ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, Nov. 2002, p. 3386–3393 Vol. 46, No. 11
0066-4804/02/$04.00+0 DOI: 10.1128/AAC.46.11.3386–3393.2002
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Cloning and Characterization of SmeT, a Repressor of the *Stenotrophomonas maltophilia* Multidrug Efflux Pump SmeDEF

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Received 7 February 2002/Returned for modification 20 May 2002/Accepted 27 July 2002

We report on the cloning of the gene *smeT*, which encodes the transcriptional regulator of the *Stenotrophomonas maltophilia* efflux pump SmeDEF. SmeT belongs to the TetR and AcrR family of transcriptional regulators. The *smeT* gene is located upstream from the structural operon of the pump genes *smeDEF* and is divergently transcribed from those genes. Experiments with *S. maltophilia* and the heterologous host *Escherichia coli* have demonstrated that SmeT is a transcriptional repressor. S1 nuclease mapping has demonstrated that expression of *smeT* is driven by a single promoter lying close to the 5′ end of the gene and that expression of *smeDEF* is driven by an unique promoter that overlaps with promoter *PsmeT*. The level of expression of *smeT* is higher in *smeDEF*-overproducing *S. maltophilia* strain D457R, which suggests that SmeT represses its own expression. Band-shifting assays have shown that wild-type strain *S. maltophilia* D457 contains a cellular factor(s) capable of binding to the intergenic *smeT-smeD* region. That cellular factor(s) was absent from *smeDEF*-overproducing *S. maltophilia* strain D457R. The sequence of *smeT* from D457R showed a point mutation that led to a Leu166Gln change within the SmeT protein. This change allowed overexpression of both *smeDEF* and *smeT* in D457R. It was noteworthy that expression of wild-type SmeT did not fully complement the *smeT* mutation in D457R. This suggests that the wild-type protein is not dominant over the mutant SmeT.

Stenotrophomonas maltophilia is an opportunistic pathogen that has been associated with different human pathologies (16). The main problem in the treatment of infections caused by *S. maltophilia* is the intrinsic antibiotic resistance phenotype displayed by this bacterial species. The basis of this resistance relies on the presence of several antibiotic resistance genes in the genome of *S. maltophilia*, including genes for beta-lactamases (30, 39, 40), aminoglycoside acetyltransferases (23), erythromycin-inactivating enzymes (3), and several multidrug resistance (MDR) efflux pumps (2, 4, 42). We have previously reported on the cloning of *smeDEF*, the first MDR pump characterized in *S. maltophilia* (2). At the time of the writing of this article, Poole and colleagues (24) reported on the cloning of another efflux pump (SmeABC), the outer membrane component of which (SmeC) also contributed to multidrug resistance in *S. maltophilia*. In a survey of clinical *S. maltophilia* isolates, we found that the *smeD* gene was present in all *S. maltophilia* strains analyzed and that mutants with overall higher levels of antibiotic resistance, in which the expression of *smeDEF* was derepressed, were frequent among the clinical isolates of this bacterial species (1). It has also been described that SmeDEF contributes to intrinsic resistance to antibiotics in *S. maltophilia* (43).

In several of the models analyzed so far, the expression of MDR pumps is tightly down-regulated by proteins encoded in genes frequently located near the structural operons of the pumps (17). Overexpression of MDR pumps is usually due to mutations in these regulatory genes. Furthermore, it has been described that expression of efflux pumps can be triggered by natural compounds, such as bile salts (26), with potential relevance for the induction of phenotypic resistance during infection (27). For these reasons, the study of the regulation of antibiotic resistance determinant expression is a relevant topic in the field of antibiotic resistance. To analyze the basis for the regulation of *smeDEF* expression, we have cloned a new gene (hereafter named *smeT*) located upstream of the *smeDEF* operon and involved in its transcriptional repression. SmeT belongs to the TetR and AcrR family of transcriptional repressors. This family of repressors comes from several members of the gram-positive and gram-negative bacteria (see http://www.ebi.ac.uk/interpro/IEntry?ac=IPR001647 for a description of this family). The primary amino acid structure of the family is not highly conserved, but all proteins of the TetR and AcrR group share structural motifs that include a helix-turn-helix (HTH) DNA-binding motif, a dimerization region, and a variable region implied in the binding of tetracycline in the case of the Tet repressors (7). Results from our work suggest that SmeT is a repressor not only of *smeDEF* transcription but also of *smeT* transcription. Mapping of the transcription start sites for *smeDEF* and *smeT* indicates that both promoters overlap, suggesting the possibility of promoter cross-interference between these two genes.

