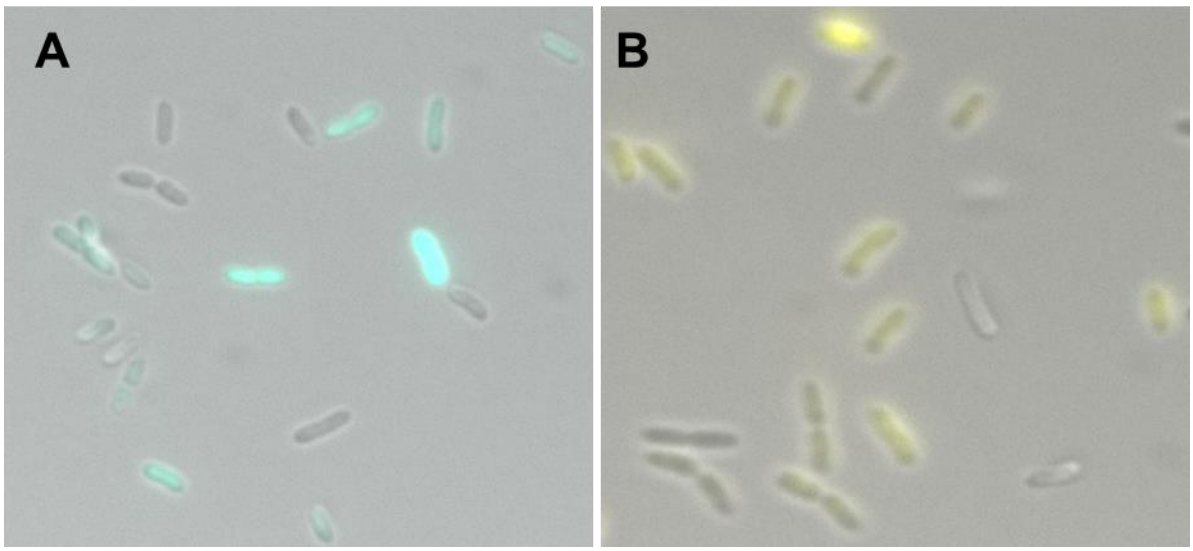


Figure B 4: Cell-free culture supernatants obtained from P*bla*$_{L2}$::*rfp* reporter strain cultures trigger phenotypic heterogeneity in SMK279a carrying the P*bla*$_{L2}$::*cfp* (A) and the P*bla*$_{L2}$::*yfp* (B) promoter gene fusion. Cells carrying either of the reporter fusion was cultured in 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'.

