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MECHANISMS OF RESISTANCE

Overexpression of the Efflux Pumps SmeVWX and SmeDEF Is a Major Cause of Resistance to Co-trimoxazole in *Stenotrophomonas maltophilia*

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ABSTRACT Co-trimoxazole is one of the antimicrobials of choice for treating *Stenotrophomonas maltophilia* infections. Most works on the molecular epidemiology of the resistance to this drug combination are based on the analysis of *sul* genes. Nevertheless, the existence of clinical co-trimoxazole-resistant *S. maltophilia* isolates that do not harbor *sul* genes has been reported. To investigate potential mutations that can reduce the susceptibility of *S. maltophilia* to co-trimoxazole, spontaneous *S. maltophilia* co-trimoxazole-resistant mutants isolated under different co-trimoxazole concentrations were studied. All mutants presented phenotypes compatible with the overexpression of either SmeVWX (94.6%) or SmeDEF (5.4%). Indeed, the analysis of a selected set of strains showed that the overexpression of either of these efflux pumps, which was due to mutations in their regulators *smeRv* and *smeT*, respectively, was the cause of co-trimoxazole resistance. No other efflux pump was overexpressed in any of the studied mutants, indicating that they do not participate in the observed resistance phenotype. The analysis of mutants overexpressing or lacking SmeDEF or SmeVWX shows that SmeDEF contributes to the intrinsic and acquired resistance to co-trimoxazole in *S. maltophilia*, whereas SmeVWX only contributes to acquired resistance. It is important to highlight that all mutants were less susceptible to other antibiotics, including chloramphenicol and quinolones. Since both SmeVWX and SmeDEF are major determinants of quinolone resistance, the potential cross-selection of resistance to co-trimoxazole and quinolones, when either of the antimicrobials is used, is of particular concern for the treatment of *S. maltophilia* infections.

KEYWORDS co-trimoxazole, SmeDEF, SmeVWX, *Stenotrophomonas maltophilia*

Stenotrophomonas maltophilia is an opportunistic pathogen that is responsible for nosocomial infections, mainly in patients with underlying diseases (1), with particular relevance in the case of cystic fibrosis patients (2–4). In addition, *S. maltophilia* is considered a prototype of intrinsically resistant organisms due to its low susceptibility to several of the antimicrobials used in clinical practice, a phenotype that is at least partly due to the presence of several genes encoding antibiotic resistance determinants in its genome (5–7). In addition, *in vitro* studies and analyses of clinical isolates have shown that strains presenting even higher levels of resistance due to mutation or to the acquisition of resistance genes are not infrequent (6–12). One of the antimicrobials used for the treatment of *S. maltophilia* is co-trimoxazole, a combination of the antibiotics trimethoprim and sulfamethoxazole, which target different steps of the folic acid biosynthesis pathway. Unfortunately, the percentage of *S. maltophilia* co-trimoxazole-resistant isolates has increased in recent years.

The presence of the *sul* genes (*sul1* and *sul2*), which encode variants of the dihydropteroate synthase that are not inhibited by sulfonamides, has been proposed to

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be a major cause of co-trimoxazole resistance in *S. maltophilia* (13–15). These elements, which are involved in sulfonamide resistance, are frequently present in integrons and in mobile elements, a situation that helps their dissemination among several different microorganisms, including *S. maltophilia*. Nevertheless, despite the presence of *sul* genes in some *S. maltophilia* co-trimoxazole-resistant isolates, resistant strains that do not carry *sul* genes have been isolated as well (16, 17). This situation indicates that *S. maltophilia* may possess mechanisms of co-trimoxazole resistance besides the presence of *sul* genes in its genome that remain to be established.

Indeed, different studies have shown that the activity of some efflux pumps might affect *S. maltophilia* co-trimoxazole susceptibility. Among these studies, it has been shown that the overexpression of SmeDEF reduces the susceptibility to co-trimoxazole of *S. maltophilia*, while the inactivation of this efflux pump increases the bacterial susceptibility to this antimicrobial (18). Other studies have shown that the deletion of the efflux pump SmeYZ or of the outer membrane protein TolC, the latter required for the activity of the efflux pump SmeOP, increases the susceptibility of *S. maltophilia* to co-trimoxazole (19–21). These findings reinforce the idea that, in addition to the acquisition of *sul* genes, there are other mechanisms, such as via efflux pumps, which could contribute to the acquisition of resistance to co-trimoxazole in *S. maltophilia*.

