Phenotypic Heterogeneity Affects \textit{Stenotrophomonas maltophilia} K279a Colony Morphotypes and \(\beta\)-Lactamase Expression

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Phenotypic heterogeneity at the cellular level in response to various stresses, e.g., antibiotic treatment has been reported for a number of bacteria. In a clonal population, cell-to-cell variation may result in phenotypic heterogeneity that is a mechanism to survive changing environments including antibiotic therapy. \textit{Stenotrophomonas maltophilia} has been frequently isolated from cystic fibrosis patients, can cause numerous infections in other organs and tissues, and is difficult to treat due to antibiotic resistances. \textit{S. maltophilia} K279a produces the L1 and L2 \(\beta\)-lactamas in response to \(\beta\)-lactam treatment. Here we report that the patient isolate \textit{S. maltophilia} K279a diverges into cellular subpopulations with distinct but reversible morphotypes of small and big colonies when challenged with ampicillin. This observation is consistent with the formation of elongated chains of bacteria during exponential growth phase and the occurrence of mainly rod-shaped cells in liquid media. RNA-seq analysis of small versus big colonies revealed differential regulation of at least seven genes among the colony morphotypes. Among those, \textit{bla}_{L1} and \textit{bla}_{L2} were transcriptionally the most strongly upregulated genes. Promoter fusions of \textit{bla}_{L1} and \textit{bla}_{L2} genes indicated that expression of both genes is also subject to high levels of phenotypic heterogeneous expression on a single cell level. Additionally, the \textit{comE} homolog was found to be differentially expressed in homogenously versus heterogeneously \textit{bla}_{L2} expressing cells as identified by RNA-seq analysis. Overexpression of \textit{comE} in \textit{S. maltophilia} K279a reduced the level of cells that were in a \textit{bla}_{L2}-ON mode to 1% or lower. Taken together, our data provide strong evidence that \textit{S. maltophilia} K279a populations develop phenotypic heterogeneity in an ampicillin challenged model. This cellular variability is triggered by regulation networks including \textit{bla}_{L1}, \textit{bla}_{L2}, and \textit{comE}.

Keywords: phenotypic heterogeneity, \textit{Stenotrophomonas maltophilia}, K279a, \(\beta\)-lactamases, colony morphotypes, antibiotic resistance, RNA-seq
INTRODUCTION

*Stenotrophomonas maltophilia* is a Gram-negative, non-fermentative bacterium, which is usually associated with the rhizosphere but can cause opportunistic infections of the respiratory tract in immunocompromised patients. In recent years, *S. maltophilia* has been frequently isolated from cystic fibrosis patients. As a nosocomial pathogen, it can also cause numerous infections in other organs and tissues, including bacteremia, endocarditis, pneumonia, peritonitis, cellulitis, and meningitis (Agger et al., 1986; Elting and Bodey, 1990; Nguyen and Muder, 1994; Gutierrez Rodero et al., 1996; Elser et al., 1997; Al-Hilali et al., 2000). Treatment is often difficult because the microorganism is intrinsically resistant against many common antibiotics including β-lactams (Denton and Kerr, 1998; Crossman et al., 2008; Ryan et al., 2009; Brooke, 2012). The intrinsic resistance of *S. maltophilia* K279a (SMK279a) against β-lactam antibiotics is primarily due to the function of two β-lactam resistance genes, *bla*<sub>L1</sub> (smlt2667) and *bla*<sub>L2</sub> (smlt3722), (Saino et al., 1982; Walsh et al., 1994, 1997). The product of *bla*<sub>L1</sub> is an Ambler class B Zn<sup>2+</sup>-dependent metalloenzyme, and the product of *bla*<sub>L2</sub> is an Ambler class A serine active site β-lactamase. Both enzymes are responsible for inactivation of a wide range of different β-lactam antibiotics (Walsh et al., 1994, 1997; Al Naiemi et al., 2006).

Expression of *bla*<sub>L1</sub> and *bla*<sub>L2</sub>, which is controlled by the activities of several proteins including AmpG, NagZ, AmpD, and the transcriptional regulator AmpR (Cullmann and Dick, 1990; Avison et al., 2002; Gould et al., 2006; Hu et al., 2008; Okazaki and Avison, 2008), has been suggested to be induced by β-lactam antibiotics. Furthermore, the expression of β-lactam resistance genes is linked to cell wall biosynthesis, a complex dynamic process affected by multiple cellular processes such as growth phase, division cycle, quorum sensing, and cell stress (Typas et al., 2012; Zeng and Lin, 2013). In addition to its autoregulation of transcription, AmpR regulates the induction of chromosomal β-lactamases in Gram-negative bacteria (Balcewich et al., 2010). A homologous *ampR-bla*<sub>L2</sub> module with a similar induction mechanism has been identified earlier in *S. maltophilia* (Lin et al., 2009). In this bacterium, *ampR* (smlt3723) is physically linked to *bla*<sub>L2</sub>, and it is assumed that AmpR can also regulate the expression of the unlinked *bla*<sub>L1</sub> gene (Okazaki and Avison, 2008). In the absence of an inducer, AmpR maintains its inactive conformational state as a result of binding to effector molecules, i.e., UDP-MurNAc-oligopeptides. However, exposure of the bacteria to β-lactam antibiotics increases cytosolic accumulation of anhydro-MurNAc-oligopeptides, a cell wall degradation product, which can displace the AmpR-associated UDP-MurNAc-oligopeptides. This leads to a conformational change of AmpR and subsequent activation of *bla*<sub>L1</sub> and *bla*<sub>L2</sub> transcription (Dietz et al., 1997; Jacobs et al., 1997; Balcewich et al., 2010).

While it is well known that the genetic background determines the ability of a bacterial strain to overcome antibiotic stress, bacteria have developed additional mechanisms to successfully overcome antibiotic treatment. These mechanisms may include smaller genetic variations such as single nucleotide polymorphisms (SNPs) and non-genetic variations. Within this context, phase variations can also be the cause of antibiotic resistance and/or phenotype switching. They occur at a rate of 1:10,000 in a population and can be the result of different events (Henderson and Owen, 1999; Tipton et al., 2015; and references herein).

Non-genetic variations that exist within an isogenic population contribute to the survival and fitness of the population (Ackermann, 2015; Grote et al., 2015). In a clonal population, cell-to-cell variation may result in a measurable phenotype termed phenotypic heterogeneity (Kaern et al., 2005; Magdanova and Golyasnaya, 2013). Phenotypic heterogeneity via heterogeneous gene expression benefits the population through division of labor and bet-hedging in a homogeneous environment as represented by laboratory culture conditions. This has been demonstrated for a number of model organisms such as *Salmonella enterica* serovar Typhimurium, *Vibrio harveyi*, *Bacillus subtilis*, *Sinorhizobium fredii*, and others (Anetzberger et al., 2009; Deris et al., 2013; Mulder and Coombes, 2013; Grote et al., 2014; Wang et al., 2014). Therefore, phenotypic heterogeneity is a way of multicellularity in bacterial populations to enhance the ability to adapt to changing environments (Shapiro, 1998; Davidson and Surette, 2008) and, consequently needs to be better understood to optimize antibiotic treatment strategies.

