High-level quinolone resistance is associated with the overexpression of *smeVWX* in *Stenotrophomonas maltophilia* clinical isolates

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Abstract

Stenotrophomonas maltophilia is the only known bacterium in which quinolone-resistant isolates do not present mutations in the genes encoding bacterial topoisomerases. The expression of the intrinsic quinolone resistance elements *smeDEF*, *smeVWX* and Sm*qnr* was analysed in 31 clinical *S. maltophilia* isolates presenting a minimum inhibitory concentration (MIC) range to ciprofloxacin between 0.5 and > 32 μg/mL; 11 (35.5%) overexpressed *smeDEF*, 2 (6.5%) presenting the highest quinolone MICs overexpressed *smeVWX* and 1 (3.2%) overexpressed Sm*qnr*. Both strains overexpressing *smeVWX* presented changes at the Gly266 position of SmeRv, the repressor of *smeVWX*. Changes at the same position were previously observed in *in vitro* selected *S. maltophilia* quinolone-resistant mutants, indicating this amino acid is highly relevant for the activity of SmeRv in repressing *smeVWX* expression. For the first time SmeVWX overexpression is associated with quinolone resistance of *S. maltophilia* clinical isolates.

Keywords: Multidrug efflux pump, quinolone resistance, *S. maltophilia*, SmeDEF, SmeVWX
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Stenotrophomonas maltophilia is an opportunistic pathogen [1,2] that presents low susceptibility to antibiotics [3], likely because it harbours several chromosomally encoded resistance determinants, including efflux pumps, antibiotic-inactivating enzymes and the quinolone resistance protein SmQnr [4–8]. In addition, *S. maltophilia* is the only known bacterium in which quinolone resistance has not been associated with mutations in genes encoding bacterial topoisomerases [9–11]. Some work has shown that overexpression of the efflux pump SmeDEF correlates with quinolone resistance of *in vitro* selected mutants [11], and several clinical *S. maltophilia* isolates overexpress this pump [12–15]. *In vitro* work also shows that *S. maltophilia* quinolone resistance also may be due to the overexpression of another efflux pump, SmeVWX [11], although nothing is known about the selection of this resistance mechanism in clinical isolates. Another unexplored possibility that might be involved in the acquisition of resistance by *S. maltophilia* clinical isolates could be SmQnr overexpression.

To ascertain the role that these resistance determinants may have in the *in vivo* acquisition of *S. maltophilia* quinolone resistance, we studied the susceptibility to quinolones and the expression of *smeDEF*, *smeVWX* and Sm*qnr* in a collection of 31 *S. maltophilia* clinical strains, isolated at the Hospital Universitario Marqués de Valdecilla, Santander (Spain). The strains were identified by MicroScan (Siemens Healthcare, Erlangen, Germany) and API 20NE (BioMérieux, Marcy l'Etoile, France) and their identification as *S. maltophilia* was further confirmed by species-specific polymerase chain reaction (PCR) [16] and by sequencing the 16S rRNA gene using PA[B27F] and PLO6-R primers [17]. To discard widely distributed clones that might

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bias our results, the phylogenetic relationship of the isolates was determined by repetitive element palindromic PCR [18]. Although some strains such as 452 and 3759 present a similar electrophoretic profile, the collection is not biased by the presence of predominant clones (Fig. 1).

Minimum inhibitory concentrations (MICs) were determined by Etest (BioMérieux, Marcy l'Etoile, France) using Mueller Hinton agar (Pronadisa, Madrid, Spain). *S. maltophilia* D457 (19) and two of its isogenic mutants, D457R [7,19] (which overexpresses *smeDEF*) and MBS287 (which overexpresses *smeVWX*) [11], were used as controls. RNA was obtained as described [11].

