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TonB dependent uptake of β-lactam antibiotics in the opportunistic human pathogen *Stenotrophomonas maltophilia*.

Running Title: TonB dependent β-lactam uptake in *S. maltophilia*

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Summary

The β-lactam antibiotic ceftazidime is one of only a handful of drugs with proven clinical efficacy against the important opportunistic human pathogen *Stenotrophomonas maltophilia*. Here, we show that mutations in the energy transducer TonB, encoded by *smlt0009* in *S. maltophilia*, confer ceftazidime resistance and that *smlt0009* mutants have reduced uptake of ceftazidime. This breaks the dogma that β-lactams enter Gram-negative bacteria only by passive diffusion through outer membrane porins. We also show that ceftazidime-resistant TonB mutants are cross-resistant to fluoroquinolone antimicrobials and a siderophore-conjugated lactivicin antibiotic designed to target TonB-dependent uptake. This implies that attempts to improve penetration of antimicrobials into *S. maltophilia* by conjugating them with TonB substrates will suffer from the fact that β-lactams and fluoroquinolones co-select resistance to these novel and otherwise promising antimicrobials. Finally, we show that *smlt0009* mutants already exist amongst *S. maltophilia* clinical isolates, and have reduced susceptibility to siderophore-conjugated lactivicin, despite the in vitro growth impairment seen in *smlt0009* mutants selected in the laboratory.

Introduction

Stenotrophomonas maltophilia is a non-fermenting Gram-negative bacillus prevalent in the environment and an important opportunistic human pathogen. In the US, it caused 4.3% of all Gram-negative bacterial infections in intensive care units between 1993 and 2004 (Lockhart et al, 2007). For pneumonia, in the US, 3.3% of infections in hospitalised patients between 2004 and 2008 were caused by *S. maltophilia* (Jones 2010). Attributable mortality rates can be high for these serious infections, up to 37.5% (Falagas et al., 2009). It also causes a wide range of other infections and colonises the lungs of >10% of adults living with Cystic Fibrosis (Brooke, 2012). *S. maltophilia* clinical isolates are resistant to almost all β-lactam antibiotics because of the production of two β-lactamases: L1, a subclass B3 metallo-β-lactamase and L2, a class A extended spectrum β-lactamase (Gould *et al*., 2006). Production of L1 and L2 is co-ordinately controlled by AmpR, a LysR-type transcriptional activator and induced during β-lactam challenge of cells (Okazaki & Avison, 2008). Despite this, many *S. maltophilia* clinical isolates remain susceptible to the β-lactam ceftazidime because it is a relatively poor substrate for these two enzymes (Calvopiña *et al*., 2017). However, mutants that have acquired ceftazidime resistance can easily be identified in the laboratory, and ceftazidime resistant isolates are commonly encountered in the clinic. In many cases, these mutants hyperproduce L1 and L2 (Okazaki & Avison, 2008, Talfan *et al*., 2013, Calvopiña & Avison, 2018) but we have previously identified ceftazidime resistant mutants that did not hyperproduce β-lactamase (Gould & Avison, 2006). It was hypothesised that these mutants might have reduced accumulation of ceftazidime (Talfan *et al*., 2013). The primary non-β-lactamase mediated mechanisms of β-lactam resistance in similar non-fermenting bacteria such as *Pseudomonas aeruginosa* are increased efflux and reduced outer membrane permeability due to a reduction in the production of outer membrane porins (Castanheira *et al*., 2014). In Gram negative bacteria, tripartite outer membrane porins are considered the only site of entry for β-lactams, and reduced porin levels can reduced β-lactam susceptibility in many species (Pfeifer *et al*., 2010). No other way of entry has

previously been suggested unless the β-lactam is conjugated to a catechol siderophore, in which case a TonB-dependent uptake system is used (Livermore, 1987). The aim of the work reported below was to identify the mechanism of non-β-lactamase mediated ceftazidime resistance in *S. maltophilia*. In so doing, we have broken the dogma that states that β-lactams can only enter Gram-negative bacteria through trimeric outer membrane porins via passive diffusion.

Results and Discussion

Disrupting a proline-rich region in the S. maltophilia TonB energy transducer Smlt0009 is associated with ceftazidime resistance.

In *S. maltophilia*, ceftazidime resistant mutants can be selected at high frequency (Avison *et al*., 2002). Around 50% of ceftazidime resistant mutants selected from *S. maltophilia* clinical isolate K279a do not hyperproduce β-lactamase (Talfan *et al*., 2013). To identify the mechanism involved, we selected additional ceftazidime resistant mutants from K279a. Mutants expressing basal β-lactamase activity were isolated at a frequency expected from our earlier work. Of these, mutants M1 and M52 are exemplars. They are not β-lactamase hyperproducers, for example in comparison with a K279a *mpl* mutant, which does hyperproduce β-lactamases as recently described (Calvopiña & Avison, 2018) (**Table 1**). Nevertheless, β-lactam susceptibility was generally reduced in M1 and M52, as shown by an observed reduction in the inhibition zone diameter around various β-lactam discs (**Fig. 1A**).

Whole genome sequencing was performed to identify the mutations present in M1 and M52. Only one gene was found to be mutated in each. It was the same in both: *smlt0009*, annotated in the K279a genome sequence as encoding a 'putative proline-rich TonB energy transducer protein' (Crossman *et al*., 2008). The mutation was confirmed using high fidelity PCR sequencing. In both M1 and M52, a proline rich region in Smlt0009 situated at around 70 amino acids into the 222 amino acid protein was shortened but there was no frameshift

(**Fig. 2A**). Assuming this shortening impairs protein function, and to confirm the role of this impairment in ceftazidime resistance, *smlt0009* was insertionally inactivated in K279a using a suicide gene replacement methodology. K279a Δ*smlt0009* was confirmed to be ceftazidime resistant (**Table 1**) and to have generally reduced β-lactam susceptibility (**Fig. 1A**). Complementation of the *smlt0009* mutation in all these mutants using a plasmid version of the wild-type gene *in trans* completely reversed ceftazidime resistance, according to disc testing (**Fig 1B**), confirming that this phenotype is caused by the *smlt0009* mutation in the mutants tested.