It has been shown that some bacteria can survive an antibiotic treatment by stochastically entering a dormant persister state during vegetative growth. The persister cells form transiently antibiotic tolerant subpopulations susceptible to re-introduction of the antibiotic stress, indicating that non-genetic mechanisms are involved in this process (Lewis, 2007, 2010). Much research has been focused on the exploration of the molecular keys linked to the persister phenomenon. For a more detailed insight into this topic, we refer to the excellent reviews given in references (Lewis, 2010; Helaine and Kugelberg, 2014; Maisonneuve and Gerdes, 2014). The persister phenotype depends on various factors such as the level of signaling nucleotide (p)pGpp, and various metabolic activities (Maisonneuve et al., 2013; Amato et al., 2014). 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., 2015).

Here we report phenotypic heterogeneity in SMK279a upon exposure to β-lactam antibiotics. 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). In the presence of ampicillin, SMK279a cells showed heterogeneity in colony and cell morphology, which goes along with colony-specific patterns of differentially expressed genes, including expression of the β-lactam resistance genes *bla*<sub>L1</sub> and *bla*<sub>L2</sub>. As demonstrated with
reporter gene fusions, \( \text{bla}_{1,1} \) and \( \text{bla}_{1,2} \) are subject to differential regulation even at the single cell level. Together with genome-wide sequences of cells from the different colony morphotypes, our data show that phenotypic heterogeneity in \( S. \text{maltophilia} \) K279a is a result of non-genetic variations in individual cells within isogenic populations.

**MATERIALS AND METHODS**

**Bacterial Strains, Plasmids, and Growth Conditions**

All strains and plasmids used in the present study are described in Table 1. \( S. \text{maltophilia} \) and \( E. \text{coli} \) strains were routinely grown either in LB medium (10 g/l tryptone, 5 g/l yeast extract and 5 g/l NaCl) or on LB agar plates at 30 or 37°C. Ampicillin (100 \( \mu \text{g/ml} \)), gentamicin (10 \( \mu \text{g/ml} \)), kanamycin (25 \( \mu \text{g/ml} \)), chloramphenicol (60 \( \mu \text{g/ml} \)), norfloxacin (5 \( \mu \text{g/ml} \)), or tetracycline (50 \( \mu \text{g/ml} \)) was added to the media as required. For analyses of phenotypic colony heterogeneity, SMK279a was spread on LB agar plates without or with ampicillin (100 \( \mu \text{g/ml} \)) and incubated at 37°C for 48 h. Pre-cultures were grown in 5 ml test tubes at 37°C. For analysis of single cell expression, cultures were grown for 12 to 15 generations in LB broth supplemented with gentamicin (50 \( \mu \text{g/ml} \)) and without \( \beta \)-lactam inducer. The Minimal Inhibitory Concentration (MIC) for ampicillin was analyzed using the microdilution technique and found to be at a level of 254 \( \mu \text{g/ml} \).

**Scanning and Fluorescence Microscopy**

Scanning electron microscopy (SEM) was performed as previously published (Krohn-Molt et al., 2013). Therefore, bacterial cells were grown overnight in 5 ml LB broth or on LB agar plates for 48 h. The cell suspension was centrifuged at 13,000 rpm for 2 min. The pellets or the colonies were resuspended in 500 \( \mu \text{l} \) sterile PBS-buffer, fixed in paraformaldehyde (1%) and glutaraldehyde (0.25%) 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 pelleted cells at the critical point with the 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 (Zeiss, Germany).

Single cell analyses were evaluated as previously described (Grote et al., 2014). The phase-contrast and fluorescence images were recorded using a Zeiss AxioCam microscope with an MRM camera mounted on the fluorescence microscope (Zeiss Axio Imager.M2; Carl Zeiss AG, Oberkochen, Germany). For fluorescence imaging, the microscope was equipped with filter BP546/12 (red), the emission filter 605/75 (red), and a Zeiss Illuminator HXP 120 C. Phase-contrast and fluorescence images were obtained from the same area and matched using the AxioVision software (release 4.8). The fluorescence phenotypes of single cells were recorded by evaluating, in general, a minimum of 400 cells per biological sample. For each treatment, at least three independent samples were analyzed by visually inspecting the obtained images.

**Molecular Cloning and Generation of SMK279a Mutants**

For construction of the reporter gene fusions, the genes \( \text{rfp} \) (DsRed2; Baird et al., 2000), \( \text{cfp} \) (mCerulean; Rizzo et al., 2004) and \( \text{yfp} \) (mVenus; Shaner et al., 2004) were fused to DNA fragments carrying the promoter of the \( \text{bla}_{1,1} \) gene or the promoter and intergenic region of the \( \text{bla}_{1,2} \) gene. The DNA fragments containing the promoter region were 318 bp for \( \text{bla}_{1,2} \), the intergenic region 180 bp for \( \text{bla}_{1,2} \) and 417 bp for \( \text{bla}_{1,1} \), and were predicted using PromBase (Rangannan and Bansal, 2011). These cassettes were cloned into the broad host range vector pBBRIMCS-5 (Kovach et al., 1995). Constructs were verified by DNA sequencing. The recombinant plasmids were transferred to SMK279a by triparental mating, using \( E. \text{coli} \) HB101 carrying the plasmid PRK2013 as a helper strain (Figurski and Helinski, 1979).

Markerless SMK279a mutants were constructed using the pPGI-SceI/pDAI-SceI-SacB system originally developed for bacteria of the genus \( \text{Burkholderia} \) (Flanagan et al., 2008; Aubert et al., 2014). The bacterial strains and plasmids of the pPGI-SceI/pDAI-SceI-SacB system were a generous gift from Miguel A. Valciano (Center for Infection and Immunity, Queen’s University, Belfast, UK). The mutagenesis method is based on the I-SceI homing endonuclease system, which relies on two independent crossover events to integrate first a deletion plasmid with an I-SceI recognition site into the genome of the recipient and then resolve the co-integrate structure by a second homologous recombination event in the presence of the I-SceI endonuclease provided in trans on plasmid pDAI-SceI-SacB. The deletion plasmid was derived from the mobilizable suicide vector pPGI-SceI-Xcm and contained upstream and downstream flanking regions of the gene to be deleted. All deletion plasmids were generated and maintained in \( E. \text{coli} \) SY327 (Miller and Mekalanos, 1988), which constitutively expresses the \( \lambda \) pir gene product for replication of plasmids containing the R6K origin of replication such as pPGI-SceI-Xcm. To construct the deletion plasmid pUDK012 targeting the smlt1134 gene of SMK279a, the primers KOsmlt1134-1, and KOsmlt1134-2 (Table 2) were used to amplify a 705 bp fragment of the flanking region upstream of smlt1134, followed by digestion of the PCR product with \( SphI/KpnI \) and cloning into the \( SphI/KpnI \) sites of pPGI-SceI-Xcm. The resulting plasmid was designated pUDK011. The flanking downstream region of smlt1134 of 741 bp was obtained by PCR with primer pair KOsmlt1134-3/KOsmlt1134-4. The PCR product was then digested with KpnI/Xbal and cloned into the Xbal/KpnI sites of pPGI-SceI-Xcm. The resulting plasmid was designated pUDK012. The flanking downstream region of smlt1134 of 741 bp was obtained by PCR with primer pair KOsmlt1134-3/KOsmlt1134-4. The PCR product was then digested with KpnI/Xbal and cloned into the Xbal/KpnI sites of pPGI-SceI-Xcm. The primer pair KOsmlt2851-3/KOsmlt2851-4 was...
### Table 1: Bacterial strains and plasmids used in this work.