To identify these potential mechanisms, we have selected *S. maltophilia* co-trimoxazole-resistant mutants at different concentrations of co-trimoxazole and have determined the underlying mechanisms of resistance in the selected mutants. The results shed light on the ignored aspects of *S. maltophilia* co-trimoxazole resistance and show that the main mechanism of mutation-driven co-trimoxazole resistance is the overexpression of efflux pumps, mainly of SmeVWX but also, in minor proportions, of SmeDEF. In both cases, the overexpression is associated with mutations in the regulator genes of these efflux pumps, *smeRv* and *smeT*, respectively.

RESULTS

Isolation and phenotypic characterization of spontaneous co-trimoxazole-resistant strains. Spontaneous co-trimoxazole-resistant mutants of *S. maltophilia* strain D457 were selected in Mueller-Hinton agar plates containing 2, 4, 8, 16, or 32 mg/liter of co-trimoxazole. Here, we use the operational definition of resistance by which a strain is considered resistant if "it has a higher MIC value for the studied compound than its parental wild-type strain" (22). Ninety-four mutants isolated from plates containing different amounts of co-trimoxazole were chosen for further analysis. After isolation, the putative mutants were regrown two sequential times in plates without antibiotics and afterwards reseeded in antibiotic-containing plates. All of them, with one exception, grew in the presence of co-trimoxazole, indicating that in most of the mutants, the observed phenotype of resistance was not due to the transient adaptation of *S. maltophilia* to the presence of the antibiotic.

To analyze the susceptibility of the 93 mutants, these strains, as well as the parental strain D457 and its derivative D457R that overexpresses SmeDEF (Table 1), were grown in a 96-well microtiter plate and seeded in antibiotic-containing plates with the aid of a 96-pin replicator as described in Material and Methods. As Fig. 1 and Table S1 in the supplemental material show, co-trimoxazole-resistant mutants display different phenotypes of susceptibility to the tested antibiotics. These differential phenotypes enabled the classification of *S. maltophilia* co-trimoxazole-resistant mutants into four groups: chloramphenicol and quinolone resistant (84 strains); quinolone resistant (4 strains); chloramphenicol, quinolone, and erythromycin resistant (3 strains), and quinolone and erythromycin resistant (2 strains). The types of mutants isolated varied according to the amount antibiotic used for the selection. As expected, the variability of the phenotypes is higher at the lowest concentration of the selective agent. The fact that resistance to co-trimoxazole is associated with a reduced susceptibility to antibiotics belonging to different structural families suggests that the overexpression of efflux pumps, able to extrude different substrates, might be the basis for the observed resistance.

TABLE 1 Bacteria and plasmid used in this work

Strain or plasmid	Description	Reference or source
Strains		
S. maltophilia		
D457	Clinical strain, wild type	29
MBS411	D457 Δ*smeE*	11, 46
PBT02	D457 with pPBT04 plasmid	31
PBT06	MBS287 with pPBT04 plasmid	31
D457R	D457 mutant overexpressing SmeDEF efflux pump	29
MBS287	D457 mutant overexpressing SmeVWX efflux pump	11
MBS704	D457 Δ*smeW*	31
MBS509-MBS602	Mutants isolated in different amounts of co-trimoxazole	This work
MBS708	MBS509 with pPBT04 plasmid	This work
MBS709	MBS512 with pPBT04 plasmid	This work
MBS713	MBS520 with pPBT04 plasmid	This work
MBS716	MBS534 with pPBT04 plasmid	This work
MBS718	MBS560 with pPBT04 plasmid	This work
E. coli		
CC118λpir	λpir lysogen from CC118, Tet^r, Δ(ara-leu) *araD* Δ*lacX74 galE galK phoA20 thi-1 rpsE rpoB argE*(Am) *recA1*	45
1047(pRK2013)	Strain containing the pRK2013 helper plasmid, Kan^r	47
Plasmid		
pPBT04	Cloning vector pSEVA237Y containing *smeVWX* promoter region	31

The acquisition of antibiotic resistance might result in fitness costs that might be reflected in a lower growth rate (23–25). To address this possibility, the growth rates of a set of mutants presenting different phenotypes of resistance were analyzed in comparison to that of the wild-type parental strain D457. As shown in Fig. S1, the acquisition of co-trimoxazole resistance was always associated with a growth impairment.