Disruption of smlt0009 leads to increased siderophore production and reduced susceptibility to siderophore-conjugated antimicrobials.

TonB-dependent uptake systems are best known for their roles in iron-siderophore import. This process requires a complex formed by a proline rich TonB energy transducer protein (Smlt0009 in this case) with ExbB (Smlt0010) and ExbD (Smlt0011), which interacts with one or more outer membrane TonB-dependent transporters (TBDTs). Specificity occurs because the TonB energy transducer only interacts with TBDTs that have bound substrate (Wilson *et al*., 2016, Klebba, 2016). In this way, proton motive force, generated in the inner membrane, is transduced by ExbBD to cause rotational motion of the N-terminus of the TonB energy transducer and specific opening of any TBDT that has bound ligand, ultimately driving ligand import (Klebba, 2016).

To understand more about the phenotype of M1 and M52, whole envelope proteomics was performed in comparison with K279a. This confirmed that the β-lactamases L1 and L2 are not overproduced. However, 162 proteins were identified that are significantly up or down regulated in both M1 and M52 relative to K279a; 83 are downregulated in both and 79 upregulated in both (**Table S1**). Proteomics for K279a Δ*smlt0009* (**Table S2**) confirmed total loss of Smlt0009; Amongst proteins upregulated in M1, M52 (and in K279a Δ*smlt0009*) were

proteins with the Uniprot accession numbers B2FHQ4, encoded by *entB*, *smlt2820* (**Fig. 3A**) and accession numbers B2FRE3-7encoded by the *smlt2053-57* (*fep*) operon (**Tables S1; S2**). These upregulated Fep proteins are involved in catechol siderophore production in *S. maltophilia* (Nas & Cianciotto, 2017) and siderophore production was found to be significantly increased in M1, M52 and K279a Δ*smlt0009* relative to K279a, as predicted from the proteomics (**Figure 3B, 3C**). The most likely explanation for this is that disruption of Smlt0009 reduces iron-siderophore uptake into *S. maltophilia* K279a, which responds to the resulting iron starvation by increases siderophore production in an attempt to obtain more iron.

Siderophore-conjugation has been used as a way of increasing the penetration of antimicrobials into Gram-negative bacteria by hijacking the TonB dependent uptake system (Kline *et al*., 2000, Choi & McCarthy, 2018). Indeed, recently we have shown that siderophore conjugation of the γ-lactam antibiotic lactivicin (to create LTV-17) dramatically improves potency against *S. maltophilia* (Calvopiña *et al*., 2016). As expected given TonB dependence of LTV-17 uptake in other species (Starr *et al*., 2014), ceftazidime resistant *smlt0009* (TonB) mutants M1 and M52 also have reduced susceptibility to LTV-17, as does K279a Δ*smlt0009* where in each case the MIC of LTV-17 increased to ≥0.25 μg.mL^{-1} (**Table 1**). A single-step mutant (KLTV) with reduced susceptibility to LTV-17 was next selected from K279a and the mutant is also resistant to ceftazidime (**Table 1**) and has reduced susceptibility to all tested β-lactams (**Fig. 1A**). KLTV whole envelope proteomics showed very similar changes to those observed in M1 and M52 (**Table S3**). In KLTV, like M1 and M52, there is upregulation of the siderophore biosynthesis enzymes and increased siderophore production (**Fig. 3**). WGS confirmed shortening of the proline-rich region in Smlt0009 in KLTV (**Fig. 2A**). TonB mutations are known to reduce susceptibility to siderophore conjugated antimicrobials in other species but have never previously been reported to affect β-lactam susceptibility (Hassett *et al*., 1996, Tomaras *et al*., 2013, Moynie *et al*., 2017). Interestingly, *S. maltophilia smlt0009* mutants do not have reduced

susceptibility to the non-siderophore conjugated parent lactivicin, LTV-13 (**Table 1**), even though this γ-lactam is structurally related to the β-lactams (Starr *et al*., 2014).

Pleotropic effects of Smlt0009 disruption in S. maltophilia including reduced uptake of a fluorescent dye, reduced susceptibility to fluoroquinolones and slower growth.

In some bacteria, TonB complexes participate in the import of TBDT-dependent ligands in addition to iron-siderophore complexes. *S. maltophilia* Smlt0009 shares 50% identity with the TonB energy transducer protein from the closely related *Xanthomonas campestris*, a species where only 15% of TBDTs are involved in iron-siderophore-complex import (Schauer *et al*., 2008). Interestingly, of 162 proteins differently regulated in *S. maltophilia* K279a M1 and M52, nineteen are putative TonB-dependent TBDT proteins (**Table S1**). Apparently, M1 and M52 are responding to a disruption of TonB-dependent energy transduction, which is associated with the import of many diverse ligands. In support of a more general role for TonB in *S. maltophilia*, envelope permeability, measured via the uptake of a fluorescent Hoescht dye, was found to be significantly reduced in *smlt0009* mutants relative to K279a. Interestingly, mutant M1, with the smallest deletion in the Smlt0009 proline-rich region had the least reduction in envelope permeability (**Fig. 2A, 2B**). Assays of susceptibility to non-β-lactam antimicrobials against K279a and the *smlt0009* mutants identified reduced fluoroquinolone susceptibility, though other classes of antimicrobial were not affected by disruption of Smlt0009 (**Fig. 2C**). Hence a TonB-dependent system is implicated in the uptake of fluoroquinolones as well as β-lactams in *S. maltophilia*.