| Strain or construct | Description | Reference |
|---------------------|-------------|-----------|
| **Escherichia coli strains** | F−, hsdS2F(r− m−)−, recA13, ara14, proA2, lacY1, galK2, pslL20 (SmR) xylF−, mtl−1, supE44, ρ− | Figurski and Helinski, 1979 |
| SMK279 | Clinical isolate from the blood of a cancer patient (Avison et al., 2000) | |
| SMK279 Δsmlt1134 | SMK279 lacking the smlt1134 gene coding for a putative DNA transport competence protein | This study |
| SMK279 Δsmlt2851 Δsmlt2852 | SMK279 lacking the smlt2851 and smlt2852 genes coding for putative transmembrane efflux proteins | This study |
| SMK279 Δsmlt3723 | SMK279 lacking the ampR (smlt3723) gene; a putative HTH and LysR family transcriptional regulator | This study |
| SMK279 Δsmlt0387 | SMK279 carrying a deletion in smlt0387 | This study |
| SMK279 EM2 | SMK279 carrying pBBR1MCS-5::P:bla2::rfp | This study |
| SMK279 EM3 | SMK279 carrying pBBR1MCS-5::P:bla1::rfp | This study |
| SMK279 EM4 | SMK279 carrying pBBR1MCS-5::P:bla1::rfp | This study |
| SMK279 EM5 | SMK279 carrying pBBR1MCS-5::P:bla2::cfp | This study |
| SMK279 EM6 | SMK279 carrying pBBR1MCS-5::P:cla1::yfp | This study |
| SMK279 EM7 | SMK279 carrying pBBR1MCS-5::P:cla1::yfp | This study |
| SMK279 EM8 | SMK279 carrying pBBR1MCS-5::P:bla2::rfp::smlt1134 | This study |
| SMK279 EM9 | SMK279 carrying pBBR1MCS-5::P:bla2::rfp::smlt2851::smlt2852 | This study |
| CF148 | Clinical isolate from the respiratory tract of a cystic fibrosis patient (Jörg Steinmann, Universitätsklinikum Essen) | |
| **Stenotrophomonas maltophilia strains** | | |
| **Plasmids** | | |
| pRK2013 | KanR; 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, GmR | Kovach et al., 1995 |
| pBBR1MCS-5::rfp | pBBR1MCS-5 carrying the rfp gene in the MCS | This study |
| pBBR1MCS-5::P:bla1::rfp | Pbla1::rfp reporter fusion in pBBR1MCS-5 | This study |
| pBBR1MCS-5::P:bla2::rfp | Pbla2::rfp reporter fusion in pBBR1MCS-5 | This study |
| pBBR1MCS-5::P:bla1::cfp | Pbla1::cfp reporter fusion in pBBR1MCS-5 | This study |
| pBBR1MCS-5::P:cla1::yfp | Pcla1::yfp reporter fusion in pBBR1MCS-5 | This study |
| pBBR1MCS-5::P:bla1::yfp | Promoterless rfp reporter fusion in pBBR1MCS-5 | This study |
| pBBR1MCS-5::P:bla2::rfp | Pbla2::rfp reporter fusion constructed with the ampR-bla2 intergenic region in pBBR1MCS-5 | This study |
| pBBR1MCS-5::P:bla2::rfp::smlt1134 | smlt1134 gene under its native promoter cloned into the pBBR1MCS-5::P:bla2::rfp reporter fusion | This study |
| pBBR1MCS-5::P:bla1::cfp::smlt2851::smlt2852 | Putative operon of smlt2851 and smlt2852 under its native promoter cloned into the pBBR1MCS-5::P:bla2::rfp reporter fusion | This study |
| pGPI-Scel-Xcm | CmR, TpR, mobilizable suicide vector; carries the R6K origin of replication, the I-SceI recognition site and a xyfE reporter gene | Hamad et al., 2010 |
| pDAI-Scel-SacB | TetR; mobilizable broad host range plasmid; carries the gene for the I-SceI homing endonuclease and the sacB gene | Rannagan et al., 2008; Hamad et al., 2010 |
| pUDK011 | pGPI-Scel-Xcm with a 705-bp SpIII/KpnI insert of SMK279a containing the flanking region upstream of smlt1134 | This study |
| pUDK012 | pUDK011 with a 741-bp KpnI/Xbarl insert of SMK279a containing the flanking region downstream of smlt1134 | This study |
| pUDK014 | pGPI-Scel-Xcm with a 779-bp NotI/KpnI insert of SMK279a containing the flanking region downstream of smlt2852 | This study |
| pUDK015 | pUDK014 with a 776-bp KpnI/XbaI insert of SMK279a containing the flanking region downstream of smlt2851 | This study |

(Continued)
TABLE 1 | Continued

| Strain or construct | Description | Reference |
|---------------------|-------------|-----------|
| pUDK017             | pGPI-SceI-Xcm with a 761-bp NotI/KpnI insert of SMK279α containing the flanking region upstream of smlt3723 | This study |
| pUDK018             | pUDK017 with a 660-bp KpnI/XbaI insert of SMK279α containing the flanking region downstream of smlt3723 | This study |

**TABLE 2 | Primers used in this study.**

| Primer            | Sequence [5′-3′]                                      |
|-------------------|------------------------------------------------------|
| Smlt3722For       | CTTAAGTGACCAGATCTCGTGCGTCAGT                      |
| Smlt3722Rev       | CGATGAATTCCGGACGATCGGCGGTCTCTCTGG                  |
| 3722rFor          | CTTAAGTACCAGCGCGCACTGATGTCG                      |
| 3722rFlRev        | CCTGAATTCATGGCGGGGTTCTCTGGG                       |
| Ma2667For         | CTTAGAATTCGTCACGTCGCTGGAGAGGTCAAGG               |
| KoSmI1134-1       | ATATTGcatgcCTCTTTCAAGGCTTCGACACGCG               |
| KoSmI1134-2       | CTTCAATCttataGTGTTGAGGGGTTGACGTGTCG               |
| KoSmI1134-3       | CCGGATCgggtaccGGTGCG                              |
| KoSmI1134-4       | CTTCTttataGTGTTGAGGGGTTGACGTGTCG               |
| KoSmI2852-1       | CCTGAgggtgccGAAGTTACCGAGGTTAAGGAGGTCAAGG          |
| KoSmI2852-2       | ATACGccaaccATGGGCCATTCCGCCAGGAGGTCAAGG          |
| KoSmI2852-3       | CATGGGcgggtaccGTACCAGGCTGACGTGTCG               |
| KoSmI2852-4       | ATACGccaaccATGGGCCATTCCGCCAGGAGGTCAAGG          |
| KoSmI3723-1       | CCTGAAGggtgccGTGTAAGGGGTTGACGTGTCG               |
| KoSmI3723-2       | TTAGGccggtaccTTCGATGGCGAGGTTGACGTG               |
| KoSmI3723-3       | CTTAGGcgggtaccGTACCAGGCTGACGTGTCG               |
| KoSmI3723-4       | TACCCgtaccAGATGCGAGGTTGACGTGTCG               |