SmeDEF extrudes chloramphenicol, tetracycline and quinolones [7]. Consistent with a potential SmeDEF overexpression, several strains presented lower levels of susceptibility to these antibiotics than the control strain D457 (Table 1). To study this possibility, *smeD* expression was analysed in the 31 clinical strains by RT-PCR with one-step reaction Illustra Ready-to-Go RT-PCR Beads Kit (GE Healthcare, Freiburg, Germany) using primers 1/2 for *smeD* [15] and FtsZ1/FtsZ2 for the reference gene *ftsZ* [11]; 35.5% (11 of 31) of the strains overexpressed *smeD*. We have previously shown that expression of the outer membrane protein of SmeDEF, SmeF, correlates with the expression of *smeD* RNA [7,15]. In other words, expression of SmeDEF at the protein level correlates with its expression at the RNA level. To further confirm our results, the expression of SmeF was analysed using specific antibodies as described [7]. The same 11 strains overexpressing *smeD* overexpressed SmeF (Table 1). These data agree with previous information, showing that SmeDEF overexpression is frequent in clinical *S. maltophilia* isolates [12–15]. The fact that other isolates such as 6632 or 7274 (Table 1) also present low susceptibility to quinolones without overexpressing SmeDEF indicates that other quinolone resistance mechanisms must be acting.

In vitro selected quinolone-resistant mutants may present increased *smeVWX* expression [11]. To decipher whether this novel mechanism of quinolone resistance is selected in clinical *S. maltophilia* isolates, the level of *smeW* expression was analysed in the 31 clinical strains by quantitative reverse