Given the pleotropic effects seen and the large number of putative TonB-dependent TBDTs encoded by *S. maltophilia*, we suspected that there would be a growth impairment for the *smlt0009* mutants even in rich medium. Growth curve assays confirmed this, with K279a growing significantly better than all mutants ($p<0.0001$ based on a T-test of OD_{600} at 12 h) and, as with the impairment of envelope permeability to the fluorescent dye, mutant M1, with

the smallest deletion of Smlt0009 suffered from significantly less impairment of growth (p<0.0001 at 12 h) than the other mutants (**Fig. 2A, 2D**).

Reduced ceftazidime uptake into S. maltophilia smlt0009 mutants and existence of these mutants amongst S. maltophilia clinical isolates.

Findings reported above led us to hypothesise that in *S. maltophilia*, uptake of β lactams is TonB dependent. Thus, mutations in the proline rich region of Smlt0009 reduce energy dependent ceftazidime uptake and confer clinically important ceftazidime resistance. This is the first time that β lactam entry via a TonB-dependent mechanism has been proposed in any bacterium. However, it is interesting to note that, unlike all other pathogens studied previously, outer membrane passive diffusion porin loss has never been found to be involved in β-lactam resistance in *S. maltophilia* (Sanchez, 2015) which supports the existence of a novel import mechanism in this species.

To test our hypothesis that disruption of Smlt0009 reduces ceftazidime uptake in *S. maltophilia*, we incubated K279a and the various *smlt0009* mutants with ceftazidime and then measured the concentration of ceftazidime remaining in the growth medium by using an *Escherichia coli* killing assay where the lower the concentration of ceftazidime, the smaller the zone of killing seen on a lawn of *E. coli* DH5α. As can be seen (**Fig. 4**) the concentration of ceftazidime outside of K279a is significantly less than the concentration outside of the *smlt0009* mutants after 24 h of incubation, confirming that ceftazidime enters K279a more readily than the *smlt0009* mutants. This adds strong support to our hypothesis that ceftazidime uptake is TonB dependent in *S. maltophilia*. Identifying which of the >20 TBDTs seen in *S. maltophilia* K279a is the one responsible for ceftazidime uptake will form the basis of future work.

Finally, we turned to our world-wide collection of 22-phylogenetic group A *S. maltophilia* clinical isolates (Gould *et al*., 2006) against which we measured the MICs of LTV-17 and

LTV-13 (**Table 2**). One isolate, number 31, stood out as having reduced susceptibility to LTV-17 (MIC = 0.25 μg.mL^{-1}) without altered susceptibility to LTV-13, a phenotype shared with K279a *smlt0009* mutants (**Tables 1, 2**). Of the tested clinical isolates, thirteen had the same predicted sequence for Smlt0009 as K279a, based on PCR sequencing; eight isolates had N169S plus A209T variants of this sequence, but given it is so common this is highly likely to be random genetic drift. Isolate number 31, with reduced LTV-17 susceptibility had an insertion of a single proline in the proline-rich region of Smlt0009 (**Table 2**).

According to our records, isolate number 31 was from a patient being treated in an intensive care unit in a Brazilian hospital in 2003. It was collected as part of the SENTRY antimicrobial surveillance programme (Toleman *et al*., 2007). Remarkably, isolate number 31 also carries an *ampD* loss of function mutation and hyper-produces both the L1 and L2 β-lactamases, which is enough to give pan β-lactam resistance without any additional mechanism (Gould *et al*., 2006, Talfan *et al*., 2013, Calvopina *et al*., 2017). We have reported, however, that isolate number 31 is unusual in its resistance to ceftazidime/β-lactamase inhibitor combinations (Calvopina *et al*., 2017), so combination therapy including a β-lactam/β-lactamase inhibitor might have selected for this mutation even in a background of β-lactamase hyper-production. Whatever the specifics of selection in this case, we have demonstrated the existence of *S. maltophilia* clinical isolates with mutations in the TonB energy transducer Smlt0009, which have reduced susceptibility to β-lactams, fluoroquinolones and siderophore-conjugated antimicrobials.

Conclusions

Examples of outer membrane tripartite porin loss leading to reduced β-lactam susceptibility in Gram-negative bacteria are numerous. They include important carbapenem resistance mutations such as OprD loss in *P. aeruginosa* (Huang *et al*., 1992) and OmpK36 loss in *Klebsiella pneumoniae* (Findlay *et al*., 2012). However, there is redundancy in β-lactam entry

porins, and they can also be highly specific. For example, OmpK36 loss causes meropenem resistance, but does not confer resistance to the closely related carbapenem, imipenem (Findlay *et al*., 2012). Because of the importance of carbapenems as last-resort antimicrobials, porin loss conferring carbapenem resistance in clinical isolates was quickly identified but *S. maltophilia* is intrinsically resistant to all carbapenems because of the production of an inducible carbapenemase, named L1 (Calvopiña *et al*., 2017). Indeed, there are only six agents for which the US Clinical and Laboratory Standards Institute provide resistance/susceptibility breakpoints; indicating potential clinical efficacy (CLSI, 2017): two β-lactams, ceftazidime and ticarcillin-clavulanate; a fluoroquinolone, levofloxacin; a tetracycline derivative, minocycline; a phenicol, chloramphenicol, and the drug of choice (and the only agent for which breakpoints are provided by the European Committee on Antimicrobial Susceptibility Testing) trimethoprim-sulfamethoxazole. We and others have identified mechanisms of resistance, including efflux pumps, modifying enzymes and bypass reactions, relevant to all these agents. Porin loss has not been found as a mechanism in in any case (Sanchez, 2015). Because of this, we have long suspected that antimicrobial entry into *S. maltophilia* may be atypical and porins may not be as important as in other clinically important species.