Acquired co-trimoxazole resistance is associated with overexpression of the efflux pumps SmeDEF and SmeVWX. As stated above, the observed multidrug-resistant phenotypes matched with the potential overexpression of efflux pumps. In particular, the phenotype of the larger group of mutants, which are chloramphenicol and quinolone resistant, is consistent with SmeVWX overexpression (11, 26). Other less-abundant mutants, presenting reduced susceptibility to chloramphenicol and quinolones and erythromycin resistance, display a phenotype consistent with the overexpression of SmeDEF (27, 28).

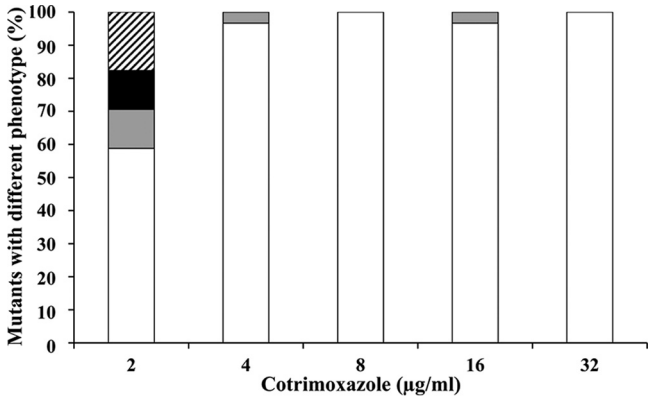

FIG 1 Phenotypes of mutants selected at different co-trimoxazole concentrations. The percentages of each category of mutants selected at each co-trimoxazole concentration are shown. White bars, mutants with chloramphenicol and quinolone resistance; gray bars, mutants with quinolone resistance; black bars, mutants with erythromycin and quinolone resistance; striped bars, mutants with erythromycin, chloramphenicol, and quinolone resistance.

TABLE 2 Expression levels of efflux pump genes *smeV* and *smeD*

Strain	Phenotype[a]	Fold change ± SEM[b]	
		smeV	*smeD*
D457		1.00 ± 0.0	1.00 ± 0.0
D457R		0.33 ± 0.03	**6.99 ± 3.61**
MBS509	Q	**55.03 ± 9.40**	0.80 ± 0.19
MBS510	Q/CHL/ERY	0.87 ± 0.11	**8.59 ± 1.55**
MBS517	Q/ERY	0.71 ± 0.17	**7.54 ± 1.14**
MBS518	Q/ERY	0.64 ± 0.28	**7.99 ± 1.58**
MBS522	Q/CHL/ERY	0.74 ± 0.26	**5.46 ± 1.01**
MBS534	Q	**30.41 ± 4.82**	0.75 ± 0.10
MBS560	Q/CHL	**48.05 ± 19.48**	0.58 ± 0.05
MBS571	Q/CHL	**91.20 ± 41.68**	0.71 ± 0.09

[a]Antibiotic resistance phenotype. Q, quinolone resistance; Q/CHL/ERY, quinolone, chloramphenicol, and erythromycin resistance; Q/CHL, quinolone and chloramphenicol resistance; Q/ERY, quinolone and erythromycin resistance.

[b]Expression levels are fold change of each mutant versus the wild-type strain, D457. Data are the means from three independent experiments. Fold changes larger than five are highlighted in bold.