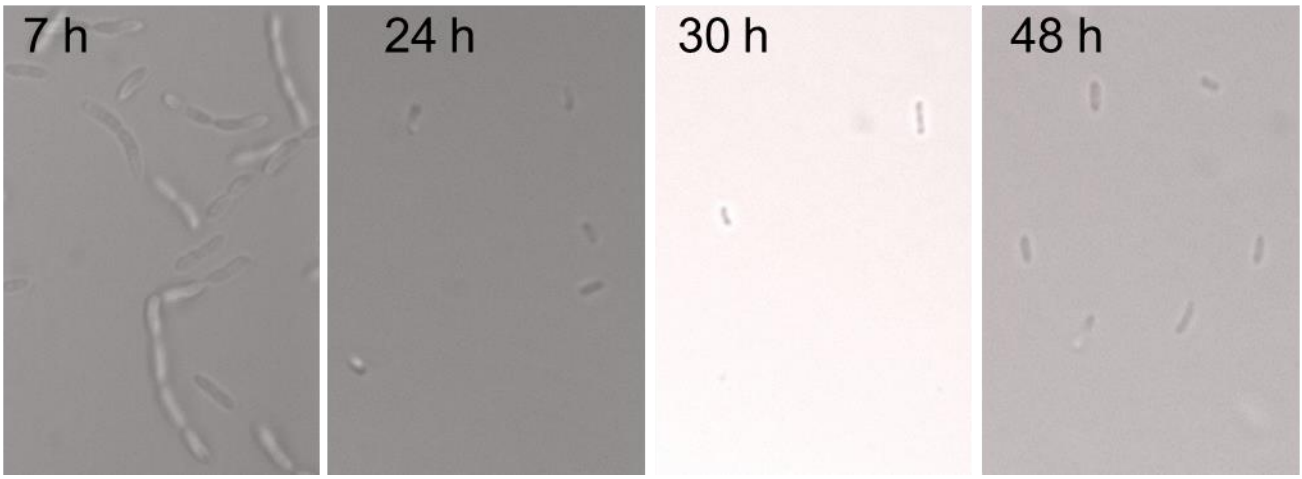


Figure B 5: Overexpression of ComE homologue abolishes heterogeneity. The *smlt1134* gene with the predicted promoter coding region was PCR amplified and inserted upstream of the RFP in the reporter plasmid in pBBR1MCS-5::P*bla*$_{L2}$::*rfp*. The construct pBBR1MCS-5::P*bla*$_{L2}$::*rfp::smlt1134* 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 *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

Sample no.	Sample type	Sample time point	No. of reads generated (x10^6)	No. of uniquely mapped reads (x10^6)
1	liquid culture	27 h	6.30	0.39
2	liquid culture	27 h	6.90	0.31
3	liquid culture	32 h	6.84	0.46
4	liquid culture	32 h	6.85	0.33