The work presented here confirm for the first time that β-lactam antibiotics can enter Gram-negative bacteria via a TonB-dependent mechanism. We also present evidence that this is true of fluoroquinolones. Whilst we have not identified the outer membrane TBDT(s) responsible for uptake, the fact that disruption of TonB reduced ceftazidime uptake to such a degree that mutants become clinically resistant, shows how important TonB-dependent uptake is to the activity of ceftazidime against this human pathogen. Since TonB-dependent uptake is very important, at the very least for iron-siderophore uptake, it was expected that disruption of TonB would cause a growth defect, and this was confirmed, even in complex medium. However, our identification of an *smlt0009* (TonB) mutant clinical isolate (isolate 31) shows that such mutants can still cause infections. It is interesting to note that isolate 31,

which is resistant to 5/6 available anti-*S. maltophilia* antimicrobials listed above, except for minocycline (Calvopina *et al*., 2016), only has a very slight modification to the Smlt0009 proline rich region: a single residue insertion. Given that the smaller the change to Smlt0009, the lower the effect on growth defect (e.g. see mutant M1 versus the others in **Fig 2D**) this hints at a trade-off between reduced entry of antimicrobials whilst allowing sufficient entry of iron-siderophores and other TonB-dependent substrates to minimise the resulting attenuation of growth.

The fact that *smlt0009* mutants exist in the clinic does not bode well for the long-term activity of catechol siderophore-conjugated antimicrobials, which are designed to target TonB-dependent uptake, including those reported to have very good activity against extensively drug resistant *S. maltophilia* isolates in animal infection models (Chen *et al*., 2019). Indeed, our finding that catechol siderophore-conjugated lactivicin has markedly reduced activity against *smlt0009* mutants, including clinical isolate 31 (**Table 1, 2**), confirms that resistance already exists in the clinical *S. maltophilia* population. In other species, TonB mutations are known to confer resistance to siderophore-conjugated antimicrobials (Tomaras *et al*., 2013), but the fact that β-lactams are shown here to be TonB dependent substrates, and it is likely that fluoroquinolones are as well, means that in *S. maltophilia*, unlike other species, TonB mutation is not only selected by the use of siderophore-conjugated antimicrobials, but also by two antimicrobial classes that have been extensively used for decades. Accordingly, as well as breaking the dogma that β-lactams only enter Gram-negative bacteria via trimeric porins, the work presented here is clinically important, both for explaining resistance to existing antimicrobials, and to consider when developing new ones.

Experimental Procedures

Bacterial isolates and materials

S. maltophilia clinical isolates used originated from the SENTRY antimicrobial resistance survey and have been previously described (Toleman *et al*., 2007) plus isolate K279a (Avison *et al*., 2000). All growth media were from Oxoid. Chemicals were from Sigma, unless otherwise stated. LTV-13 was re synthesized according to the literature protocol (Starr *et al*., 2014) and kindly provided by Prof. C. Schofield, University of Oxford. LTV-17 was kindly supplied by Pfizer.

Selection of resistant mutants

K279a ceftazidime resistant mutants were selected after exposure of lawns of bacteria to 30 µg ceftazidime discs on Muller-Hinton Agar (MHA) by picking the colonies within the zone of inhibition after using a bacterial suspension that was 100-fold higher than the recommended value according to the CLSI guidelines (CLSI, 2012). Mutants with reduced susceptibility to LTV-17 were selected by plating 100 µL of an overnight culture grown in Nutrient Broth (NB) on MHA containing increasing concentrations of LTV-17. Colonies from the highest LTV-17 concentration plate where growth was seen were picked.

β-lactamase assays

100 µL of an overnight NB culture was diluted in 10 mL of NB and incubated at 37°C with shaking until OD_{600} was 0.4. Cells were pelleted by centrifugation (4,000 x *g*, 10 min) and pellets resuspended in 100 µL of BugBuster (Ambion). Pellets were transferred to 1.5 mL microtube (Eppendorf) before rocking at 70 rpm for 30 min at room temperature. Cell debris and unlysed cells were pelleted by centrifugation (13,000 x *g*, 5 min) and the supernatant retained as a source of crude cell protein. Protein concentrations in cell extracts were determined using the BioRad protein assay dye reagent concentrate according to the manufacturer's instructions. β-Lactamase activity in crude cell extracts was determined using a POLARstar Omega plate spectrophotometer (BMG Labtech). Nitrocefin (40 µM)

solution was used as a substrate, prepared in 0.2 µm syringe-filtered assay buffer (60 mM $Na_2HPO_4 \cdot 7H_2O$ pH 7.0, 40 mM $NaH_2PO_4 \cdot H_2O$, 10 mM KCl, 1 mM $MgSO_4 \cdot 7H_2O$, 100 µM $ZnCl_2$). Nitrocefin hydrolysis assays were performed in Corning Costar 96-well flat-bottomed cell culture plates with a combination of 1 µL of cell extract and 179 µL of nitrocefin solution. Product accumulation was measured at 482 nm for 5 min or until the end of the linear phase of the reaction. Final β-lactamase activity ($nmol.min^{-1}.\mu g^{-1}$ of protein in cell extract) was calculated via change in absorbance per minute taken from the linear phase of the reaction in Omega Data Analysis. An extinction coefficient of 17400 $M^{-1}cm^{-1}$ was used for nitrocefin. The path length for liquid in a well in the 96-well plate was set at 0.56 cm.