Two mutants presenting each of the phenotypes were chosen to analyze the possibility that the overexpression of any of the eight efflux pumps described in *S. maltophilia* (5, 6) could be responsible of these phenotypes. The expression of the efflux pumps encoded by *smeABC*, *smeDEF*, *smeGH*, *smeIJK*, *smeMN*, *smeOP*, *smeVWX*, and *smeYZ* was analyzed by real-time reverse transcriptase PCR (RT-PCR). As shown in Table 2, only the efflux pumps SmeDEF and SmeVWX were overexpressed in the selected mutants (data not shown for the other analyzed efflux pumps). Mutants presenting either chloramphenicol and quinolone or just quinolone resistance phenotypes (MBS509, MBS534, MBS560, and MBS571) overexpressed the efflux pump SmeVWX, while mutants presenting either chloramphenicol, quinolone, and erythromycin resistance or quinolone and erythromycin resistance (MBS510, MBS517, MBS518, and MBS522) overexpressed the SmeDEF efflux pump. Taking into consideration the distribution of phenotypes shown in Fig. 1, these results indicate that mutants overexpressing SmeDEF are selected mainly at low co-trimoxazole concentrations, while SmeVWX mutants are selected in the full range of concentrations of the antimicrobial.

To further confirm that the selected mutants overexpressed these efflux pumps, two different approaches were utilized. The overexpression of the efflux pump SmeDEF was confirmed by Western blotting (27) as described in Materials and Methods. As shown in Fig. 2, MBS510, MBS517, MBS518, and MBS522 presented levels of SmeF similar to those of the D457R mutant, which is known to overexpress SmeDEF (11, 27, 29, 30), while the expression levels of SmeF in MBS509, MBS534, MBS560, and MBS571 were similar to those found in the parental wild-type D457. These results are in agreement with the data obtained by real-time RT-PCR.

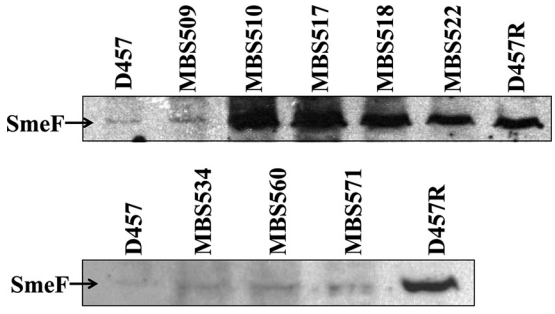

FIG 2 Expression of SmeF by co-trimoxazole-resistant mutants. The level of expression of SmeDEF was measured by Western blotting using an anti-SmeF antibody. D457, wild-type *S. maltophilia* strain; D457R, SmeDEF-overexpressing mutant. In agreement with the real-time RT-PCR data, the mutants MBS510, MBS517, MBS518, and MBS522 overexpress SmeDEF.

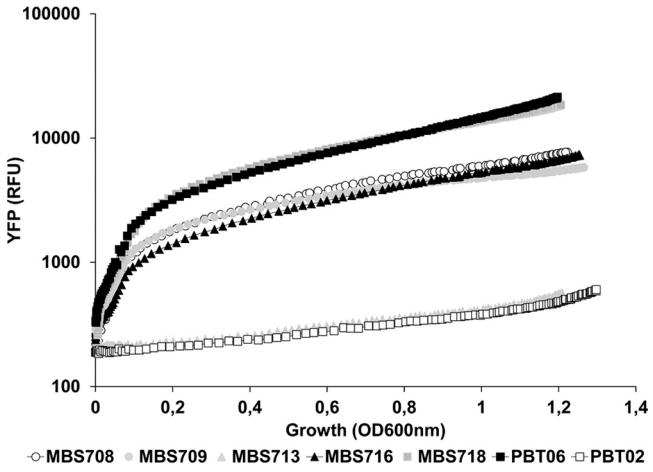

FIG 3 Analysis of *smeVWX* expression by co-trimoxazole-resistant mutants. The level of expression of smeVWX was analyzed using a fluorescent reporter as described in Materials and Methods. MBS708, MBS709, MBS716, MBS718, and PBT06 were derived from MBS509, MBS512, MBS534, MBS560, and MBS287, respectively. In agreement with real-time RT-PCR data, the expression of the fluorescent reporter YFP was higher in these mutants than in the control strains PBT02 (a derivative of the wild-type strain D457) and MBS713 (derived from MBS520, a mutant that overexpresses SmeDEF but not SmeVWX).