-A: SphI site is shown in lower case letters.
-B: KpnI site is shown in lower case letters.
-C: XbaI site is shown in lower case letters.
-D: NotI site is shown in lower case letters.

The successful construction of all plasmids was verified by DNA sequence analysis of the inserts. The deletion plasmids were transferred to SMK279α by triparental mating as described previously (Aubert et al., 2014), using *E. coli* DH5α carrying the plasmid pRK2013 as a helper strain, and *E. coli* SY327/pUDK012, SY327/pUDK015, and SY327/pUDK018 as the donor strains, respectively. Selection for SMK279α co-integrants was performed at 37°C on LB agar plates containing 60 μg/ml chloramphenicol and 5 μg/ml norfloxacin to counter-select against the *E. coli* helper and donor strains. Streaks of SMK279α exconjugants were subsequently sprayed with 0.45 M pyrocatechol to confirm integration of the deletion plasmids into the genomes of co-integrants. Due to the activity of the pyrocatechol 2,3-dioxygenase encoded by the *xylE* reporter gene of the deletion plasmids, a bright yellow color of the biomass was indicative of the presence of pUDK012, pUDK015, and pUDK018 in the genomes of the strains, respectively.

As a next step, the plasmid pDAI-SceI-SacB was introduced into the SMK279α co-integrants by triparental mating with DH5α/pRK2013 and DH5α/pDAI-SceI-SacB as helper and donor strains, respectively. Exconjugants were selected at 30°C on LB agar plates containing 50 μg/ml tetracycline and 5 μg/ml norfloxacin, followed by screening for excision of the deletion plasmids. The inability of pyrocatechol to turn the color of the exconjugants into bright yellow and sensitivity of the strains to 60 μg/ml chloramphenicol indicated the loss of the integrated plasmids. Finally, the plasmid pDAI-SceI-SacB was cured by sucrose counter-selection as described (Aubert et al., 2014). As a result, the unmarked mutants SMK279α smlt1134, SMK279α smlt2851 Δsmlt2852 and SMK279α smlt2852 were obtained. The deleted regions of the mutant strains were verified by PCR and DNA sequence analysis.

The mutant strain SMK279α Δsmlt0387 was generated according to Hoang et al. (1998) and Alavi et al. (2013).

**RNA-seq and Data Analysis**

To prepare the cell material for RNA-seq analyses, SMK279α 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. About 10–15 mg of wet biomass were generally used for each biological replicate, 10 mg of cell material corresponded to an OD₆₀₀ of 6.35. Additionally, for RNA extraction of liquid cultures, the strain SMK279α carrying the *Pba12::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 RNAs. The cDNA libraries for sequencing were constructed by vertis Biotechnology AG, Germany as described by 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 0.13.1. The alignment of reads, coverage calculation, gene wise read quantification and differential gene expression was performed with READemption (Fürstner 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 genome wasperformed with READemption (Förstner et al., 2014) version 0.0.131. The alignment of reads, coverage calculation, 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 to the National Center for Biotechnology Information’s Gene Expression Omnibus under accession 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: GSE17353. A shell script that covers the main RNA-Seq data processing steps was deposited at https://zenodo.org/record/34153 (DOI:10.5281/zenodo.34153).

RT-qPCR

Selected differentially regulated genes identified in SMK279a RNA-seq analyses were verified by RT-qPCR experiments as described previously (Krysciak et al., 2014). Gene-specific primers used for RT-qPCR are shown in Table 3. Samples for RNA extraction were taken from colonies that had been grown for 48 h in the presence of ampicillin (100 μg/ml) and were obtained from three independent experiments. The SuperScript VILO cDNA synthesis kit (Invitrogen, TX, USA) was used to generate cDNA using 1.2 μg RNA. The expression values were normalized against rpoD and 16S rRNA.

TABLE 3 | Gene-specific primers used for RT-qPCR.

| Primer | Sequence [5’-3’] | Length of amplified product/gene |
|--------|-----------------|-------------------------------|
| qsmmit1416s-Bfor | AAGGGCGCTGGAACAGGGCTCA | 145 bp/rpoD* |
| qsmmit1416s-Brev | CCACATCGGCGGAACCTCA | 200 bp/RNA |
| q16S-Afor | AGAGTTTGTCTGCTGGCTCAAG | 157 bp/16S rRNA* |
| q16S-Arev | CTACGGGACATGGGGTCACT | 194 bp/16S rRNA |
| qsmmit2667-for | CGTCACGCTGCTGGAACAACAG | 240 bp/16S rRNA |
| qsmmit2667-rev | CACGCGCGTCTGGCATTTTTG | 157 bp/16S rRNA |
| qsmmit3722-Afor | CGTCGGCGGATCCGCGTCAAG | 144 bp/16S rRNA |
| qsmmit3722-Arev | TCTCCGCGCAGCGAGAATCAC | 157 bp/16S rRNA |
| qsmmit3721-for | GAGCGCATGACTGCTGACCA | 196 bp/smr3721 |
| qsmmit3721-rev | GAACACGAGACAGGGAGGAAA | 196 bp/smr3721 |
| qsmmit0018-for | CGGAAGCAGTGGTCACCTCTT | 168 bp/smr0018 |
| qsmmit0018-rev | TGATGCAGATTCCGCGTACCC | 168 bp/smr0018 |
| qsmmit0019-for | TAAATAAAGCGGCGCGCACTT | 205 bp/smr0019 |
| qsmmit0019-rev | TCCCGCTGACGCTGAGTCT | 205 bp/smr0019 |
| qsmmit1007-for | GAGGGTTTCACCCGAGCTCC | 126 bp/smr1007 |
| qsmmit1007-rev | GATGCGCTGCTGCTGAGATCTAT | 126 bp/smr1007 |

*Genes used for normalization of gene expression.