Determining minimal inhibitory concentrations (MICs) of antimicrobials and disc susceptibility testing

The CLSI protocol was followed for disc susceptibility testing (CLSI, 2006). The clearance zone was measured after 20 h of incubation and bacteria reported as susceptible or resistant according to CLSI published breakpoints, where available (CLSI, 2017).

MICs were determined using CLSI broth microtitre assays (CLSI, 2012) and interpreted using published breakpoints (CLSI, 2017). Briefly, a PBS bacterial suspension was prepared to obtain a stock of OD_{600}=0.01. The final volume in each well of a 96-well cell culture plate (Corning Costar) was 200 µL and included 20 µL of the bacterial suspension. Bacterial growth was determined after 20 h of incubation by measuring OD_{600} values using a POLARstar Omega spectrophotometer (BMG Labtech).

Whole genome sequencing to Identify mutations

Whole genome resequencing was performed by MicrobesNG (Birmingham, UK) on a HiSeq 2500 instrument (Illumina, San Diego, CA, USA). Reads were trimmed using Trimmomatic (Bolger *et al*., 2014) and assembled into contigs using SPAdes 3.10.1 (http://cab.spbu.ru/software/spades/). Assembled contigs were mapped to reference genome

for *S. maltophilia* K279a (Crossman *et al*., 2008) obtained from GenBank (accession number NC_010943) using progressive Mauve alignment software (Darling *et al*., 2010).

Mutations were checked by PCR using Phusion High Fidelity DNA Polymerase (New England Biolabs). To generate template DNA, a bacterial colony was resuspended in 100 µL of molecular biology grade water and heated at 100°C for 5 min. The sample was centrifuged at 13000 rpm for 5 min. PCR reactions were set up using 5 µL of 5X Phusion GC Buffer, 0.5 µL of dNTPs (10 mM), 1.25 µL of forward primer (10 µM), 1.25 µL of reverse primer (10 µM), 0.75 µL of DMSO, 0.25 µL of Phusion DNA Polymerase, 1 µL of DNA template, and 15 µL of molecular biology grade water. The cycling conditions were the following: 1 cycle of 98°C for 30 s, 30 cycles of: 98°C for 10 s, 60°C for 15 s, and 72°C for 30 s, 1 cycle of 72°C for 10 min for final extension.

The primers used were: *smlt0009* F 5'-GTGTGAAGAACCAGGCTGATGCCA-3' and *smlt0009* R 5'-AGGGTGTAGCTAAGCTAAACAAT-3'. PCR products were purified using the QIAquick PCR Purification Kit (Qiagen) according to the manufacturer's instructions. DNA concentration of purified samples was quantified using NanoDrop Lite spectrophotomer (Thermo Scientific). PCR products were sequenced by Eurofins. Sequences obtained were analysed with ClustalW OMEGA or MultiAlignPro. Alignments were represented using ESPript 3.0.

Insertional inactivation of smlt0009

The K279a Δ*smlt0009* mutant was constructed by gene inactivation mediated by the pKNOCK suicide plasmid (Alexeyev, 1999). The *smlt0009* DNA fragment was amplified with Phusion High-Fidelity DNA Polymerase (NEB, UK) from *S. maltophilia* genomic DNA by using primers *smlt0009* KO FW (5′-GTGAAGAATCTGTCGCCGC-3′) and *smlt0009* KO RV (5′-GGATCACTTCGCCCTGGATA-3′). The PCR product was ligated into the pKNOCK-GM at SmaI site. The recombinant plasmid was then transferred into wild-type *S. maltophilia* cells by conjugation. The mutant was selected for gentamicin resistance and the mutation

was confirmed by PCR using primers *smlt0009* full length FW (5'-AAAGAATTCAGTAGGAATAACGCCTGAATGC-3') and *smlt0009* full length RV (5'-AAAGAATTCTGACGCTTACCTTTGTTGTGTG-3').

Cloning smlt0009 and trans-complementation

The *smlt0009* gene and its promoter was amplified with Phusion High-Fidelity DNA Polymerase (NEB, UK) from *S. maltophilia* genomic DNA by using primers *smlt0009* full length FW (5'-AAAGAATTCAGTAGGAATAACGCCTGAATGC-3') and *smlt0009* full length RV (5'-AAAGAATTCTGACGCTTACCTTTGTTGTGTG-3'). The PCR product was ligated into the vector pBBR1MCS at a SmaI site. The recombinant plasmid was then transferred into *S. maltophilia* cells by conjugation from *E. coli* SM10. The transconjugants were selected for chloramphenicol resistance and the presence of plasmids was confirmed by PCR using primers M13 FW (5'- GTAAAACGACGGCCAGT-3') and M13 RV (5'-CAGGAAACAGCTATGAC-3').

Proteomics

500 µL of an overnight NB culture were transferred to 50 mL NB and cells were grown at 37˚C to 0.6 OD_{600}. Cells were pelleted by centrifugation (10 min, 4,000 × *g*, 4°C) and resuspended in 30 mL of 30 mM Tris-HCl, pH 8 and broken by sonication using a cycle of 1 s on, 0.5 s off for 3 min at amplitude of 63% using a Sonics Vibracell VC-505TM (Sonics and Materials Inc., Newton, Connecticut, USA). The sonicated samples were centrifuged at 8,000 rpm (Sorval RC5B PLUS using an SS-34 rotor) for 15 min at 4°C to pellet intact cells and large cell debris; For envelope preparations, the supernatant was subjected to centrifugation at 20,000 rpm for 60 min at 4°C using the above rotor to pellet total envelopes. To isolate total envelope proteins, this total envelope pellet was solubilised using 200 µL of 30 mM Tris-HCl pH 8 containing 0.5% (w/v) SDS.

