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1 **TonB dependent uptake of β-lactam antibiotics in the opportunistic human pathogen**

2 ***Stenotrophomonas maltophilia*.**

3

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

5

6

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15

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.

40 **Introduction**

41 *Stenotrophomonas maltophilia* is a non-fermenting Gram-negative bacillus prevalent in the
42 environment and an important opportunistic human pathogen. In the US, it caused 4.3% of
43 all Gram-negative bacterial infections in intensive care units between 1993 and 2004
44 (Lockhart et al, 2007). For pneumonia, in the US, 3.3% of infections in hospitalised patients
45 between 2004 and 2008 were caused by *S. maltophilia* (Jones 2010). Attributable mortality
46 rates can be high for these serious infections, up to 37.5% (Falagas et al., 2009). It also
47 causes a wide range of other infections and colonises the lungs of >10% of adults living with
48 Cystic Fibrosis (Brooke, 2012). *S. maltophilia* clinical isolates are resistant to almost all β-
49 lactam antibiotics because of the production of two β-lactamases: L1, a subclass B3 metallo-
50 β-lactamase and L2, a class A extended spectrum β-lactamase (Gould *et al.*, 2006).
51 Production of L1 and L2 is co-ordinately controlled by AmpR, a LysR-type transcriptional
52 activator and induced during β-lactam challenge of cells (Okazaki & Avison, 2008). Despite
53 this, many *S. maltophilia* clinical isolates remain susceptible to the β-lactam ceftazidime
54 because it is a relatively poor substrate for these two enzymes (Calvopiña *et al.*, 2017).
55 However, mutants that have acquired ceftazidime resistance can easily be identified in the
56 laboratory, and ceftazidime resistant isolates are commonly encountered in the clinic. In
57 many cases, these mutants hyperproduce L1 and L2 (Okazaki & Avison, 2008, Talfan *et al.*,
58 2013, Calvopiña & Avison, 2018) but we have previously identified ceftazidime resistant
59 mutants that did not hyperproduce β-lactamase (Gould & Avison, 2006). It was hypothesised
60 that these mutants might have reduced accumulation of ceftazidime (Talfan *et al.*, 2013).
61 The primary non-β-lactamase mediated mechanisms of β-lactam resistance in similar non-
62 fermenting bacteria such as *Pseudomonas aeruginosa* are increased efflux and reduced
63 outer membrane permeability due to a reduction in the production of outer membrane porins
64 (Castanheira *et al.*, 2014). In Gram negative bacteria, tripartite outer membrane porins are
65 considered the only site of entry for β-lactams, and reduced porin levels can reduced β-
66 lactam susceptibility in many species (Pfeifer *et al.*, 2010). No other way of entry has

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

73

74 **Results and Discussion**

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

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

87 Whole genome sequencing was performed to identify the mutations present in M1 and M52.
88 Only one gene was found to be mutated in each. It was the same in both: *smlt0009*,
89 annotated in the K279a genome sequence as encoding a 'putative proline-rich TonB energy
90 transducer protein' (Crossman *et al*., 2008). The mutation was confirmed using high fidelity
91 PCR sequencing. In both M1 and M52, a proline rich region in Smlt0009 situated at around
92 70 amino acids into the 222 amino acid protein was shortened but there was no frameshift

93 (**Fig. 2A**). Assuming this shortening impairs protein function, and to confirm the role of this
94 impairment in ceftazidime resistance, *smlt0009* was insertionally inactivated in K279a using
95 a suicide gene replacement methodology. K279a Δ*smlt0009* was confirmed to be
96 ceftazidime resistant (**Table 1**) and to have generally reduced β-lactam susceptibility (**Fig.
97 1A**). Complementation of the *smlt0009* mutation in all these mutants using a plasmid version
98 of the wild-type gene *in trans* completely reversed ceftazidime resistance, according to disc
99 testing (**Fig 1B**), confirming that this phenotype is caused by the *smlt0009* mutation in the
100 mutants tested.