Whole Genome Sequencing and Variant Detection

Usually a single colony was transferred to fresh LB and cultured overnight on a Petri dish at 37°C. Addition of ampicillin (100 μg/ml) resulted in the formation of colony heterogeneity, with colonies differing in size and appearance (Figure 1) and could be observed as soon as they were visible on the plates. This was usually after 16–18 h of growth at 37°C for big colonies. Small colonies appeared between 24 and 36 h of growth. Colonies were grouped into three categories based on size and origin, i.e., small and big colonies in the presence of ampicillin (Figure 1B) and uniform colonies (Figure 1A) in the absence of ampicillin (Table 4). Formation of different colony morphotypes was independent of the presence or absence of ampicillin in the pre-cultures. When grown in the presence of high concentrations of ampicillin (600 μg/ml), small and big colonies were observed (Figure 1C) and, furthermore, colonies slightly changed the color and appearance (Figure 1D). Among these colonies, some were pointed at the center while others appeared to have a flat colony surface with a wrinkled texture.

RESULTS

Transcript Colony Heterogeneity and Formation of Outer Membrane Vesicles in Response to Antibiotic Treatment

In the absence of antibiotics, the SMK279a wild-type strain forms cream-colored, round and uniform colonies with a diameter of approximately 1.75–2.0 mm after 24 h of growth on LB agar plates at 37°C. Addition of ampicillin (100 μg/ml) resulted in the formation of colony heterogeneity, with colonies differing in size and appearance (Figure 1) and could be observed as soon as they were visible on the plates. This was usually after 16–18 h of growth at 37°C for big colonies. Small colonies appeared between 24 and 33 h of growth. Colonies were grouped into three categories based on size and origin, i.e., small and big colonies in the presence of ampicillin (Figure 1B) and uniform colonies (Figure 1A) in the absence of ampicillin (Table 4). Formation of different colony morphotypes was independent of the presence or absence of ampicillin in the pre-cultures. When grown in the presence of high concentrations of ampicillin (600 μg/ml), small and big colonies were observed (Figure 1C) and, furthermore, colonies slightly changed the color and appearance (Figure 1D). Among these colonies, some were pointed at the center while others appeared to have a flat colony surface with a wrinkled texture.
Abda et al. Phenotypic Heterogeneity Affects \textit{S. maltophilia} K279a

FIGURE 1 | Phenotypic heterogeneity of SMK279a cells during growth on solid media. (A) SMK279a forms 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).

When we streaked small colonies onto fresh plates without antibiotics, they formed uniform large colonies after overnight incubation, indicating the small colony morphotype was not caused by a genetic mutation. More importantly, small colonies formed both big and small colonies when recultivated in the presence of ampicillin. However, in the presence of ampicillin, cells from big colonies formed again a uniform colony at 24 and 48 h after transfer to fresh agar plates containing ampicillin. This reversible variation in colony morphology was also observed for other strains such as \textit{S. maltophilia} CF148, an isolate from the respiratory tract of a cystic fibrosis patient, and \textit{S. maltophilia} DSM-50170 isolated from the human oropharyngeal region (Table 1).

Together with these findings, we observed that cells grown in the presence of ampicillin formed more frequently long bacterial chains (Figure 2). Furthermore, SMK279a cells from both colony morphotypes showed the formation of outer membrane vesicles (OMVs). However, this feature was much more pronounced in the presence of ampicillin (Figures 2B–D) in comparison to cells grown in the absence of the antibiotic that showed only very few or no vesicles (Figure 2A). Intriguingly, the size of the OMVs varied considerably, ranging from less than 100 nm up to 677 nm in diameter in some SMK279a cells, which was twice the size as those reported for other Gram-negative bacteria (Beveridge, 1999). Most likely, these vesicles are packed with \textit{bla\textsubscript{L1}} and \textit{bla\textsubscript{L2}} active enzymes (Devos et al., 2015).

These initial data suggested that the observed colony morphotypes resulted either from gene expression differences at the cellular level within isogenic populations or from heterogeneous expression of resistance genes at a single cell level. Since colony morphotypes were reversible, they most likely were not a result of permanent mutations such as SNPs. To test this hypothesis, we sequenced DNA from 24 individual colonies with diverse morphotypes using NGS technology (Supplementary Figure S1). Overall, we analyzed the genomes of 13 small, 9 big, and 2 uniform colonies with an average 51.4-fold coverage. Compared with the published SMK279a genome sequence, we identified 2–15 SNPs per colony, up to seven deletions and four insertions within all colonies (Supplementary Table S1). Most remarkably, none of these were identified within the known resistome of SMK279a, further supporting the notion that the colony morphotypes were not a primary result of genetic alterations.

A more detailed genome analysis identified SNPs or smaller deletions in 15 genes and ORFs, and none of these appeared to be linked to a gene that is essential for growth (Supplementary Table S2). Only one of the observed SNPs may be linked to \textit{β}-lactam treatment, namely \textit{smlt3885}, a gene which codes for a putative UDP-N-acetylmuramate-1-alanyl-gamma-D-glutamyl-meso-diaminopimelate ligase. This enzyme is most likely involved in recycling of cell wall precursors during bacterial cell wall synthesis but is not essential for growth (Mengin-Lecreulx et al., 1996). Two other regions in the SMK279a genome contained high densities of SNPs and indels. These loci are assigned to genes coding for the hypothetical protein Smlt1844B and a two-component regulatory system-sensor histidine kinase (Smlt3944). The \textit{smlt3944} genes carried non-synonymous mutations at seven or nine base positions, respectively (Supplementary Table S2). The product of \textit{smlt3944} has a length of 880 amino acids and may be transcribed within an operon with the \textit{smlt3943} gene coding for the phosphate-selective porin OprP (Supplementary Figure S2). OprP is mainly expressed under conditions of phosphate.
of cell material for each colony type. We used colonies for RNA extraction as we expected that gene expression profile might reflect a different expression level given the different phenotypes. Given the above observation that the small colonies needed 24–33 h to appear on plates, a harvest at 48 h ensured us sufficient biomass and highly reproducible colony phenotypes for the RNA extraction.

After RNA extraction, cDNA libraries were constructed and sequenced using NGS. For each colony variant, we analyzed two independent biological replicates. Between 7.35 and 12.35 million reads were generated for the two biological replicates and aligned to the reference genome sequence of SMK279a (GenBank: NC_010943.1). The metadata of the samples and RNA-seq analyses are summarized in Table 5.

For comparative analyses of RNA-seq data, we considered genes with a fold-change of $\geq 2.0$, a Likelihood value $\geq 0.9$ and an adjusted $P$-value of $\leq 0.05$ as statistically significant and differentially expressed between the colony variants. The RNA-seq data unveiled that isogenic populations of SMK279a cells differentially regulate only a small number of genes between the colony morphotypes. Surprisingly, only 12 genes were significantly and differentially regulated between big and small colony variants (Table 6, Figure 3A). Among the differentially expressed genes, $\text{bla}_{\text{smt}2667}$ and $\text{bla}_{\text{smt}3722}$ were 15.3- and 6.9-fold up-regulated in cells forming big colonies in comparison to those forming small colonies, respectively (Table 6, Figure 3B). Upon $\beta$-lactam challenge, the transcriptional response in a small colony morphotype further included an altered expression level of genes involved in diverse cellular functions. The level of altered expression of these genes ranged from 2.0 to 3.6-fold, including down-regulation of eight of 14 genes, and up-regulation of six out of the 14 regulated genes (Figure 3B).