Protein concentrations in all samples were quantified using Biorad Protein Assay Dye Reagent Concentrate according to the manufacturer's instructions. Proteins (5 µg/lane for

envelope protein analysis) were separated by SDS-PAGE using 11% acrylamide, 0.5% bis-acrylamide (Biorad) gels and a Biorad Min-Protein Tetracell chamber model 3000X1. Gels were resolved at 200 V until the dye front had moved approximately 1 cm into the separating gel. Proteins in all gels were stained with Instant Blue (Expedeon) for 20 min and de-stained in water.

The 1 cm of gel lane was subjected to in-gel tryptic digestion using a DigestPro automated digestion unit (Intavis Ltd). The resulting peptides from each gel fragment were fractionated separately using an Ultimate 3000 nanoHPLC system in line with an LTQ-Orbitrap Velos mass spectrometer (Thermo Scientific). In brief, peptides in 1% (v/v) formic acid were injected onto an Acclaim PepMap C18 nano-trap column (Thermo Scientific). After washing with 0.5% (v/v) acetonitrile plus 0.1% (v/v) formic acid, peptides were resolved on a 250 mm × 75 μm Acclaim PepMap C18 reverse phase analytical column (Thermo Scientific) over a 150 min organic gradient, using 7 gradient segments (1-6% solvent B over 1 min, 6-15% B over 58 min, 15-32% B over 58 min, 32-40% B over 5 min, 40-90% B over 1 min, held at 90% B for 6 min and then reduced to 1% B over 1 min) with a flow rate of 300 nL/min. Solvent A was 0.1% formic acid and Solvent B was aqueous 80% acetonitrile in 0.1% formic acid. Peptides were ionized by nano-electrospray ionization MS at 2.1 kV using a stainless-steel emitter with an internal diameter of 30 μm (Thermo Scientific) and a capillary temperature of 250°C. Tandem mass spectra were acquired using an LTQ-Orbitrap Velos mass spectrometer controlled by Xcalibur 2.1 software (Thermo Scientific) and operated in data-dependent acquisition mode. The Orbitrap was set to analyze the survey scans at 60,000 resolution (at m/z 400) in the mass range m/z 300 to 2000 and the top twenty multiply charged ions in each duty cycle selected for MS/MS in the LTQ linear ion trap. Charge state filtering, where unassigned precursor ions were not selected for fragmentation, and dynamic exclusion (repeat count, 1; repeat duration, 30 s; exclusion list size, 500) were used. Fragmentation conditions in the LTQ were as follows: normalized collision energy,

40%; activation q, 0.25; activation time 10 ms; and minimum ion selection intensity, 500 counts.

The raw data files were processed and quantified using Proteome Discoverer software v1.4 (Thermo Scientific) and searched against the UniProt *S. maltophilia* strain K279a database (4365 protein entries; UniProt accession UP000008840) using the SEQUEST (Ver. 28 Rev. 13) algorithm. Peptide precursor mass tolerance was set at 10 ppm, and MS/MS tolerance was set at 0.8 Da. Search criteria included carbamidomethylation of cysteine (+57.0214) as a fixed modification and oxidation of methionine (+15.9949) as a variable modification. Searches were performed with full tryptic digestion and a maximum of 1 missed cleavage was allowed. The reverse database search option was enabled and all peptide data was filtered to satisfy false discovery rate (FDR) of 5 %. The Proteome Discoverer software generates a reverse “decoy” database from the same protein database used for the analysis and any peptides passing the initial filtering parameters that were derived from this decoy database are defined as false positive identifications. The minimum cross-correlation factor filter was readjusted for each individual charge state separately to optimally meet the predetermined target FDR of 5 % based on the number of random false positive matches from the reverse decoy database. Thus, each data set has its own passing parameters. Protein abundance measurements were calculated from peptide peak areas using the Top 3 method (Silva *et al*., 2006) and proteins with fewer than three peptides identified were excluded. The proteomic analysis was repeated three times for each parent and mutant strain, each using a separate batch of cells. Data analysis was as follows: all raw protein abundance data were uploaded into Microsoft Excel. Raw data from each sample were normalised by division by the average abundance of all 30S and 50S ribosomal protein in that sample. A one-tailed, unpaired T-Test was used to calculate the significance of any difference in normalised protein abundance data in the three sets of data from the parent strains versus the three sets of data from the mutant derivative. A p-value of <0.05 was considered significant. The fold change in abundance for each protein in the mutant

compared to its parent was calculated using the averages of normalised protein abundance data for the three biological replicates for each strain.

Siderophore Detection

100 µL of an overnight culture in Cation-Adjusted Muller-Hinton Broth (CA-MHB) was used to set up a fresh subculture in 10 mL of CA-MHB which was then incubated until the OD_{600} reached 0.5. Cells were centrifuged (4,000 x *g*, 10 min) and the resulting pellet was resuspended in 10 mL of Phosphate Buffered Saline (PBS) and centrifuged again (4,000 x *g*, 10 min). The supernatant was discarded, and the pellet was again resuspended in fresh PBS (10 mL) and centrifuged (4,000 x *g*, 10 min). This washed bacterial pellet was then diluted in PBS to prepare a bacterial suspension of OD_{600} 0.2. Ten microliters of the bacterial suspension were spotted on Chrome Azurol S (CAS) agar. CAS agar was made up mixing up 90 mL of MHA and 10 mL of freshly made CAS solution. 100 mL of the CAS solution was made up based on the following description: 60.5 mg of CAS in 50 mL of water, 72.9 mg of hexadecyltrimethyl ammonium bromide in 40 mL of water, and 10 mL of 1 mM $FeCl_3$, 10 mM HCl) (Garcia *et al*., 2012). CAS agar control included 100 µM $FeCl_3$ where no colour change was expected.