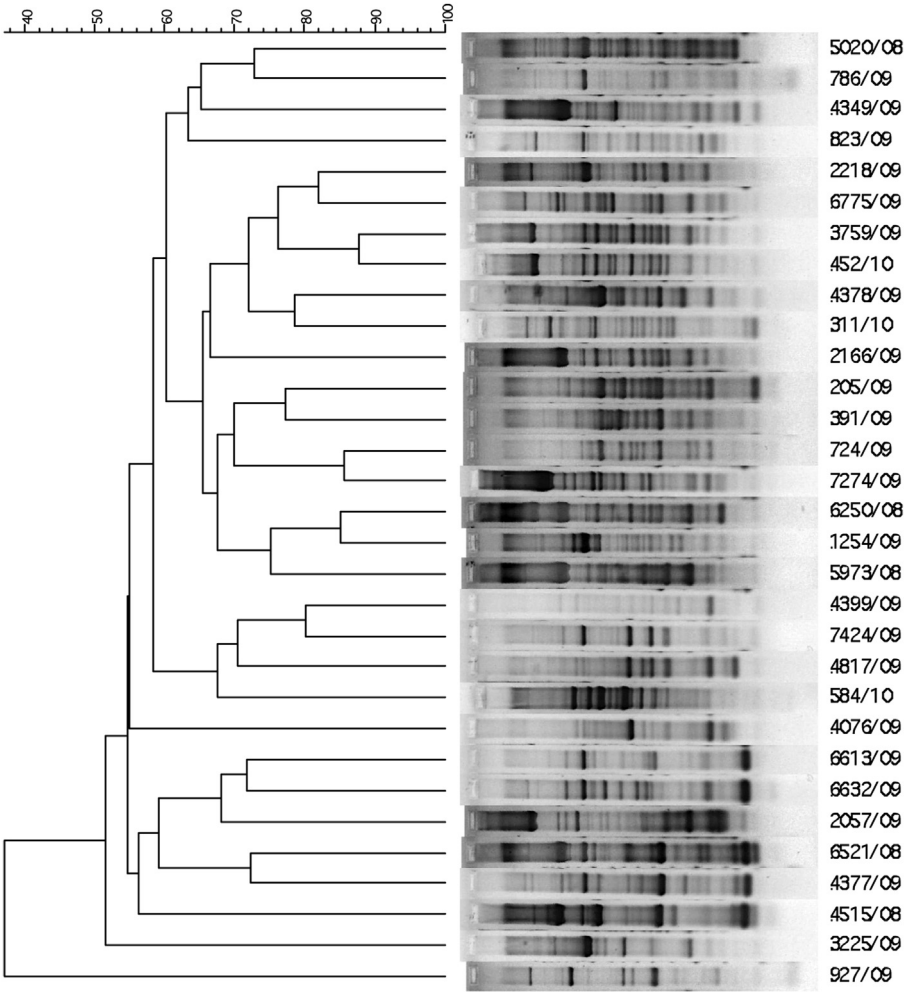

FIG. 1. Analysis of the phylogenetic relationship of the studied *S. maltophilia* clinical isolates. The phylogenetic relationship of the strains was determined by analysing the band patterns obtained by repetitive element palindromic polymerase chain reaction. The electrophoretic profiles were analysed using FPQuest II software version 4.5 (BioRad). As shown in the figure, although some isolates presented similar patterns, there were not predominant clones in the studied strains.

TABLE 1. Antibiotic susceptibilities and expression of efflux pumps *smeDEF* and *smeVWX* of *S. maltophilia* clinical strains

Strain	Origin	MIC (μg/mL)					Overexpression*	
		NAL	CIP	MOX	TET	CHL	*smeVWX*	*smeDEF*
2057	Sputum	>256	>32	>32	>256	24	+	−
6775	Sputum	>256	>32	>32	4	>256	+	−
205	Urine	>256	2	0.125	>256	96	−	+
1254	Tracheal aspirate	64	1.5	0.250	96	12	−	+
2218	Sputum	48	2	0.250	24	8	−	+
4349	Surgical wound exudate	48	6	0.125	>256	64	−	+
4377	Skin ulcer	32	3	0.19	>256	24	−	+
391	Skin ulcer	32	2	0.125	>256	192	−	+
6613	Surgical wound exudate	24	0.75	0.064	>256	16	−	+
4378	Diabetes foot exudate	16	0.75	0.064	>256	32	−	+
5020	Blood culture	12	1	0.125	>256	24	−	+
4076	Sputum	8	1	0.094	24	12	−	+
311	Tracheal aspirate	8	0.5	0.032	24	16	−	+
7274	Urine	>256	6	0.5	>256	8	−	−
6632	Skin ulcer	>256	6	0.125	>256	32	−	−
724	Sputum	96	1.5	0.125	>256	32	−	−
7424	Diabetes foot exudate	48	3	0.094	>256	24	−	−
3759	Sputum	32	1	0.125	32	4	−	−
6521	Sputum	32	1	0.125	>256	8	−	−
4399	Articular biopsy	32	1.5	0.125	>256	16	−	−
927	Brain biopsy	24	0.5	0.023	>256	4	−	−
786	Blood culture	24	1.5	0.094	>256	32	−	−
3225	Surgical wound exudate	16	1	0.064	12	3	−	−
823	Bronchoalveolar lavage	16	3	0.5	64	8	−	−
4515	Surgical wound exudate	16	2	0.5	>256	12	−	−
4817	Surgical wound exudate	12	0.75	0.064	32	16	−	−
2166	Urine	8	0.75	0.094	32	4	−	−
452	Pleural fluid	8	0.5	0.032	>256	12	−	−
584	Surgical wound exudate	8	0.75	0.064	24	48	−	−
5973	Sputum	4	0.75	0.047	64	4	−	−
6250	Abdominal drainage	3	0.5	0.064	32	2	−	−
D457	Bronchial aspirate	12	1.5	0.190	6	4	−	−
D457R	D457 mutant in *smeT*	128	>32	0.750	48	96	−	+
MBS287	D457 mutant in *smeRv*	>256	>32	0.750	6	>256	+	−

NAL, nalidixic acid; CIP, ciprofloxacin; MOX, moxifloxacin; TET, tetracycline; CHL, chloramphenicol.
*Efflux pump with (+) or without (−) overexpression.