Moreover, as summarized in Table 6, 34 genes were identified to be differentially regulated in small colonies when compared to big and uniform colonies. Thus, four hypothetical proteins (Smilt0018, Smilt0019, Smilt1766, and Smilt2668), a putative Na$^+/H^+$ antiporter (Smilt3721, AmpH) and two fibramia adhesin proteins (Smilt0706, Smf1, and Smilt0709) are associated with the formation of the different colony morphotype. The fimbrial operon includes $\text{smf}-1$ and $\text{smt}0707$ and is involved in haemagglutination, biofilm formation, and adherence to cultured mammalian cells (de Oliveiera-Garcia et al., 2003). Notably, $\text{smilt}0018$ and $\text{smilt}0019$ most likely form an operon. Furthermore, although $\text{ampH}$ is physically linked to $\text{bla}_{\text{smt}22}$ and the flanking $\text{ampR}$ gene ($\text{smt}3723$; Figure 3C), the function of $\text{ampH}$ remains to be determined.

Expression analysis by RT-qPCR was used to confirm the RNA-seq data. We analyzed the expression profiles of five different genes ($\text{bla}_{\text{smt}22}$; $\text{ampH}$; $\text{bla}_{\text{smt}0019}$; $\text{smt}0018$) that

### Table 4 | SMK279a colony morphotypes observed after 48 h of growth on LB agar plates with and without ampicillin.

| Treatment      | Concentration [μg/ml] | Colony morphotype | % of all colonies | Colony size [diameter, mm] |
|----------------|-----------------------|-------------------|-------------------|---------------------------|
| Ampicillin     | 100                   | Small             | 80                | 1.4 ± 0.26                |
| Ampicillin     | 100                   | Big               | 20                | 3.2 ± 0.27                |
| No ampicillin  | –                     | Uniform           | 100               | 3.0 ± 0.06                |

**FIGURE 2** | Representative scanning electron micrographs (SEM) 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). (A) SEM image of cells obtained from colonies cultured in the absence of 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 big colonies cultured in the presence of 100 μg/ml ampicillin. (D) SEM image of cells obtained from big colonies cultured in the presence of 300 μg/ml ampicillin. Cells from (A–C) predominantly formed long filamentous cells (indicated by letter F) and OMVs (indicated by arrows). In the presence of ampicillin, SMK279a cells became enlarged and formed OMVs with sizes up to 677 nm in diameter.
were differentially transcribed between cells obtained from big and small colonies, including rpoD and 16S rRNA as internal reference genes. We analyzed expression data based on the normalized gene expression (2^{-ΔΔCt} method), and the results confirmed the data obtained by RNA-seq (Figure 3D). The β-lactamase genes bla_{L1} and bla_{L2}, as well as the putative Na^+/H^+ antiporter gene ampH were among the strongly upregulated genes in cells forming big colonies.

Since AmpR is the central regulator for the expression of β-lactamases, we analyzed the basal expression of ampR (smlt3723) in big vs. small colony variants. Therefore, we constructed an ampR-deletion mutant. The SMK279aDelta3723 deletion was verified by PCR and whole genome sequencing. Although the SMK279aDelta3723 mutant failed to grow on LB agar plates in the presence of 100 μg/ml ampicillin, it grew well in the absence of any added antibiotic. Therefore, this deletion mutant could not be used to analyze colony morphotypes in the presence of ampicillin.

Heterogeneous Expression of bla_{L1} and bla_{L2} in Single Cells

In addition to the observed phenotypic heterogeneity with respect to colony morphologies and OMV formation, we speculated that β-lactamase expression might be subject to heterogeneous expression at a single cell level. To address this hypothesis, we constructed different reporter fusions 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 (Table 1). All obtained constructs were verified by sequencing and introduced into SMK279a by tri-parental mating using E. coli HB101 with pRK2013 as a helper plasmid (Figurski and Helinski, 1979). In the presence of ampicillin, SMK279a cells grew in long chains during the exponential growth phase rather than in individual rods or aggregates as observed during stationary growth phase. Furthermore, the ampicillin susceptibility of this bacterium was unchanged indicating that the native AmpR binding ligand remained intact and was not affected in trans by the Pbla_{L2}:rfp promoter construct (data not shown).

A detailed analysis of single cells by fluorescence microscopy indicated that bla_{L1} and bla_{L2} were heterogeneously expressed between individual bacterial cells (Figure 4). In an isogenic culture, we usually detected two stable subpopulations, i.e., individual cells in the bla-ON showing strong fluorescence and those in the bla-OFF mode that display no fluorescence. Noteworthy, individual cells within filaments (or aggregates) of exponentially growing cultures displayed a bla-OFF mode, while adjacent cells were in a bla-OFF mode (Figure 4C). This phenomenon was observed in both cells expressing the bla_{L1} and the bla_{L2} gene fusion. Further tests in which the promoter of bla_{L2} was fused to a yellow and cyan fluorescent protein also confirmed heterogeneous expression of the β-lactamase within actively growing cells in the exponential phase (Figures 4D,E; Supplementary Figure S3).

Additionally, we used SMK279a carrying the native promoter of the ampR-bla_{L2} intergenic region fused to rfp (Pbla_{L2}:rfp; Table 1). However, cells 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.

RNA-seq Indicates Few Significantly Differentially Regulated Genes in Homogenous Versus Heterogeneous bla_{L2}-Expressing 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 majority of cells expressed the red fluorescent reporter and, thus, was in the bla_{L2}-ON mode (Table 7). This response was independent of the presence of ampicillin. These observations prompted us to analyze the transcriptomes of cells that grew for 27 or 32 h in liquid cultures using RNA-seq. These two time points were selected because growth experiments and single cell counting indicated that less than 5% of all cells were in a bla_{L2}-ON mode after 27 h, whereas more than 90% of all cells were in a bla_{L2}-ON mode after 32 h. Data were analyzed as described above, and the transcriptomic metadata are summarized in Table 5. Two biological replicates were analyzed for each time point. Interestingly, only three genes were differentially expressed between the two time points of 27 and 32 h: smlt2851 and smlt2852, encoding a putative antibiotic resistance transporter and forming an operon, and a

| Sample no. | Sample type | Sample time point | No. of reads generated (x 10^6) | No. of uniquely mapped reads (x 10^6) |
|------------|-------------|-------------------|---------------------------------|----------------------------------|
| 1          | Big colony  | 48 h              | 8.80                            | 0.69                             |
| 2          | Big colony  | 48 h              | 9.88                            | 0.80                             |
| 3          | Small colony| 48 h              | 9.72                            | 0.63                             |
| 4          | Small colony| 48 h              | 12.35                           | 0.98                             |
| 5          | Uniform colony | 48 h          | 7.35                            | 0.38                             |
| 6          | Uniform colony | 48 h           | 9.60                            | 1.25                             |
| 7          | Liquid culture| 27 h              | 6.30                            | 0.39                             |
| 8          | Liquid culture| 27 h              | 6.90                            | 0.31                             |
| 9          | Liquid culture| 32 h              | 6.84                            | 0.46                             |
| 10         | Liquid culture| 32 h              | 6.85                            | 0.33                             |

