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Expression of Multidrug Efflux Pump SmeDEF by Clinical Isolates of *Stenotrophomonas maltophilia*

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The presence of the multidrug efflux pump SmeDEF was assessed in a collection of clinical isolates of *Stenotrophomonas maltophilia*. All isolates encoded this pump, as demonstrated by PCR. Forty-seven percent of the strains overproduced a protein of the same size that was immunoreactive against an anti-SmeF antibody, and 33% overexpressed the gene *semD* when they were tested by reverse transcription-PCR. A correlation between *smeDEF* overexpression and antibiotic resistance was observed.

Stenotrophomonas maltophilia is an opportunistic pathogen that has been associated with different human pathologies (5) and that is considered an archetype of antibiotic-resistant bacteria. It has been demonstrated that *S. maltophilia* produces beta-lactamases (10, 13, 14), aminoglycoside acetyltransferases (7), and erythromycin-inactivating enzymes (1), with an obvious role in the antibiotic resistance phenotype of this bacterial species. Like all bacterial species studied so far (9, 11), *S. maltophilia* probably contains several multidrug resistance (MDR) efflux pumps, as demonstrated by the analysis of multidrug-resistant mutants that can be obtained upon antibiotic selective pressure (15). In a recent article, we have described *smeDEF*, the first MDR pump so far cloned and characterized in this bacterial species (2). In the present work, we have analyzed both the presence and the levels of expression of the SmeDEF pump in a collection of clinical isolates of *S. maltophilia* and correlated the results of those analyses with those obtained from the study of the susceptibilities of such isolates to antibiotics.

TABLE 1. Clinical strains of *S. maltophilia* used in the present work

Strain	Isolation date	Sample
D457	January 1992	Bronchial aspirate
C048	November 1990	Urine
C357	March 1991	Urine
D388	December 1991	Urine
E301	October 1992	Urine
E539	January 1993	Pus from a wound
E729	March 1993	Urine
E759	March 1993	Sputum
E824	April 1993	Blood
E923	June 1993	Sputum
E999	July 1993	Respiratory secretion
F227	November 1993	Blood
F375	January 1994	Blood
F861	August 1994	Sputum
G51	November 1994	Blood

The clinical strains of *S. maltophilia* used in the present work were isolated from different sources at the Hospital of Mostoles (Madrid, Spain) (Table 1). *S. maltophilia* strain D457R is a single-step multidrug-resistant spontaneous mutant that is derived from *S. maltophilia* D457 and that overexpresses the SmeDEF multidrug efflux system (3). Bacteria were routinely cultured at 37°C in Luria-Bertani medium (4). The MICs of antibiotics were determined in Mueller-Hinton agar (4) by the E-test method (AB Biodisk), according to the manufacturer's instructions.

To assess the presence of the *smeDEF* efflux system among *S. maltophilia* isolates, chromosomal DNA (obtained with the Genome DNA Kit; Bio 101) from 15 clinical strains obtained from different clinical sources was subjected to PCR amplification with primers specific for the *smeD* gene. An internal fragment of 150 bp from the *smeD* gene was amplified with primers 1 (5′-CCAAGAGCCTTTCCGTCAT-3′) and 2 (5′-TCTCGGACTTCAGCGTGAC-3′). A total of 100 ng of chromosomal DNA was used as the template for each PCR. The reaction mixture (50 μl) contained each deoxynucleotide (dCTP, dGTP, dATP and dTTP) at a concentration of 0.2 mM, 0.5 μM each primer, 1.5 mM $MgCl_2$, 10 mM Tris-HCl (pH 8.3), 50 mM KCl, and 1.0 U of *Taq* DNA polymerase. The mixture was heated at 94°C for 90 s, followed by 35 cycles of 30

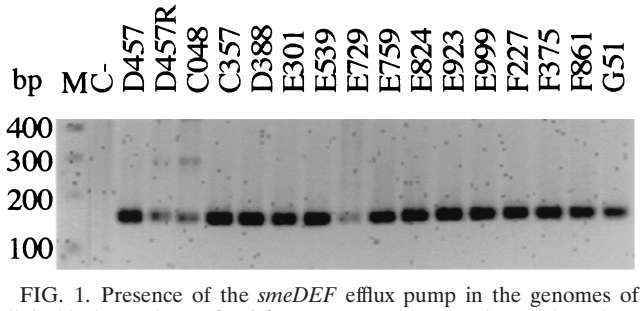

FIG. 1. Presence of the *smeDEF* efflux pump in the genomes of clinical isolates of *S. maltophilia*. Lane M, molecular size markers; lane C−, negative control, for which the same PCR reaction was performed but without the addition of DNA. A band of the predicted size was observed in all isolates.