Concerning *smeVWX*, its increased expression was confirmed using a fluorescent reporter under the control of the *smeV* promoter as described previously (31). As Fig. 3 shows, MBS708, MBS716, and MBS718 presented higher fluorescence levels than PBT02 (a derivative of the wild-type strain D457) and MBS713 (derived from the MBS520 mutant that overexpresses SmeDEF) and similar levels to those observed in PBT06 (a derivative of MBS287, which overexpresses SmeVWX).

Overexpression of the efflux pumps SmeDEF and SmeVWX are associated with mutations in the genes *smeT* and *smeRv* encoding their respective local regulators. It has been described that the overexpression of SmeDEF and SmeVWX is associated with mutations in their regulators genes, *smeT* and *smeRv*, respectively (9, 11, 30).

The *smeT* and *smeRv* genes of 24 co-trimoxazole-resistant mutants (Table 3), including those analyzed above, were amplified by PCR, and the resulting amplicons were sequenced. All five mutants overexpressing SmeDEF presented mutations in *smeT*. Three of the studied mutants presented the amino acid change Leu166Gln, which has been previously described in other SmeDEF-overproducing mutants (30). The other two mutants presented a Lys insertion in position 161 (Lys160_Ile161insLys) (Table 3).

Concerning the 19 analyzed mutants presenting phenotypes compatible with SmeVWX overexpression, 11 of them presented mutations previously associated with the overexpression of this efflux pump (11), namely, Gly266Asp (5 strains), Gly266Ser (5 strains), and Cys310Phe (1 strain) (Table 3), while seven presented novel mutations, Ala265Thr, Ala265Asp, Gly266Cys, Asp302Gly, Cys310Trp, Ala308Pro, and Asp302Asn. In one of the mutants, MBS512, the inactivation of *smeRv* was due to the insertion of an IS3 family insertion sequence, which is present at six different positions in the genome of *S. maltophilia* D457 (accession number NC_017671.1).

SmeVWX is not involved in the intrinsic co-trimoxazole resistance of *S. maltophilia*. We have previously described that SmeDEF contributes to intrinsic and acquired co-trimoxazole resistance in *S. maltophilia* (18). The results presented in the current work indicate that SmeVWX, when overexpressed, is a relevant element in the acquisition of *S. maltophilia* co-trimoxazole resistance as well. Nevertheless, information on the role of SmeVWX in intrinsic resistance is absent. To address this issue, the susceptibility to co-trimoxazole of the MBS704 strain, which presents a deletion of the *smeW* gene (31), was determined. The susceptibility of the wild-type D457 strain, as well as mutants overexpressing either SmeDEF or SmeVWX or were defective in SmeE, was

TABLE 3 Mutations identified in co-trimoxazole-resistant *S maltophilia* strains

Co-trimoxazole conc. (mg/liter)[a]	Strain	Phenotype[b]	Mutation[c]	
			SmeRv	SmeT
2	MBS509[d]	Q	GCC→ACC (Ala265Thr)	
2	MBS510[e]	Q/CHL/ERY		CTG→CAG (Leu166Gln)
2	MBS512	Q/CHL	IS3 insertion	
2	MBS513	Q/CHL	GAC→GGC (Asp302Gly)	
2	MBS514	Q/CHL	TGC→TGG (Cys310Trp)	
2	MBS515	Q/CHL	GGC→AGC (Gly266Ser)	
2	MBS516	Q/CHL	GAC→AAC (Asp302Asn)	
2	MBS517[e]	Q/ERY		CTG→CAG (Leu166Gln)
2	MBS518[e]	Q/ERY		CTG→CAG (Leu166Gln)
2	MBS520	Q/CHL/ERY		Lys160_Ile161insLys
2	MBS522[e]	Q/CHL/ERY		Lys160_Ile161insLys
2	MBS523	Q	TGC→TTC (Cys310Phe)	
4	MBS530	Q/CHL	GGC→GAC (Gly266Asp)	
4	MBS534[d]	Q	GGC→GAC (Gly266Cys)	
4	MBS540	Q/CHL	GCG→CCG (Ala308Pro)	
8	MBS560[d]	Q/CHL	GGC→AGC (Gly266Ser)	
8	MBS563	Q/CHL	GGC→AGC (Gly266Ser)	
8	MBS567	Q/CHL	GGC→GAC (Gly266Asp)	
16	MBS571[d]	Q/CHL	GGC→GAC (Gly266Asp)	
16	MBS572	Q	GCC→GAC (Ala265Asp)	
16	MBS580	Q/CHL	GGC→AGC (Gly266Ser)	
32	MBS583	Q/CHL	GGC→GAC (Gly266Asp)	
32	MBS584	Q/CHL	GGC→GAC (Gly266Asp)	
16	MBS593	Q/CHL	GGC→AGC (Gly266Ser)	