101

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

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

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

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

128 Siderophore-conjugation has been used as a way of increasing the penetration of
129 antimicrobials into Gram-negative bacteria by hijacking the TonB dependent uptake system
130 (Kline *et al*., 2000, Choi & McCarthy, 2018). Indeed, recently we have shown that
131 siderophore conjugation of the γ-lactam antibiotic lactivicin (to create LTV-17) dramatically
132 improves potency against *S. maltophilia* (Calvopiña *et al*., 2016). As expected given TonB
133 dependence of LTV-17 uptake in other species (Starr *et al*., 2014), ceftazidime resistant
134 *smlt0009* (TonB) mutants M1 and M52 also have reduced susceptibility to LTV-17, as does
135 K279a Δ*smlt0009* where in each case the MIC of LTV-17 increased to ≥0.25 $\mu g.mL^{-1}$ (**Table**
136 **1**). A single-step mutant (KLTV) with reduced susceptibility to LTV-17 was next selected
137 from K279a and the mutant is also resistant to ceftazidime (**Table 1**) and has reduced
138 susceptibility to all tested β-lactams (**Fig. 1A**). KLTV whole envelope proteomics showed
139 very similar changes to those observed in M1 and M52 (**Table S3**). In KLTV, like M1 and
140 M52, there is upregulation of the siderophore biosynthesis enzymes and increased
141 siderophore production (**Fig. 3**). WGS confirmed shortening of the proline-rich region in
142 Smlt0009 in KLTV (**Fig. 2A**). TonB mutations are known to reduce susceptibility to
143 siderophore conjugated antimicrobials in other species but have never previously been
144 reported to affect β-lactam susceptibility (Hassett *et al*., 1996, Tomaras *et al*., 2013, Moynie
145 *et al*., 2017). Interestingly, *S. maltophilia smlt0009* mutants do not have reduced

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

148

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

151 In some bacteria, TonB complexes participate in the import of TBDT-dependent ligands in
152 addition to iron-siderophore complexes. *S. maltophilia* Smlt0009 shares 50% identity with the
153 TonB energy transducer protein from the closely related *Xanthomonas campestris*, a species
154 where only 15% of TBDTs are involved in iron-siderophore-complex import (Schauer *et al.*,
155 2008). Interestingly, of 162 proteins differently regulated in *S. maltophilia* K279a M1 and
156 M52, nineteen are putative TonB-dependent TBDT proteins (**Table S1**). Apparently, M1 and
157 M52 are responding to a disruption of TonB-dependent energy transduction, which is
158 associated with the import of many diverse ligands. In support of a more general role for
159 TonB in *S. maltophilia*, envelope permeability, measured via the uptake of a fluorescent
160 Hoescht dye, was found to be significantly reduced in *smlt0009* mutants relative to K279a.
161 Interestingly, mutant M1, with the smallest deletion in the Smlt0009 proline-rich region had
162 the least reduction in envelope permeability (**Fig. 2A, 2B**). Assays of susceptibility to non-β-
163 lactam antimicrobials against K279a and the *smlt0009* mutants identified reduced
164 fluoroquinolone susceptibility, though other classes of antimicrobial were not affected by
165 disruption of Smlt0009 (**Fig. 2C**). Hence a TonB-dependent system is implicated in the
166 uptake of fluoroquinolones as well as β-lactams in *S. maltophilia*.

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

172 the smallest deletion of Smlt0009 suffered from significantly less impairment of growth

173 (*p*<0.0001 at 12 h) than the other mutants (**Fig. 2A, 2D**).