Fluorescent Hoescht (H) 33342 dye accumulation assay

Envelope permeability in living bacteria was tested using a dye accumulation assay protocol (Coldham *et al*., 2010) where the dye only fluoresces if it crosses the entire envelope and interacts with DNA. Overnight cultures (in NB) at 37°C were used to prepare NB subcultures, which were incubated at 37°C until a 0.6 OD_{600} was reached. Cells were pelleted by centrifugation (4000 rpm, 10 min) (ALC, PK121R) and resuspended in 500 µL of PBS. The optical densities of all suspensions were adjusted to 0.1 OD_{600}. Aliquots of 180 µL of cell suspension were transferred to a black flat-bottomed 96-well plate (Greiner Bio-one, Stonehouse, UK). Eight technical replicates, for each strain tested, were in each column of the plate. The plate was transferred to a POLARstar spectrophotometer (BMG Labtech) and

incubated at 37°C. Hoescht dye (H33342, 250 µM in water) was added to bacterial suspension of the plate using the plate-reader's auto-injector to give a final concentration of 25 µM per well. Excitation and emission filters were set at 355 nm and 460 nm respectively. Readings were taken in intervals (cycles) separated by 150 s. 31 cycles were run in total. A gain multiplier of 1300 was used. Results were expressed as absolute values of fluorescence versus time.

Growth curves

OD_{600} measurements of bacterial cultures were performed using a Spectrostar Nano Microplate Reader (BMG, Germany) in Costar Flat Bottom 96-well plates. Overnight cultures (in NB) were adjusted to OD_{600} = 0.01 and 200 µL of the diluted culture were taken to the plate together with a blank, NB. The plate was incubated at 37°C with double orbital shaking and OD_{600} was measured every 10 min for 24 h.

Indirect ceftazidime uptake assay

Overnight cultures of the strains being assayed for ceftazidime uptake were sub-cultured in NB to 0.5-0.7 OD_{600} and bacteria pelleted by centrifugation. Pellets were resuspended in NB to a density of 1.0 OD_{600} and ceftazidime (5 $\mu g.mL^{-1}$ or 10 $\mu g.mL^{-1}$) was introduced. After 24 h incubation at 37°C with shaking, bacteria were pelleted and 10 µL of filter sterilized (0.2 µm pore) supernatant were spotted on a freshly spread lawn of *E. coli* DH5α, using MHA, and made as if for disc susceptibility testing (CLSI 2006). The plate was incubated for 20 h at 37°C and zones of inhibition were measured using a ruler. To calibrate the assay, relating inhibition zone diameter to ceftazidime concentration, fixed concentrations of ceftazidime were made in NB and spotted onto the *E. coli* lawn and incubated and the inhibitions zones measured as above.

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Author Contributions

Conception and design: MBA, KC.

Acquisition of data: KC, PD, KJH supervised by MBA.

Analysis and Interpretation of data: ALL AUTHORS.

Drafting the manuscript; ALL AUTHORS.

Conflicts of Interest

The authors declare that they have no conflict of interest.

Tables

Table 1. Comparison of MICs (μg.mL^{-1}) of ceftazidime and lactivicin derivatives against *S. maltophilia* ceftazidime and lactivicin mutants and the levels of β-lactamase produced.

Strain	Mean β-lactamase activity ±SEM	MIC of Ceftazidime	MIC of LTV-13	MIC of LTV-17
K279a	0.02±0.004	4	64	0.03
M1	0.02±0.002	256	128	0.5
M52	0.04±0.013	256	128	0.5
KLTV	0.05±0.005	256	64	0.25
K279a Δ*smlt0009*	0.01±0.002	128	128	0.5
K279a *mpl*	0.72 ± 0.01	64	ND	ND

β-Lactamase activity was determined using nitrocefin hydrolysis (nmol.min^{-1}.μg^{-1}) in cell extracts from bacteria grown in the absence of antibiotic. Data for the β-lactamase hyper-producing K279a *mpl* are taken from Calvopiña & Avison, 2018.

Shaded MIC values represent a more than two doubling reduced susceptibility in reference to K279a and in the case of ceftazidime, shading show clinical resistance according to CLSI breakpoints. ND, Not Determined

513 **Table 2. MICs (µg.mL^{-1}) of lactivicin derivatives against *S. maltophilia* clinical isolates**

514 **with different Smlt0009 sequences**

Isolate	Smlt0009 sequence (as compared with K279a)	LTV-13 MIC	LTV-17 MIC
K279a	Wild-type (by definition)	64	0.03
10	N169S, A209T	128	0.03
12	Wild-type	64	0.03
14	Wild-type	64	0.03
16	Wild-type	64	0.03
17	Wild-type	64	0.03
19	Wild-type	128	0.06
21	Wild-type	64	0.03
22	Wild-type	128	0.03
23	N169S, A209T	256	0.03
26	N169S, A209T	64	0.03
27	N169S, A209T	128	0.06
28	Wild-type	64	0.03
29	Wild-type	64	0.03
30	N169S, A209T	128	0.03
31	Insertion of Proline between P69 and P70	128	0.25
32	N169S, A209T	128	0.13
35	N169S, A209T	64	0.06
36	Wild-type	64	0.03
37	Wild-type	64	0.06
39	Wild-type	128	0.03
40	N169S, A209T	64	0.03
43	Wild-type	128	0.06

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516

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Figure Legends

Figure 1. β-Lactam susceptibilities of ceftazidime resistant mutants versus K279a.