[a]Co-trimoxazole concentration used to isolate the mutant (data refer to trimethoprim concentration, as the ratio of trimethoprim to sulfamethoxazole is 1:5).
[b]Antibiotic phenotype. Q, quinolone resistance; Q/CHL/ERY, quinolone, chloramphenicol, and erythromycin resistance; Q/CH, quinolone and chloramphenicol resistance; Q/ERY, quinolone and erythromycin resistance.
[c]Mutations identified in *smeRv* or *smeT* gene in each mutant.
[d]Overexpression of *smeV* confirmed by real time RT-PCR (see Table 2).
[e]Overexpression of *smeD* confirmed by real time RT-PCR (see Table 2).

measured. As show in Table 4, the inactivation of SmeW did not increase the susceptibility of *S. maltophilia* to co-trimoxazole, indicating that in contrast to SmeDEF, the efflux pump SmeVWX is involved in just acquired resistance, without any role in the intrinsic resistance to co-trimoxazole of *S. maltophilia*.

DISCUSSION

S. maltophilia is an opportunistic pathogen of increasing relevance at hospitals (1) and is characterized by its low susceptibility to several antibiotics (5, 6). Co-trimoxazole is one of the antimicrobials of choice for treating *S. maltophilia* infections, but unfortunately, *S. maltophilia* resistant strains are increasingly isolated from patients (15, 32) Most epidemiological surveys concentrate their analyses on the presence of *sul* genes in *S. maltophilia* co-trimoxazole-resistant clinical isolates (13–15). Nevertheless, it has been reported that some co-trimoxazole-resistant *S. maltophilia* strains do not contain *sul* genes (16, 17). This indicates that there must exist mechanisms other than the presence of *sul* with relevance for *S. maltophilia* co-trimoxazole resistance. For example, using *in vitro*-constructed mutants, we previously described that the efflux pump SmeDEF may contribute to the intrinsic and acquired (when overexpressed) resistance

TABLE 4 MICs of mutants without or overexpressing the efflux pump SmeDEF or SmeVWX

Strain	Phenotype	SXT[a] MIC (mg/liter)
D457	Wild type	0.25
MBS411	Defective in SmeE	0.19
D457R	SmeDEF overexpression	2
MBS704	Defective in SmeW	0.25
MBS287	SmeVWX overexpression	2

[a]SXT, co-trimoxazole (trimethoprim-sulfamethoxazole, 1:19) test trips.

to co-trimoxazole in *S. maltophilia* (18). Nevertheless, this study does not provide evidence on the mechanisms that are actually selected when *S. maltophilia* is challenged with co-trimoxazole.

Our results show that the mutations selected in the presence of co-trimoxazole are found in *smeT* and *smeRv*, which encode the transcriptional regulators of *smeDEF* and *smeVWX*, respectively. The regulator SmeT belongs to TetR family of transcriptional repressors, while SmeRv belongs to the LysR family of transcriptional regulators, which can be activators and repressors. In both cases, the selected mutations were localized in the C-terminal region of each regulatory protein, outside the DNA binding domain. In the case of SmeT, the C-terminal domain is mainly involved in ligand binding and dimerization, and mutations were localized in the dimerization region (33). For SmeRv, this part of the protein is predicted to be the effector binding motif, and mutations in this region, as those selected in the current work, will most likely modify the activity of this regulator. Indeed, the analyzed mutations correlate with an increased expression of the corresponding efflux pump and with changes in the susceptibility to antibiotics belonging to different structural families, a result that fits with previous results showing that the overexpression either SmeDEF or SmeVWX is associated with multidrug resistance (MDR) to several antibiotics (9, 11). In addition, our results indicate that none of the other efflux pumps described so far are overexpressed in the studied co-trimoxazole-resistant mutants.