Table 5: Overall transcriptome metadata for the analyzed SMK279a colony morphotypes and liquid culture samples.
The table shows genes with a fold-change of $\geq 2.0$, a Likelihood value $\geq 0.9$ and an adjusted P-value of $\leq 0.05$. 

| 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 Smf-1               | –2.3                      | –2.0                       | –                             |
| Smlt0709    | Fimbria adhesin protein                     | –2.0                      | –                          | –                             |
| Smlt1735    | tRNA delta(2)-isopentenyl pyrophosphate MiaA| +2.4                      | –                          | –                             |
| Smlt1766    | Hypothetical protein                        | +2.1                      | –                          | –                             |
| Smlt2667    | β-lactamase Bla$_{L1}$                      | +15.3                     | +9.9                       | –                             |
| Smlt2668    | Hypothetical protein                        | +5.9                      | +5.1                       | –                             |
| Smlt3721    | Putative Na$^+$/H$^+$ antiporter AmpH        | +3.6                      | +3.5                       | –                             |
| Smlt3722    | β-lactamase Bla$_{L2}$                      | +6.9                      | +7.4                       | –                             |
| Smlt1844    | Modification methylase                      | –2.0                      | –                          | –                             |
| Smlt2601    | Poly (beta-D-mannuronate) lyase             | –2.1                      | –                          | –                             |
| Smlt0003    | Hypothetical protein                        | –                         | –                          | +2.1                          |
| Smlt0028    | DNA helicase II                             | –                         | –                          | +2.0                          |
| Smlt0044    | RH5-repeat-containing protein               | –                         | –                          | +2.0                          |
| Smlt0201    | Hypothetical protein                        | –                         | –                          | +2.2                          |
| Smlt0265    | Acyl CoA dehydrogenase                      | –                         | –                          | –2.1                          |
| Smlt0336    | Hypothetical protein                        | –                         | –                          | +2.3                          |
| Smlt0363    | 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 super family transmembrane protein | – | – | –2.6 |
| Smlt2041    | 50S ribosomal protein L36 RpmJ              | –                         | –                          | +2.0                          |
| Smlt3041    | Hypothetical protein                        | –                         | –                          | +2.3                          |
| Smlt3066    | 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                          |

putative *comE* homolog (*smlt1134*). The *smlt2851* and *smlt2852* genes were 29- and 7.2-fold up-regulated, respectively, while *smlt1134* was 5.4-fold down-regulated at the 32-h sampling time point (*Figure 5A*). Additional RT-qPCR data supported the observation made for the *comE* homolog. RT-qPCR indicated a $1.74 \pm 0.29$-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. For the *smlt2851* and *smlt2852* loci, RT-qPCR data did not confirm the RNA-seq data.

To analyze the impact of *Smlt2851*, *Smlt2852*, and *Smlt1134* (*ComE* homolog) on phenotypic heterogeneous *bla$_{L2}$* expression, the genes were overexpressed in SMK279a. The respective operons and predicted promoter regions were PCR amplified and inserted upstream of *Pbla$_{L2}$::rfp* in *pBBR1MCS-5*, resulting in *pBBR1MCS-5::Pbla$_{L2}$::rfp::smlt1134* and *pBBR1MCS-5::Pbla$_{L2}$::rfp::smlt2851::smlt2852* (*Figure 5B*). The recombinant plasmids were transferred to SMK279a cells and challenged with 100 $\mu$g/ml ampicillin. Only the overexpression of the *comE*
FIGURE 3 | RNA-seq and RT-qPCR data analysis for different SMK279a colony morphotypes. (A) 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 the absence of ampicillin on LB agar plates. A twofold change in the transcription of genes with adjusted P-value of ≤0.05 is considered as significantly, differentially regulated. Cells forming big colonies differentially regulated several genes mainly involved in degradation of antibiotics. The numbers in the plot indicate most strongly regulated genes: 1, smlt0018; 2, smlt2667; 3, smlt3772; 4, smlt2668; 5, smlt0019; 6, smlt3721; 7, smlt1760; and 7, smlt0596. (B) Differentially regulated genes in small colonies in comparison to big and uniform colonies. Down-regulation of blaL1 and blaL2 affected genes involved in regulation, metabolism, virulence, transport and genetic information processing/translation (for details see Table 6). (C) Transcriptome profiles of blaL2 and the flanking genes ampR (smlt3723) and ampH (smlt3721) among the colony morphotypes. The blaL2 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. (D) RT-qPCR analysis of genes, which were identified in the RNA-seq data set. Expression profiles of five different genes (blaL2; ampH; blaL1; smlt0019; smlt0018) were obtained from three independent experiments and analyzed based on the normalized gene expression \left[2^{-\Delta \Delta C_{t}}\right] method, using rpoD and 16S rRNA as internal reference genes.

homolog in SMK279a resulted in an altered transcriptomic profile with respect to phenotypic heterogeneity at a single cell level (Figure 5C; Table 7). The expression of extra copies of the comE homolog in SMK279a reduced the frequency of blaL2-ON cells to 1 % or lower after 32 h of growth. The overexpression of both putative transporter genes (smlt2851; smlt2852) under their native promoter did not alter phenotypic heterogeneous expression of the blaL2 gene (Figure 5D). In corresponding control sets, usually >90% of all cells were in a blaL2-ON mode (Table 7).

Furthermore, we constructed deletion mutants of both loci, smlt1134 (comE homolog) and smlt2851–smlt2852 (Supplementary Figure S4). However, phenotypic heterogeneous gene expression of the blaL2 reporter fusion was not altered in the background of any of these deletion mutants, suggesting that the lack of expression is not linked to heterogeneous expression. Furthermore, the use of a deletion mutant strain with a mutation in the smlt0387 gene (ax21 homolog) did not show phenotypic heterogeneity (Table 7). The Ax21 peptide was suggested to act as a cell–cell signal to regulate a diverse range of functions, including virulence, in Stenotrophomonas (McCarthy et al., 2011). Taken together, the data indicate that the ComE homolog appears to be involved in the regulation of phenotypic heterogeneity of blaL2 gene expression in S. maltophilia.