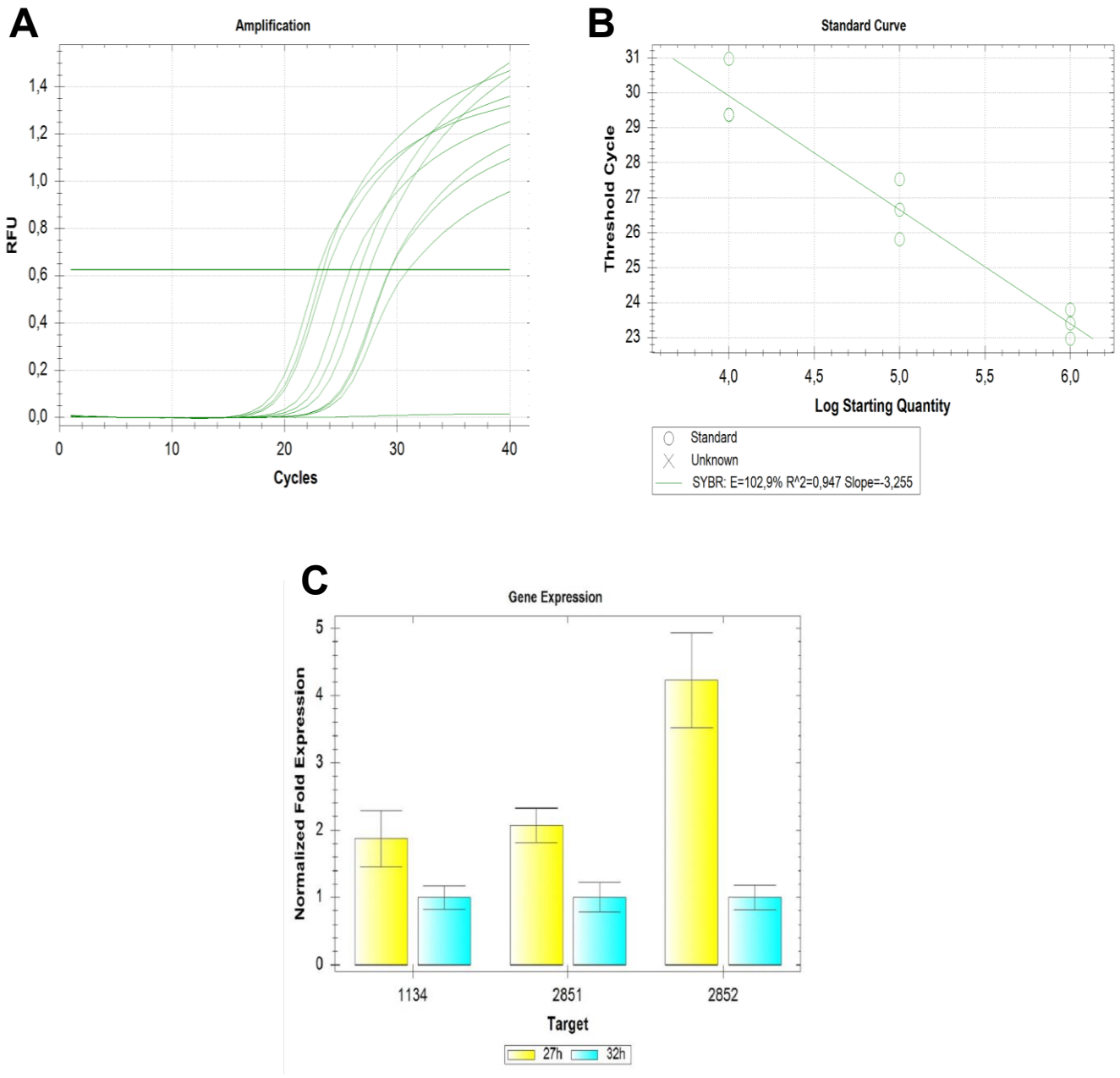


Figure B 6: RT-qPCR data analysis of *bla*$_{L2}$ ON vs. OFF cells (A) Amplification curves for *rpoD* gene generated from a 10-fold serial dilutions of cDNA. It was used to evaluate the primer efficiency and for data analysis. (B) The standard curve generated for *rpoD* gene using the CFX Manager™ software. 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, SMK279aΔ*smlt1134*, SMK279aΔ*smlt2851*Δ*smlt2852* and SMK279aΔ*smlt3723*.

SMK279a reference base position	ref. base	Strain												Gene	Function
		SMK279a wild-type control			SMK279aΔ*smlt1134*			SMK279a Δ*smlt2851*Δ*smlt2852*			SMK279aΔ*smlt3723*				
		Type[b]	Base	Predicted effect	Type[b]	Base	Predicted effect	Type[b]	Base	Predicted effect	Type[b]	Base	Predicted effect		
60299	A	DEL	Gap	Unknown	DEL	Gap	Unknown	DEL	Gap	Unknown	DEL	Gap	Unknown	None	No feature annotated; intergenic region between *smlt0054* & *smlt0056*
60300	T	DEL	Gap	Unknown	DEL	Gap	Unknown	DEL	Gap	Unknown	DEL	Gap	Unknown	None	No feature annotated; intergenic region between *smlt0054* & *smlt0056*
70880	T	SNP	C	Unknown	SNP	C	Unknown	SNP	C	Unknown	SNP	C	Unknown	None	No feature annotated; intergenic region between *smlt0069* & *smlt0073*
70883		SNP	G	Unknown	SNP	G	Unknown	SNP	G	Unknown	SNP	G	Unknown	None	No feature annotated; intergenic region between *smlt0069* & *smlt0073*
70892	T	DEL	Gap	Unknown	DEL	Gap	Unknown	DEL	Gap	Unknown	DEL	Gap	Unknown	None	No feature annotated; intergenic region between *smlt0069* & *smlt0073*
70893	C	SNP	A	Unknown	SNP	A	Unknown	SNP	A	Unknown	SNP	A	Unknown	None	No feature annotated; intergenic region between *smlt0069* & *smlt0073*
502982	T	SNP	C	Unknown	SNP	C	Unknown	SNP	C	Unknown	SNP	C	Unknown	None	No feature annotated; intergenic region between *smlt0490* & *smlt0493*
502987	C	SNP	T	Unknown	SNP	T	Unknown	SNP	T	Unknown	SNP	T	Unknown	None	No feature annotated; intergenic region between *smlt0490* & *smlt0493*
504209	C	SNP	A	Unknown	SNP	A	Unknown	SNP	A	Unknown	SNP	A	Unknown	None	No feature annotated; intergenic region between *smlt0494* & *smlt0496*
504211i1	A	INS	T	Unknown	INS	T	Unknown	INS	T	Unknown	INS	T	Unknown	None	No feature annotated; intergenic region between *smlt0494* & *smlt0496*
504212	T	SNP	G	Unknown	SNP	G	Unknown	SNP	G	Unknown	SNP	G	Unknown	None	No feature annotated; intergenic region between *smlt0494* & *smlt0496*
504215i1	A	INS	G	Unknown	INS	G	Unknown	INS	G	Unknown	INS	G	Unknown	None	No feature annotated; intergenic region between *smlt0494* & *smlt0496*
600456	G	DEL	Gap	Frameshift	DEL	Gap	Frameshift	DEL	Gap	Frameshift	DEL	Gap	Frameshift	*smlt0590*	GntR family transcriptional regulator (Smlt0590)
1139035	G	SNP	A	R239H	SNP	A	R239H	SNP	A	R239H	SNP	A	R239H	*smlt1089*	Twitching mobility protein (PilT)