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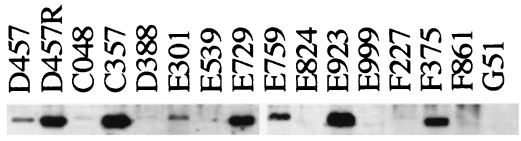

FIG. 2. Expression of SmeF by clinical isolates of *S. maltophilia*. Whole-cell protein extracts of *S. maltophilia* were obtained and electrophoresed in SDS–10% polyacrylamide gels. Western blot analysis was performed with an anti-SmeF antibody.

s at 94°C, 60 s at 58°C, and 90 s at 72°C and, finally, one 10-min extension step at 72°C before the end of the reaction. PCR products were run on 2.0% agarose gels and stained with ethidium bromide. Figure 1 shows that a PCR product of the expected size was detected in all strains analyzed. This demonstrates that the *smeDEF* system was ubiquitously found in *S. maltophilia* isolates.

It has been described that overexpression of the outer membrane protein SmeF, the gene of which is a part of the *smeDEF* operon, is associated with increased levels of resistance for in vitro-selected multidrug-resistant mutants of *S. maltophilia* (2, 3). To test whether overexpression of the *smeDEF* operon was also associated with increased levels of resistance in clinical isolates of *S. maltophilia*, we analyzed both the expression of the SmeF protein by Western blotting and *smeD* RNA by reverse transcription-PCR (RT-PCR). Whole-cell extracts from clinical *S. maltophilia* isolates were electrophoresed on sodium dodedcyl sulfate (SDS)-polyacrylamide gels and immunoblotted as described previously with a polyclonal antibody raised against the outer membrane efflux component of SmeDEF, SmeF (2). As shown in Fig. 2, an immunoreactive band of the same size as SmeF was detected in 7 of 15 clinical isolates (strain D457R is not a clinical isolate but is a mutant derivative that has been included in the analysis as a control).

Some outer membrane components of efflux MDR pumps, such as OprM of *Pseudomonas aeruginosa* (8, 16) and TolC of *Escherichia coli* (6), are associated with more than one MDR system. In order to assess if SmeF expression was linked to the expression of the *smeDEF* operon, we analyzed the expression of *smeD*, the first gene of the operon, by RT-PCR analysis. RNA was obtained with the TriReagent-LS Kit (Molecular Research Center Inc.) according to the manufacturer's instructions. Residual DNA was eliminated with RNase-free DNase (Boehringer Mannheim), followed by acid phenol treatment and RNA precipitation with ethanol. The RNA concentration and purity were estimated by spectrophotometric analysis at 260 and 280 nm, and the RNA quality was analyzed by electrophoresis on 1% agarose gels under denaturing conditions (12). For RT-PCR analysis, a two-step reaction was carried out. First, 5 μg of RNA was incubated for 1 h at 37°C with primer 2 and avian myeloblastosis virus (AMV) reverse transcriptase (U.S. Biochemicals) to obtain cDNA. cDNA was then PCR amplified with primers 1 and 2 as described above. Amplicons were analyzed on 2% agarose gels and stained with ethidium bromide. The same reaction protocol performed for RT-PCR was carried out for each sample, but without AMV reverse transcriptase, as a control to detect DNA contamination in the RNA preparations, with negative results in all cases. The amount of *smeD* RNA was analyzed from bacteria grown to the early exponential or stationary growth phase because it was previously demonstrated that *smeDEF* expression is induced at the exponential phase of growth and is very low at the stationary phase of growth (2). As shown in Fig. 3, an RT-PCR product of the predicted size was found in 5 of the 15 isolates analyzed. All of the isolates with a positive RT-PCR result also expressed a protein immunoreactive against the anti-SmeF antiserum, indicating that expression of *smeD* and SmeF was linked in these strains. Two isolates (isolates E301 and E759), however, overproduced a protein that was of the same size as SmeF and that was immunoreactive against the SmeF antibody, but a RT-PCR band was not detected in our analysis. Two possibilities might explain these results: (i) the immunoreactive band detected in E301 and E759 was SmeF, but its expression was associated with other efflux systems in these strains, so that SmeF was expressed but *smeD* was not; (ii) the immunoreactive band detected in E301 and E759 was not SmeF but another protein which shares some structural features with SmeF and which is then recognized with the polyclonal anti-SmeF antibody, so that neither SmeF nor *smeD* was expressed in these isolates. In any case, it is thus clear that *smeDEF* is not expressed by these two strains.