DISCUSSION

Stenotrophomonas maltophilia encodes in its genome for two β-lactamases, L1 and L2. Expression of both β-lactamases is mainly controlled through the function of the master regulator AmpR in the presence of β-lactam antibiotics. The activity of AmpR is modulated via its activator (anhydro-MurNAc-oligopeptide) and repressor (UDP-MurNAc-oligopeptide) ligands (Dietz et al., 1997; Jacobs et al., 1997; Balcewich et al., 2010). Here we provide the first evidence, that beside these
well-known regulatory elements, phenotypic heterogeneity plays a crucial role in β-lactamase gene expression and has profound effects on colony morphotypes. Non-genetically determined phenotypic heterogeneity has been observed in many bacteria as extensively reviewed (Ackermann, 2015; Grote et al., 2015). Well-studied systems with respect to phenotypic heterogeneity include the phenomenon of persister cells, activation of autoinducer synthase genes, flagella biosynthesis, and others (Anetzberger et al., 2009; Deris et al., 2013; Mulder and Coombes, 2013; Grote et al., 2014; Wang et al., 2014). The term ‘phenotypic heterogeneity’ thereby describes the more general phenomenon of heterogeneous behavior within isogenic cultures and is often linked with the term ‘bet-hedging’, which describes the evolutionary strategy for phenotypic heterogeneity.

The data presented in this study imply that SMK279a forms phenotypic heterogeneous subpopulations with respect to the expression of both β-lactamase genes. Two lines of evidence support this hypothesis. Firstly, cells from small and big colony variants differ mainly in the expression of 12 genes, with both β-lactamase genes \(\text{bla}_{L1}\) and the \(\text{bla}_{L2}\) being among the strongest differentially regulated genes between the two different colony morphotypes (Table 6). Several distinct expression patterns were stable enough to allow differentiation into different colony morphotypes. SEM analyses further revealed that many cells rather grew as long cell chains and produced OMVs in response to β-lactam treatment (Figures 2B,C). This phenomenon, however, was only observed in part of the populations. A second line of evidence resulted from data obtained with promoter fusions of the \(\text{bla}_{L1}\) and \(\text{bla}_{L2}\) genes. High levels of heterogeneous β-lactamase expression were observed in liquid cultures. 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 \(\text{bla}_{L1}\) and \(\text{bla}_{L2}\) expression (Figures 4A–E). Indeed, a weak and constitutive expression of the \(\text{bla}_{L1}\) and \(\text{bla}_{L2}\) genes was present.
β-diversity is reversible but not based on
from one state to another. Furthermore, this phenotypic
population mainly involving genetics switches during alternations
result of heterogeneous behavior within a syngeneic bacterial
These data imply that the colony morphotypes were indeed a
hitherto known genesthat are part of the SMK279a resistome.
S3). Remarkably, none of the SNPs were observed in any of
hypothetical protein Smlt1844B (Supplementary Tables S2 and
regulatory system-sensor histidine kinase Smlt3944 and the
of SNPs were affiliated with genes encoding the two-component
stressful antibiotic challenges.
in untreated cells that were grown in an environment free of
stressful antibiotic challenges.
Furthermore, genome-wide sequence analysis of 24 colony
variants identified a significant number of SNPs. The majority
of SNPs were affiliated with genes encoding the two-component
system-sensor histidine kinase Smlt1394 and the
hypothetical protein Smlt1844B (Supplementary Tables S2 and
S3). Remarkably, none of the SNPs were observed in any of
hitherto known genes that are part of the SMK279a resistome.
These data imply that the colony morphotypes were indeed a
result of heterogeneous behavior within a syngeneic bacterial
population mainly involving genetic switches during alternations
from one state to another. Furthermore, this phenotypic
diversity is 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 Rodero et al., 1996). Our study also revealed that
the phenotypic heterogeneity described herein differs from
previously described slow colony variants (SCVs) isolated
for many pathogens, including S. maltophilia (Proctor et al.,
2006; Anderson et al., 2007). SCVs represent a subpopulation
of slow-growing bacteria with growth characteristics and
morphotypes distinct from those of the wild-type counterparts.
Our data further indicate that ComE is part of a network
involved in the regulation of phenotypic heterogeneity in
SMK279a. Consequently, overexpression of the comE homolog
resulted in a decreased heterogeneity of blaL2 expression.
The comE gene codes for a DNA-binding protein that has
been identified previously as part of a regulatory cascade
responsible for bacterial heterogeneity in the Gram-positive
bacterium Streptococcus pneumoniae (Johnsборг and Havarstein,
2009). It is noteworthy that ComE contains a helix-hairpin-
helix (HhH) motif, which is mainly involved in non-sequence-
specific DNA-binding (Shao and Grishin, 2000). Thereby,
post-transcriptional regulation of the comE gene involves in
part the RNA-binding protein (Hfq) that regulates a wide
variety of cellular responses via interacting with small RNAs
(sRNAs) and mRNAs (Roscetto et al., 2012), indicating the
complex network for comE regulation and its diverse roles
in cells. Within this framework, comE might also be involved
in regulating phenotypic heterogeneity with respect to the
blaL2 gene expression (Figure 5C, Table 7). Finally, it is
important to consider that the underlying mechanism for
modulating phenotypic heterogeneity is complex, involving in

FIGURE 5 | Expression of the comE homolog (smlt1134) under its native promoter. (A) Transcriptome profiles of smlt1134 (black arrow) and flanking genes
smlt1133; smlt1135 (gray arrows) in an ‘ON’ state, 32 h (blue) and an ‘OFF’ state, 27 h (red). The comE homolog was found to be 5.4-fold down-regulated in the
‘ON’ state (32 h). Transcriptome profile images of the leading strand and the lagging strand (generated with the IGB software [Nicol et al., 2009]) were merged and
rearranged on the leading strand for a simplified visualization. (B) Physical map and orientation of the comE homolog and both putative transporter genes in
pBBR1MCS-5. The promoter regions were inserted upstream of P
bla
L2 gene. Here, 5.5
-ON mode. Right and left panels are a bright-field and a fluorescence microscopic image, respectively. (D) Expression of both putative transporter genes
(smlt2851; smlt2852) in SMK279a under their native promoter P
comE. Cells were cultivated for 7 h in LB medium supplemented with 100 μg/ml ampicillin.
Expression of comE clearly affects blaL2-heterogeneous expression resulting in non-fluorescing cells (blaL2-OFF mode), here less than 1% of cells were in
the bla ON mode. Right and left panels are a bright-field and a fluorescence microscopic image, respectively. Images (C) and (D) were recorded as described in
Figure 4.
some cases several genes from the regulatory circuit (Morand and Mühlemann, 2007).

In summary, phenotypic heterogeneity affects β-lactamase expression in SMK279a. We speculate that phenotypic heterogeneity in SMK279a cells provides a selective advantage in natural environments and during the infection of human epithelia to respond to 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 will identify the underlying molecular switches involved in triggering phenotypic heterogeneity in S. maltophilia.

AUTHOR CONTRIBUTIONS

EA is the contributing author. EA, DK and WRS wrote and edited the manuscript. UM provided mutants and edited the manuscript. TK and SN provided the SNP analyses; KF provided the RNA-seq data; IK-M, CS, US also contributed to this research manuscript. TK and SN provided the SNPanalyses; KF provided edited the manuscript. UM provided mutants and edited the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fmicb.2015.01373

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