In (**A**) growth inhibition zone diameters (mm) versus ceftazidime resistant mutants (M1 and M52) the LTV-17 resistant mutant (KLTV) and K279a Δ*smlt0009* are reported in comparison with the parental strain (K279a). Smaller zone diameters mean reduced susceptibility. The following antibiotics were tested, with the amounts present in each disc noted: cefoxitin (FOX 30 µg), ceftazidime (CAZ 30 µg), cefepime (FEP 30 µg), piperacillin-tazobactam (TZP 110 µg), doripenem (DOR 10 µg), meropenem (MEM 10 µg). In (**B**) the inhibition zone diameters versus K279a and mutants carrying control plasmid (pBBR1MCS), white bar (p), or pBBR1MCS::*smlt0009* black bar (pSmlt0009), where *smlt0009* mutation is complemented *in trans*, are reported. Zones of inhibition around a CAZ (30 µg) disc are reported as mean values, *n*=3. Error bars represent standard error of the mean (SEM). Zone diameters are measured across the disc, so the minimum zone diameter is 6 mm, which is the diameter of the disc.

Figure 2. Sequence of Smlt0009 in ceftazidime resistant mutants and impact on growth and permeability phenotypes.

(**A**) is an alignment of translated high fidelity PCR sequences that confirmed mutation in the proline-rich region in ceftazidime resistant mutants M1, M52 and KLTV. Alignment was performed with CLUSTAL Omega and GeneDoc, showing amino acids 61-96; all other residues are identical across the variants. (**B**) reports the rate of fluorescent dye accumulation, which is reduced in all *smlt0009* mutants relative to K279a ($p<0.0001$ based on a T-test at 15 cycles), but greater in M1 than the other mutants ($p<0.01$ at 15 cycles). The assay followed injection of Hoescht (H) 33342 dye (25 µM final) to a cell suspension of 0.1 OD_{600} in PBS over 20 cycles (50 min) of incubation at 37°C. Each curve plots mean data for

three biological replicates with four technical replicates for each biological replicate. Error bars represent one Standard Deviation (SD). (**C**) reports growth inhibition zone diameters (mm) versus *smlt0009* mutants in comparison with K279a. Smaller zone diameters mean reduced susceptibility. Non-β-lactams tested were with the amount in each disc noted: amikacin (AK 30 µg), ciprofloxacin (CIP 5 µg), norfloxacin (NOR 10 µg), tigecycline (TGC 15 µg), minocycline (MH 30 µg), trimethoprim-sulfamethoxazole (SXT 25 µg), chloramphenicol (C 30 µg). Zones of inhibition are reported as mean values, *n*=3. Error bars represent SEM. Zone diameters are measured across the disc, so the minimum zone diameter is 6 mm, which is the diameter of the disc. (**D**) reports growth curves for K279a and the *smlt0009* mutants in NB over 24 h. The curves show OD_{600} versus time. Each curve plots mean data for eight replicates. Error bars represent one SD.

Figure 3. Increased siderophore production in ceftazidime resistant mutants versus K279a.

(**A**) Protein abundance data for EntB (Uniprot: B2FHQ4) derived from LC-MS/MS proteomics analysis were normalised using the average abundance of 30S and 50S ribosomal proteins in each sample. Values are reported as mean +/- SEM (*n*=3). In each case the change relative to K279a in each mutant is statistically significant ($p<0.05$). Full proteomics data are shown **Tables S1-S3**. (**B**) illustrates the diffusion of siderophore produced by K279a and *smlt0009* mutants. Diffusion of siderophore can be seen after spotting 10 µL of a PBS washed bacterial suspension (OD_{600} 0.2) onto a modified CAS agar. The image is representative of three experiments. (**C**) reports the diameter of diffusion of siderophore produced by strains tested as in (**B**). Values are reported as mean +/- SEM of three biological repeats and all mutants are significantly greater than K279a ($p<0.01$ based on a T-test).

Figure 4 Reduced uptake of ceftazidime in *smlt0009* mutants.

In (**A**) a standard curve is reported, where fixed concentrations of ceftazidime (made in NB) were applied to a freshly spread lawn of *E. coli* DH5α on an MHA plate in 10 µL spots, with the zone of growth inhibition being measured following 20 h of growth at 37°C. Zone of growth inhibition was then plotted versus the ceftazidime concentration applied. Data are means (*n*=3). The regression (solid line) with 95% confidence intervals (dashed line) was fitted with an R^2 of 0.99. (**B**) shows zones of inhibition in the *E. coli* DH5α lawn, incubated as in (**A**) but after spotting 10 µL of clarified (by centrifugation) and filter-sterilized culture medium that remained following 24 h of growth of *S. maltophilia* K279a or the four *smlt0009* mutant derivatives, initially inoculated in the presence of 10 $\mu g.mL^{-1}$ of ceftazidime. This image is representative of three experiments and the zone diameter is correlated with the concentration of ceftazidime remaining in the culture medium. (**C**) reports mean data (*n*=3) +/- SEM for the concentrations of ceftazidime remaining in these culture media, calculated by spotting each culture medium onto an *E. coli* DH5α lawn as in (**B**). The zones of inhibition measured were used to estimate the concentration of ceftazidime present in each culture medium applied to the lawn by reference to standard curve presented in (**A**). Data in (**C**) show values for experiments using *S. maltophilia* cultures inoculated initially in the presence of 5 $\mu g.mL^{-1}$ or 10 $\mu g.mL^{-1}$ ceftazidime. In all cases, concentrations in the supernatants of cultures growing mutants were greater than those growing K279a ($p<0.01$ based on a Mann-Whitney test).

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