transcriptase PCR (RT-qPCR) as described [11]. Strains 2057 and 6775, which present the highest MICs to quinolones (Table 1), showed higher levels of *smeVWX* expression than our control strain D457 (fold changes 2.65 ± 0.79 and 19 ± 10.66, respectively). To the best of our knowledge this is the first time that overexpression of *smeVWX* has been associated with high-level quinolone resistance in *S. maltophilia* clinical strains. *In vitro* selected *S. maltophilia* mutants overexpressing *smeVWX* contain mutations in the gene encoding the repressor of the pump, *smeRv* [11]. Consequently, the *smeRv* alleles from 2057 and 6775 were PCR-amplified and sequenced as described [11]. Both isolates presented mutations at *smeRv* in comparison with the sequence of the wild-type allele of the control strain D457; 2057 presented one Gly266Ser mutation, whereas 6775 presented three mutations: Gly46Asp, Thr222Pro and Gly266Asp. The mutations at positions Gly266, Gly266Ser and Gly266Asp have been described previously in mutants obtained *in vitro* [11]. Together with our results, it indicates that this amino acid is relevant for SmeRv activity. This substitution of glycine for serine or aspartic acid results in *smeVWX* overexpression and consequently high-level quinolone resistance in *S. maltophilia*.

Five of the studied strains presented nalidixic acid MICs >256. Two of them (2057 and 6775) overexpressed *smeVWX*, and another (205) overexpressed *smeDEF*. In addition to SmeDEF and SmeVWX, *S. maltophilia* encodes in its genome six RND efflux pumps, SmeABC, SmeGH, SmeIJK, SmeMN, SmeOP and SmeYZ [4]. To determine whether the high-level resistance to quinolones observed in strains 7274 and 6632 was due to the overexpression of any of these efflux pumps, their level of expression was analysed by RT-qPCR as described [20]. None of these pumps was overexpressed in strains 7274 and 6632. Although it has been reported that *S. maltophilia*–resistant mutants do not present mutations in the genes encoding bacterial topoisomerases, it would be possible that this type of mutations could be responsible for the resistance of strains 7274 and 6632. To address this possibility, the quinolone resistance determining regions (QRDRs) of the topoisomerase genes (*gyrA*, *gyrB*, *parC* and *parE*) were amplified and sequenced in the strains 7274 and 6632, as described [21]. In agreement with previous studies [9,21], we did not find significant mutations in the studied QRDRs.

Irrespective of efflux pumps, the deletion of Sm*qnr* has been reported to increase quinolone susceptibility in *S. maltophilia*, whereas its overexpression under control of a heterologous promoter in a plasmid reduces such susceptibility [22]. Sm*qnr* expression was analysed in the 31 clinical isolates by RT-qPCR as described [22] using primers RTqnr7 (5′

AGTGGTCGACCAGCAGTTC 3′) and RTqnr8 (5′ CATCGTAGAAGCTGCAGTTGATG 3′). Only *S. maltophilia* 6775 showed higher Sm*qnr* expression than our wild-type control strain D457 (2.4 ± 0.2). Because 6775 also overexpresses SmeVWX, it is difficult to evaluate the role that *Smqnr* overexpression may have in the high-level quinolone resistance of this isolate. These results are in agreement with studies on *in vitro* selected *S. maltophilia* quinolone-resistant mutants; none of which presented increased Sm*qnr* expression [11].

Our data indicate that overexpression of the efflux pumps SmeDEF or SmeVWX is frequent in clinical isolates of *S. maltophilia*, whereas overexpression of the chromosomally encoded Qnr protein SmQnr does not seem to be a relevant mechanism for the acquisition of quinolone resistance in the studied isolates. To the best of our knowledge this is the first study reporting that overexpression of efflux pump *smeVWX*, due to mutations in its regulator SmeRv, is associated with high-level quinolone resistance in *S. maltophilia* clinical isolates.

Transparency declaration

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