				(cgc/cAc)			(cgc/cAc)			(cgc/cAc)			(cgc/cAc)		
1174560	C	None	C	None	None	C	None	SNP	A	Unknown	SNP	A	Unknown	None	No feature annotated; intergenic region between *smlt1129* & *smlt1130*
1897615i1	C	INS	G	Unknown	INS	G	Unknown	INS	G	Unknown	INS	G	Unknown	None	No feature annotated; intergenic region between *smlt1846* & *smlt1846A*
1897616	A	SNP	T	Unknown	SNP	T	Unknown	SNP	T	Unknown	SNP	T	Unknown	None	No feature annotated; intergenic region between *smlt1846* & *smlt1846A*
3410155	C	None	C	None	None	C	None	SNP	T	None (tga/tAa)	None	C	None	*smlt3373*	Phenylalanyl-tRNA synthetase subunit beta (PheT)
4046251	C	SNP	G	A840G (gcc/gGc)	SNP	G	A840G (gcc/gGc)	SNP	G	A840G (gcc/gGc)	SNP	G	A840G (gcc/gGc)	*smlt3944*	Two-component regulatory system, sensor histidine kinase (Smlt3944)
4046259i1	G	INS	G	Frameshift	INS	G	Frameshift	INS	G	Frameshift	INS	G	Frameshift	*smlt3944*	Two-component regulatory system, sensor histidine kinase (Smlt3944)
4046260	A	SNP	C	D843A (gat/gCt)	SNP	C	D843A (gat/gCt)	SNP	C	D843A (gat/gCt)	SNP	C	D843A (gat/gCt)	*smlt3944*	Two-component regulatory system, sensor histidine kinase (Smlt3944)
4046263	C	DEL	Gap	Frameshift	DEL	Gap	Frameshift	DEL	Gap	Frameshift	DEL	Gap	Frameshift	*smlt3944*	Two-component regulatory system, sensor histidine kinase (Smlt3944)
4046274i1	G	INS	G	Frameshift	INS	G	Frameshift	INS	G	Frameshift	INS	G	Frameshift	*smlt3944*	Two-component regulatory system, sensor histidine kinase (Smlt3944)
4046275	A	SNP	T	E848V (gag/gTg)	SNP	T	E848V (gag/gTg)	SNP	T	E848V (gag/gTg)	SNP	T	E848V (gag/gTg)	*smlt3944*	Two-component regulatory system, sensor histidine kinase (Smlt3944)
4177234	C	None	C	None	SNP	T	V575M (gtg/Atg)	None	C	None	None	C	None	*smlt4071*	Acriflavin resistance protein B (AcrB)

[a]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* & *smlt2852* and *smlt3723* genes are not included.