We previously showed that under quinolone selective pressure, SmeDEF overexpression is the main mechanism of resistance among the selected mutants, while the role of SmeVWX overexpression is less relevant (11). However, when co-trimoxazole is used as the selective pressure, we observe the opposite; most mutants present a phenotype compatible with SmeVWX overexpression and just a few mutants, isolated in the presence of low co-trimoxazole concentrations, overexpress SmeDEF. These data strongly suggest that the overexpression of either SmeVWX or SmeDEF may imply a differential fitness cost when *S. maltophilia* is growing in the presence of either of the antibiotics, despite the fact that SmeDEF and SmeVWX can extrude both quinolones and co-trimoxazole. We also found that the acquisition of co-trimoxazole resistance is associated with impaired growth when resistant mutants are compared with the wild-type strain. This fitness cost may reduce the chances for the maintenance and the spread of these mutants after their selection (34). Nevertheless, it is important to recall that the overexpression of either SmeDEF or SmeVWX has been shown to be a relevant cause of *S. maltophilia* antibiotic resistance in clinical resistant isolates (9, 35–37).

It is worth mentioning that one of the risk factors for the acquisition of co-trimoxazole resistance by *S. maltophilia* is previous treatment with fluoroquinolones (38). Our results provide one explanation for these findings: SmeDEF is the major determinant of quinolone resistance in *S. maltophilia* (11, 37, 39) and its expression confers resistance to co-trimoxazole, whereas SmeVWX is also able of extruding both antibiotics when overexpressed, although its contribution to intrinsic resistance is minor.

The findings that co-trimoxazole selects multidrug-resistant mutants and that resistance to this antibiotic is not specific or confined to the presence of *sul* genes are of particular concern, given the ample use of co-trimoxazole in the treatment of *S. maltophilia* infections. Although the present results are based on the analysis of *in vitro*-selected mutants, it is important to highlight that *S. maltophilia* quinolone-resistant isolates overexpressing either SmeDEF or SmeVWX have been isolated from infected patients (9, 37, 40), providing evidence of the relevance of our *in vitro* results for understanding the selection of antibiotic-resistant mutants at hospitals.

MATERIALS AND METHODS

Strains, plasmids, and bacterial growth. The strains and plasmid used in this work are described in Table 1. To select resistant mutants, *S. maltophilia* was seeded on Mueller-Hinton (MH) agar plates (41) containing different concentrations of co-trimoxazole (trimethoprim-sulfamethoxazole [1:5], Soltrim, injectable; Laboratorios Almofarma SL, Barcelona, Spain). The concentrations of co-trimoxazole described in this article refer to the amount of trimethoprim, being the trimethoprim-sulfamethoxazole ratio is 1:5

unless otherwise stated. *Escherichia coli* strains were grown in LB (41) with kanamycin (25 mg/liter for strain 1047 carrying pRK2013 and 50 μg/ml for strains carrying pPBT04). *S. maltophilia* strains containing the plasmid pPBT04 were grown in LB with 500 μg/ml kanamycin. All strains were grown at 37°C.

Isolation of co-trimoxazole *S. maltophilia* spontaneous mutants. The concentration of co-trimoxazole most suitable for selection was determined by applying 5 μl of an overnight culture of *S. maltophilia*, as well as serial dilutions (10^{-1} to 10^{-3}) of the culture, to MH agar plates containing different amounts of the antimicrobial. The results were recorded after 24 h at 37°C.