174

175 *Reduced ceftazidime uptake into S. maltophilia smlt0009 mutants and existence of these*

176 *mutants amongst S. maltophilia clinical isolates.*

177 Findings reported above led us to hypothesise that in *S. maltophilia*, uptake of β lactams is

178 TonB dependent. Thus, mutations in the proline rich region of Smlt0009 reduce energy

179 dependent ceftazidime uptake and confer clinically important ceftazidime resistance. This is

180 the first time that β lactam entry via a TonB-dependent mechanism has been proposed in

181 any bacterium. However, it is interesting to note that, unlike all other pathogens studied

182 previously, outer membrane passive diffusion porin loss has never been found to be involved

183 in β-lactam resistance in *S. maltophilia* (Sanchez, 2015) which supports the existence of a

184 novel import mechanism in this species.

185 To test our hypothesis that disruption of Smlt0009 reduces ceftazidime uptake in *S.*

186 *maltophilia*, we incubated K279a and the various *smlt0009* mutants with ceftazidime and

187 then measured the concentration of ceftazidime remaining in the growth medium by using an

188 *Escherichia coli* killing assay where the lower the concentration of ceftazidime, the smaller

189 the zone of killing seen on a lawn of *E. coli* DH5α. As can be seen (**Fig. 4**) the concentration

190 of ceftazidime outside of K279a is significantly less than the concentration outside of the

191 *smlt0009* mutants after 24 h of incubation, confirming that ceftazidime enters K279a more

192 readily than the *smlt0009* mutants. This adds strong support to our hypothesis that

193 ceftazidime uptake is TonB dependent in *S. maltophilia*. Identifying which of the >20 TBDTs

194 seen in *S. maltophilia* K279a is the one responsible for ceftazidime uptake will form the basis

195 of future work.

196 Finally, we turned to our world-wide collection of 22-phylogenetic group A *S. maltophilia*

197 clinical isolates (Gould *et al.*, 2006) against which we measured the MICs of LTV-17 and

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

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

218

219 *Conclusions*

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

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

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

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

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

273

274

275 **Experimental Procedures**

276 *Bacterial isolates and materials*

277 *S. maltophilia* clinical isolates used originated from the SENTRY antimicrobial resistance
278 survey and have been previously described (Toleman *et al*., 2007) plus isolate K279a
279 (Avison *et al*., 2000). All growth media were from Oxoid. Chemicals were from Sigma, unless
280 otherwise stated. LTV-13 was re synthesized according to the literature protocol (Starr *et al*.,
281 2014) and kindly provided by Prof. C. Schofield, University of Oxford. LTV-17 was kindly
282 supplied by Pfizer.

283 *Selection of resistant mutants*

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

291 *β-lactamase assays*

292 100 µL of an overnight NB culture was diluted in 10 mL of NB and incubated at 37°C with
293 shaking until OD_{600} was 0.4. Cells were pelleted by centrifugation (4,000 x *g*, 10 min) and
294 pellets resuspended in 100 µL of BugBuster (Ambion). Pellets were transferred to 1.5 mL
295 microtube (Eppendorf) before rocking at 70 rpm for 30 min at room temperature. Cell debris
296 and unlysed cells were pelleted by centrifugation (13,000 x *g*, 5 min) and the supernatant
297 retained as a source of crude cell protein. Protein concentrations in cell extracts were
298 determined using the BioRad protein assay dye reagent concentrate according to the
299 manufacturer's instructions. β-Lactamase activity in crude cell extracts was determined
300 using a POLARstar Omega plate spectrophotometer (BMG Labtech). Nitrocefin (40 µM)

301 solution was used as a substrate, prepared in 0.2 μm syringe-filtered assay buffer (60 mM
302 $Na_2HPO_4 \cdot 7H_2O$ pH 7.0, 40 mM $NaH_2PO_4 \cdot H_2O$, 10 mM KCl, 1 mM $MgSO_4 \cdot 7H_2O$, 100 μM
303 $ZnCl_2$). Nitrocefin hydrolysis assays were performed in Corning Costar 96-well flat-bottomed
304 cell culture plates with a combination of 1 μL of cell extract and 179 μL of nitrocefin solution.
305 Product accumulation was measured at 482 nm for 5 min or until the end of the linear phase
306 of the reaction. Final β-lactamase activity ($nmol.min^{-1}.\mu g^{-1}$ of protein in cell extract) was
307 calculated via change in absorbance per minute taken from the linear phase of the reaction
308 in Omega Data Analysis. An extinction coefficient of 17400 $M^{-1}cm^{-1}$ was used for nitrocefin.
309 The path length for liquid in a well in the 96-well plate was set at 0.56 cm.