C Programs and databases

Programs

BioEdit (Sequence alignment editor)	*http://www.mbio.ncsu.edu/bioedit/bioedit.html*
CFX Manager™ (RT—qPCR)	*http://www.bio-rad.com/en-us/product/cfx-manager*
Chromas Lite (Process of chromatogram)	*http://www.technelysium.com.au/chromas_lite.html*
Clone Manager Suite 7 (Edition of sequence files)	*http://www.scied.com/in_508.htm*
EndNote (Literature)	*http://www.endnote.com*
Mega 6 (Phylogenetic analysis)	*http://www.megasoftware.net/*
Quality One (Gel Documentation software)	http://www.bio-rad.com/
GAP4 software (DNA sequence edit / assembly)	*http://staden.sourceforge.net/*
IGB (Integrated Genome Browser)	*http://bioviz.org/igb/index.html*

Databases

BRENDA (Enzyme database)	*http://www.brenda-enzymes.info/*
EMBL-EBI (InterPro Scan)	*http://www.ebi.ac.uk/embl/*
ExPASy (Proteomics server)	*http://expasy.org/*
KEGG (Encyclopedia of Genes/Genomes)	*http://www.genome.jp/kegg/*
NCBI (Database)	*http://www.ncbi.nlm.nih.gov/*
RAST (Annotation server)	*http://rast.nmpdr.org/*
RCSB PDB (Protein Data Bank)	*http://www.rcsb.org/pdb/explore.do/*
Signal IP (Signal peptide prediction)	*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

Acknowledgements

My sincere appreciation and thanks goes to my advisor and mentor, Prof. Wolfgang R. Streit. He kindly accepted my request for carrying out scientific exploration in his laboratory and helped me all the way up for successful accomplishment of this dissertation. I am truly indebted for opportunities provided to explore scientific experiments on my own as well as for the guidance and constant encouragement throughout the project when my steps were faltered. Thank you also for rigorously editing and structuring this work.

I am also thankful to Dr. Christel Vollstedt and Angela Jordan for undeserved support especially at the start of the PhD work, Dr. Andreas Pommerening-Röser for reading the dissertation, Dr. Uwe Mamat for construction of mutants, Dr. Dagmar Krysciak for handling issues related to transcriptome analysis and Dr. Ines Krohn-Molt for methodological support. I am also grateful to the researcher and collaborator at Research Center Borstel, especially the working group Prof. Ulrich E. Schaible and Prof. Stefan Nieman for whole-genome analysis aimed to detect SNPs and variants.

Special thanks go to those who proofread my work and shared their experiences to improve the dissertation write-up. Thanks to Philipp, Patrick, Maria, Mariita, Jenny.C, Fantahun, Boris and Christel for reading the different sections of this dissertation. Amanda Le Gros and Pascal Heitzmann deserved to be thanked for the English proofreading. I thank also Anna De LA Roca and Pali for their friendly constant support though out my studies.

I am always happy for time and scientific thought exchanged with Simon, Birhanu, Dominik, Jenny Chow, Frederike, Jessica, Gao, Andrea, Katrin, Philipp, Jenny H, Dagmar, Katja, Andreas, Gabi, Gesche, Simone, Nele, Arek, Claudia, Janine, Christian, Ulrich, Steffi and Jeremy. Thanks to Dr. Jenny Chow and Philipp Jenike for making my stay in lab awesome. And also the staff in the department of microbiology and biotechnology: Regina Liebram, Uschi Reinitz, Christiane Debus, Martina Schmidt and Nurcan Taskin.

It gives me also a great pleasure and happiness to thank my office mates for their academic and non-academic support. Thanks to Boris, Simon, Simone, and Philipp.

I would like to express my heartfelt gratitude to my Mom-Etaghehu Alemu, Dad-Mama Abda, brothers and sister. Your support, encouragement and love gave me strength during my studies. In the end, I thank God for blessing me to complete this work successfully.