To isolate spontaneous co-trimoxazole mutants, 100 μl of dilutions 10^{-1} (10^9 CFU) and 10^{-2} (10^8 CFU) of *S. maltophilia* overnight cultures were spread on MH agar plates containing the concentration of co-trimoxazole that inhibits growth under these conditions or concentrations above this value (2×, 4×, 8×, and 16×). Colonies were selected after 48 to 72 h of incubation at 37°C. Each chosen colony was replicated two times on MH plates without antibiotic and then again in plates with antibiotic to discard false-positive or transient adaptations to the presence of the antimicrobial.

Antibiotic susceptibility assay. MICs of erythromycin, chloramphenicol, norfloxacin, ofloxacin, nalidixic acid, and ciprofloxacin were determined by 2-fold agar dilution in MH agar plates, using a 96-pin replicator. The plates were incubated for 24 h at 37°C, and the data were the results from at least three independent assays. The co-trimoxazole MICs of the analyzed strains were determined on MH agar plates by using MIC test trips (Liofilchem).

RNA extraction and estimation of gene expression. Overnight cultures were used to inoculate 40 ml of LB at an optical density at 600 nm (OD_{600}) of 0.05. These new cultures were grown until an OD_{600} of 0.6 to 0.7, and 30 ml of each culture was recovered and centrifuged, and the RNA was extracted using an RNase kit (Qiagen) according to the manufacturer's instructions. The RNA was treated with DNase Turbo (Ambion) to remove any DNA present and cleaned using the RNase kit according to the manufacturer's instructions. Afterwards, the expression of the best-known *S. maltophilia* efflux pumps (*smeABC*, *smeDEF*, *smeGH*, *smeIJK*, *smeMN*, *smeOP*, *smeVWX*, and *smeYZ*) was analyzed by real-time RT-PCR. For this, 400 ng of RNA was retrotranscribed using the High-Capacity cDNA reverse transcription kit (Applied Biosystems). The expression of the first gene of each of the operons (*smeA*, *smeD*, *smeG*, *smeI*, *smeM*, *smeY*, *smeO*, and *smeV*) was analyzed using the primers and conditions of amplification described in reference 42. The primers for *ftsZ1/2*, which is used as a reference for normalization, are described in reference 11. The data obtained from three independent experiments were analyzed using the $2^{-\Delta\Delta CT}$ method (43).

Western blotting. It was shown that the level of SmeF expression is a good marker of SmeDEF expression (27). Hence, to compare the levels of expression between the selected mutants and the wild-type strain, whole-cell extracts from overnight cultures were loaded in 12% SDS-PAGE gels (44). After electrophoresis, the proteins were transferred to an Immobilon-P membrane (Millipore, Billerica, MA, USA), and the Western blot analysis was performed as described previously (27). SmeF was detected with a polyclonal anti-SmeF antibody (27) at a 1:5,000 dilution and with a secondary goat anti-rabbit-horseradish peroxidase (HRP) conjugate (Bio-Rad) at 1:40,000. The bands were detected by chemiluminescence using the Immobilon Western kit (Millipore).

Analysis of *smeVWX* expression. The plasmid pPBT04 (Table 1) that contains a gene encoding yellow fluorescent protein (YFP), whose expression is under the control of the promoter of *smeVWX*, was introduced into different co-trimoxazole-resistant mutants by triple conjugation as described previously (45). Transconjugants containing the plasmid were selected in LB with 500 mg/liter kanamycin and 20 mg/liter imipenem to remove *E. coli*.

Strains containing the plasmid were grown at 37°C for 22 h, and the OD_{600} of the cultures and their fluorescence (excitation at 508 nm and emission at 540 nm) were measured in a Tecan Infinite 200 plate reader.

Identification of mutations in *smeT* and *smeRv*. The complete *smeT* and *smeRv* genes were amplified using a PCR master mix (Promega) and the primers SmeRvR/SmeRvF (11) for *smeRv* and 27/43 (30) for *smeT*. The PCR products were purified using a Qiagen kit according to the manufacturer's instructions and were sequenced by Macrogen (Amsterdam, The Netherlands) using the same primers.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at https://doi.org/10.1128/AAC.00301-18.

SUPPLEMENTAL FILE 1, PDF file, 0.2 MB.

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