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

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

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

321 *Whole genome sequencing to Identify mutations*

322 Whole genome resequencing was performed by MicrobesNG (Birmingham, UK) on a HiSeq
323 2500 instrument (Illumina, San Diego, CA, USA). Reads were trimmed using Trimmomatic
324 (Bolger *et al.*, 2014) and assembled into contigs using SPAdes 3.10.1
325 (http://cab.spbu.ru/software/spades/). Assembled contigs were mapped to reference genome

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

328 Mutations were checked by PCR using Phusion High Fidelity DNA Polymerase (New
329 England Biolabs). To generate template DNA, a bacterial colony was resuspended in 100 µL
330 of molecular biology grade water and heated at 100°C for 5 min. The sample was
331 centrifuged at 13000 rpm for 5 min. PCR reactions were set up using 5 µL of 5X Phusion GC
332 Buffer, 0.5 µL of dNTPs (10 mM), 1.25 µL of forward primer (10 µM), 1.25 µL of reverse
333 primer (10 µM), 0.75 µL of DMSO, 0.25 µL of Phusion DNA Polymerase, 1 µL of DNA
334 template, and 15 µL of molecular biology grade water. The cycling conditions were the
335 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
336 s, 1 cycle of 72°C for 10 min for final extension.

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

344 *Insertional inactivation of smlt0009*

345 The K279a Δ*smlt0009* mutant was constructed by gene inactivation mediated by the
346 pKNOCK suicide plasmid (Alexeyev, 1999). The *smlt0009* DNA fragment was amplified with
347 Phusion High-Fidelity DNA Polymerase (NEB, UK) from *S. maltophilia* genomic DNA by
348 using primers *smlt0009* KO FW (5′-GTGAAGAATCTGTCGCCGC-3′) and *smlt0009* KO RV
349 (5′-GGATCACTTCGCCCTGGATA-3′). The PCR product was ligated into the pKNOCK-GM
350 at SmaI site. The recombinant plasmid was then transferred into wild-type *S. maltophilia*
351 cells by conjugation. The mutant was selected for gentamicin resistance and the mutation

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

355 *Cloning smlt0009 and trans-complementation*

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

365 *Proteomics*

366 500 µL of an overnight NB culture were transferred to 50 mL NB and cells were grown at
367 37˚C to 0.6 OD_{600}. Cells were pelleted by centrifugation (10 min, 4,000 × *g*, 4°C) and
368 resuspended in 30 mL of 30 mM Tris-HCl, pH 8 and broken by sonication using a cycle of 1
369 s on, 0.5 s off for 3 min at amplitude of 63% using a Sonics Vibracell VC-505TM (Sonics and
370 Materials Inc., Newton, Connecticut, USA). The sonicated samples were centrifuged at
371 8,000 rpm (Sorval RC5B PLUS using an SS-34 rotor) for 15 min at 4°C to pellet intact cells
372 and large cell debris; For envelope preparations, the supernatant was subjected to
373 centrifugation at 20,000 rpm for 60 min at 4°C using the above rotor to pellet total envelopes.
374 To isolate total envelope proteins, this total envelope pellet was solubilised using 200 µL of
375 30 mM Tris-HCl pH 8 containing 0.5% (w/v) SDS.