The expression of *smeDEF* was correlated with the suscep-

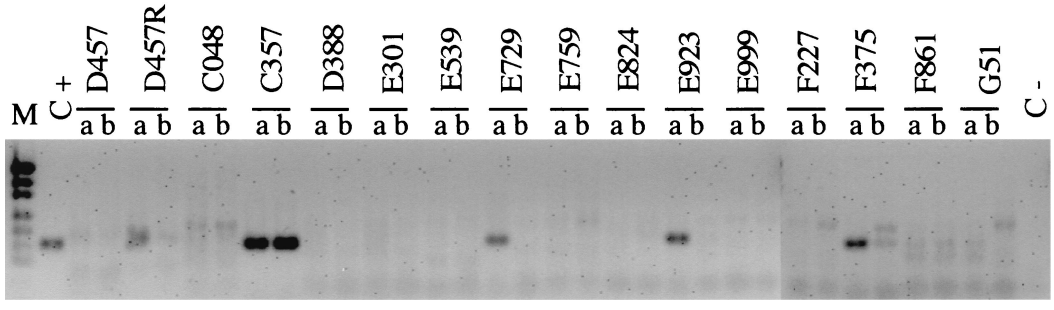

FIG. 3. Expression of *smeDEF* RNA by clinical isolates of *S. maltophilia*. The expression of *smeDEF* was analyzed by RT-PCR. RNAs obtained at the exponential (lanes a) and stationary (lanes b) growth phases were analyzed. A clear band of the predicted size was observed for strains D457R, C357, E729, E923, and F375; and a very faint band was observed for strain D457. Note that strain D457R is not a clinical isolate but a multidrug-resistant mutant derived from strain D457 (3), and it has been included in the present analysis as a control for *smeDEF* expression. Lane M, molecular size markers (from top to bottom, 404, 331, 242, 190, 157, 147, and 112 bp); C+, positive control, for which PCR was performed with chromosomal DNA from *S. maltophilia* D457 as the template; C−, negative control, for which PCR was performed without DNA.

TABLE 2. Antibiotic susceptibilities of *S. maltophilia* clinical strains

Strain	MIC (μg/ml)[a]							
	TET	CHL	AMK	TIC	ERY	NAL	NOR	OFX
D457	6	1.5	24	4	48–64	8	6	3
D457R	16	12	16	6	>256	128	64	>32
C048	4	1.5	6	0.75	128	12	3	2
C357	32	96	4	24	>256	128	256	>32
D388	16	12	96	32	96	8	8	2
E301	12	4	3	16	128	12	3	1.0
E539	8	4	>256	32	96	12	4	2
E729	32	16	16	1	256	32	>256	8
E759	16	16	24	>256	>256	12	8	4
E824	4	8	16	4	192	3	2	0.38
E923	12	64	12	96	>256	16	16	3
E999	12	12	256	8	>256	16	8	3
F227	4	4	24	48	16	4	3	0.38
F375	12	16	128	16	>256	128	32	16
F861	3	2	32	96	24	4	2	0.5
G51	6	3	12	192	96	16	2	1

[a] TET, tetracycline; CHL, chloramphenicol; AMK, amikacin; TIC, ticarcillin-clavulanic acid; ERY, erythromycin; NAL, nalidixic acid; NOR, norfloxacin; OFX, ofloxacin.

tibilities to antibiotics of the collection of clinical isolates described in Table 1. The MICs of tetracycline, chloramphenicol, erythromycin, and the quinolones were, overall, higher for the strains in which expression of SmeF was detectable by Western blotting and in which *smeD* was detectable by RT-PCR (33% of the isolates) than for the isolates that did not express the protein at detectable levels. This relationship was not observed for the MICs of amikacin or ticarcillin-clavulanic acid. Noteworthy increased levels of resistance to tetracycline, chloramphenicol, erythromycin, and the quinolones, with no effect on the MIC of amikacin or ticarcillin-clavulanic acid, were observed in the multidrug-resistant mutant *S. maltophilia* strain D457R (which overexpresses SmeF) compared to its isogenic wild-type *S. maltophilia* strain D457 (Table 2). The same substrate range was observed when *smeDEF* was cloned in a low-copy-number plasmid and introduced in the heterologous host *E. coli* (3).

Our data indicate that *smeDEF* is overexpressed by 33% of the clinical isolates analyzed in our work and suggest that its overexpression might contribute to increased levels of resistance to tetracycline, chloramphenicol, erythromycin, and quinolones in clinical isolates of *S. maltophilia*.

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