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

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

383 The 1 cm of gel lane was subjected to in-gel tryptic digestion using a DigestPro automated
384 digestion unit (Intavis Ltd). The resulting peptides from each gel fragment were fractionated
385 separately using an Ultimate 3000 nanoHPLC system in line with an LTQ-Orbitrap Velos
386 mass spectrometer (Thermo Scientific). In brief, peptides in 1% (v/v) formic acid were
387 injected onto an Acclaim PepMap C18 nano-trap column (Thermo Scientific). After washing
388 with 0.5% (v/v) acetonitrile plus 0.1% (v/v) formic acid, peptides were resolved on a 250 mm
389 × 75 μm Acclaim PepMap C18 reverse phase analytical column (Thermo Scientific) over a
390 150 min organic gradient, using 7 gradient segments (1-6% solvent B over 1 min, 6-15% B
391 over 58 min, 15-32% B over 58 min, 32-40% B over 5 min, 40-90% B over 1 min, held at
392 90% B for 6 min and then reduced to 1% B over 1 min) with a flow rate of 300 nL/min.
393 Solvent A was 0.1% formic acid and Solvent B was aqueous 80% acetonitrile in 0.1% formic
394 acid. Peptides were ionized by nano-electrospray ionization MS at 2.1 kV using a stainless-
395 steel emitter with an internal diameter of 30 μm (Thermo Scientific) and a capillary
396 temperature of 250°C. Tandem mass spectra were acquired using an LTQ-Orbitrap Velos
397 mass spectrometer controlled by Xcalibur 2.1 software (Thermo Scientific) and operated in
398 data-dependent acquisition mode. The Orbitrap was set to analyze the survey scans at
399 60,000 resolution (at m/z 400) in the mass range m/z 300 to 2000 and the top twenty
400 multiply charged ions in each duty cycle selected for MS/MS in the LTQ linear ion trap.
401 Charge state filtering, where unassigned precursor ions were not selected for fragmentation,
402 and dynamic exclusion (repeat count, 1; repeat duration, 30 s; exclusion list size, 500) were
403 used. Fragmentation conditions in the LTQ were as follows: normalized collision energy,

404 40%; activation q, 0.25; activation time 10 ms; and minimum ion selection intensity, 500
405 counts.

406 The raw data files were processed and quantified using Proteome Discoverer software v1.4
407 (Thermo Scientific) and searched against the UniProt *S. maltophilia* strain K279a database
408 (4365 protein entries; UniProt accession UP000008840) using the SEQUEST (Ver. 28 Rev.
409 13) algorithm. Peptide precursor mass tolerance was set at 10 ppm, and MS/MS tolerance
410 was set at 0.8 Da. Search criteria included carbamidomethylation of cysteine (+57.0214) as
411 a fixed modification and oxidation of methionine (+15.9949) as a variable modification.
412 Searches were performed with full tryptic digestion and a maximum of 1 missed cleavage
413 was allowed. The reverse database search option was enabled and all peptide data was
414 filtered to satisfy false discovery rate (FDR) of 5 %. The Proteome Discoverer software
415 generates a reverse "decoy" database from the same protein database used for the analysis
416 and any peptides passing the initial filtering parameters that were derived from this decoy
417 database are defined as false positive identifications. The minimum cross-correlation factor
418 filter was readjusted for each individual charge state separately to optimally meet the
419 predetermined target FDR of 5 % based on the number of random false positive matches
420 from the reverse decoy database. Thus, each data set has its own passing parameters.
421 Protein abundance measurements were calculated from peptide peak areas using the Top 3
422 method (Silva *et al.*, 2006) and proteins with fewer than three peptides identified were
423 excluded. The proteomic analysis was repeated three times for each parent and mutant
424 strain, each using a separate batch of cells. Data analysis was as follows: all raw protein
425 abundance data were uploaded into Microsoft Excel. Raw data from each sample were
426 normalised by division by the average abundance of all 30S and 50S ribosomal protein in
427 that sample. A one-tailed, unpaired T-Test was used to calculate the significance of any
428 difference in normalised protein abundance data in the three sets of data from the parent
429 strains versus the three sets of data from the mutant derivative. A *p*-value of <0.05 was
430 considered significant. The fold change in abundance for each protein in the mutant

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

433 *Siderophore Detection*

434 100 µL of an overnight culture in Cation-Adjusted Muller-Hinton Broth (CA-MHB) was used
435 to set up a fresh subculture in 10 mL of CA-MHB which was then incubated until the OD_{600}
436 reached 0.5. Cells were centrifuged (4,000 x *g*, 10 min) and the resulting pellet was
437 resuspended in 10 mL of Phosphate Buffered Saline (PBS) and centrifuged again (4,000 x *g*,
438 10 min). The supernatant was discarded, and the pellet was again resuspended in fresh
439 PBS (10 mL) and centrifuged (4,000 x *g*, 10 min). This washed bacterial pellet was then
440 diluted in PBS to prepare a bacterial suspension of OD_{600} 0.2. Ten microliters of the bacterial
441 suspension were spotted on Chrome Azurol S (CAS) agar. CAS agar was made up mixing
442 up 90 mL of MHA and 10 mL of freshly made CAS solution. 100 mL of the CAS solution was
443 made up based on the following description: 60.5 mg of CAS in 50 mL of water, 72.9 mg of
444 hexadecyltrimethyl ammonium bromide in 40 mL of water, and 10 mL of 1 mM $FeCl_3$, 10 mM
445 HCl) (Garcia *et al.*, 2012). CAS agar control included 100 µM $FeCl_3$ where no colour change
446 was expected.

447 *Fluorescent Hoescht (H) 33342 dye accumulation assay*

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

457 incubated at 37°C. Hoescht dye (H33342, 250 µM in water) was added to bacterial
458 suspension of the plate using the plate-reader's auto-injector to give a final concentration of
459 25 µM per well. Excitation and emission filters were set at 355 nm and 460 nm respectively.
460 Readings were taken in intervals (cycles) separated by 150 s. 31 cycles were run in total. A
461 gain multiplier of 1300 was used. Results were expressed as absolute values of
462 fluorescence versus time.

463 *Growth curves*

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

469 *Indirect ceftazidime uptake assay*

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

480

481

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491 **Author Contributions**

492 Conception and design: MBA, KC.

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

494 Analysis and Interpretation of data: ALL AUTHORS.

495 Drafting the manuscript; ALL AUTHORS.

496

497 **Conflicts of Interest**

498 The authors declare that they have no conflict of interest.

499 **Tables**

500

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

504

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

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

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

511

512

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

515

516

517

518 **Figure Legends**

519

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

521 In (**A**) growth inhibition zone diameters (mm) versus ceftazidime resistant mutants (M1 and
522 M52) the LTV-17 resistant mutant (KLTV) and K279a Δ*smlt0009* are reported in comparison
523 with the parental strain (K279a). Smaller zone diameters mean reduced susceptibility. The
524 following antibiotics were tested, with the amounts present in each disc noted: cefoxitin (FOX
525 30 μg), ceftazidime (CAZ 30 μg), cefepime (FEP 30 μg), piperacillin-tazobactam (TZP 110
526 μg), doripenem (DOR 10 μg), meropenem (MEM 10 μg). In (**B**) the inhibition zone diameters
527 versus K279a and mutants carrying control plasmid (pBBR1MCS), white bar (p), or
528 pBBR1MCS::*smlt0009* black bar (pSmlt0009), where *smlt0009* mutation is complemented *in*
529 *trans*, are reported. Zones of inhibition around a CAZ (30 μg) disc are reported as mean
530 values, n=3. Error bars represent standard error of the mean (SEM). Zone diameters are
531 measured across the disc, so the minimum zone diameter is 6 mm, which is the diameter of
532 the disc.

533

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

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

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

555

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

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

569

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

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

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