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MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. The bacterial strains and plasmids used in the present work are shown in Table 1. In all cases, bacteria were grown in Luria-Bertani (LB) medium (5) supplemented with the following antibiotics, if needed, at the indicated concentrations: ampicillin, 100 μg/ml;

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TABLE 1. Bacteria and plasmids

Strain or plasmid	Description[a]	Reference or source
Strains		
E. coli		
TG1	*supE hsdΔ5 thi* Δ(*lac-proAB*) F′[*traD36 proAB*$^+$ *lacI*^{q} *lacZ*ΔM15]	33
HB101	*supE44 hdsS20*(rβmβ) *recA13 ara-14 proA2 lacY1 galK2 rpsL20 xyl-5 mtl-1*	33
M15(pREP4)	A K-12-derived *E. coli* strain that carries plasmid pREP4; Nal^s Str^s Rif^s *lac ara gal mtl* F$^-$ *recA*$^+$ *uvr*$^+$	Qiagen, Inc.
S. maltophilia		
D457	From a bronchial aspirate	4
D457R	Single-step spontaneous SmeDEF-overproducing mutant derived from D457	2, 4
Plasmids		
pLAFR3	Low-copy-number cloning cosmid	38
pAS1	pLAFR3 containing the *smeDEF* operon	2
pAS5	pLAFR3 containing an *S. maltophilia* D457R genome fragment that overlaps with *smeDEF*	This study
pGEMT-Easy	Ap^r, vector for cloning of PCR products	Promega
pVLT31	Tc^r, RSF1010-*lacI*^{q}/*Ptac* hybrid broad-host-range expression vector, MCS of pUC18	12
pBluescript II KS(+)	Ap^r, cloning vector	33
pPS1	pVLT31 derivative plasmid that carries the wild-type *smeT* gene from D457	This study
pUJ8	Ap^r, ori ColE1, *tpr′-′lacZ* promoter probe plasmid vector, *lacZ* fusions type I	13
pPS3	PUJ8 derivative plasmid that carries a 224-pb *Eco*RI fragment containing *PsmeDEF*::*lacZ*	This study
pALTER-Ex2	Tc^r, ori p15a	Promega
pPS4	PALTER-Ex2 derivative plasmid that carries wild-type *smeT* with its own promoter sequence	This study
pPS5	pALTER-Ex2 derivative plasmid that carries L166Q *smeT* from *S. maltophilia* D457R with its own promoter sequence	This study
pQE31	Expression vector to create fusion proteins tagged with the six-histidine tag, Ap^r	Qiagen, Inc.
pPREP4	Plasmid that carries the *lacI* gene encoding the *lac* repressor; used to regulate expression of recombinant proteins tagged with the six-histidine tag	Qiagen, Inc.
pPS6	pQE31 derivative plasmid that carries a *Kpn*I-*Pst*I restriction fragment countering *smeT*	This study
pRK600	Cm^r, ori ColE1, RK2-Tra$^+$, helper plasmid in triparental mating	21

[a] Km, kanamycin; Rif, rifampin; Ap, ampicillin; Cm, chloramphenicol; Tc, tetracycline; Nal, nalidixic acid; Str, streptomycin.

chloramphenicol, 25 μg/ml; and kanamycin and tetracycline, 12 μg/ml. Selection of *S. maltophilia* D457 and D457R exconjugants was made in LB supplemented with 20 μg of imipenem per ml and 30 or 60 μg of tetracycline per ml, respectively.

Cloning of *smeT*. Chromosomal DNA of *S. maltophilia* D457R was obtained with the Genome DNA Kit (Bio 101) according to the instructions of the manufacturer. A cosmid (pLAFR3)-based chromosomal DNA library was constructed from *S. maltophilia* D457R and introduced into phage particles as described previously (2). The phage particles were used to infect *Escherichia coli* HB101, and tetracycline-resistant colonies were transferred to nylon membranes and subjected to denaturation and renaturation as described previously (33). An internal 150-bp fragment from the *smeD* gene was amplified as described previously (1), with two modifications: dATP was replaced by 0.32 mM [α-^{32}P]dATP (50 mCi), and 100 ng of chromosomal DNA from *S. maltophilia* D457R was used as the template. The labeled fragment was used as a probe for the screening of the aforementioned library by Southern blotting by previously described procedures (33). Restriction and Southern analyses of cosmids from positive clones were performed (33), and cosmid pAS5 was selected for further analysis. DNA sequencing of a 1-kbp region upstream of the *smeDEF* operon was done by primer walking as described previously (2).

Primers *sme* 27 (5′-TGCCAGCGACAGTGCAAAGGGTC-3′) and *sme* 43 (5′-CCAGGATCATCGATCTGCC-3′) were used to amplify a 980-bp fragment which contains the *smeT* gene and the *smeD-smeT* intergenic region from strains D457 and D457R, as described previously (1). *Vent* DNA polymerase (New England BioLabs) was used for the amplification. In all cases, both strands were sequenced.

The DNA sequences of the open reading frames were analyzed with the CodonPreference program from the University of Wisconsin Genetics Computer Group. Screening of the EMBL database was performed by using the BLAST network service of the Swiss Institute of Bioinformatics. Predictive structural analysis of SmeT was done at the PredictProtein server (http://maple.bioc.columbia.edu/predictprotein/).

DNA manipulations. PCR amplification of *smeT* together with its own promoter was performed with chromosomal DNA from *S. maltophilia* strains D457 and D457R, which were obtained as described above, by using primers *sme* 42 (5′-GTGAAAGCCCGCAGATCG-3′) and *sme* 43 (see above). The *smeT* gene without its promoter was amplified by using primers *smeT* 4 (5′-GCAGCCTCG TTCACGCCTC-3′) and *smeT* 5 (5′-ATGGCCCGCAAGACCAAAGAG-3′). In both cases the PCR mixture (0.2 mM each deoxynucleotide triphosphate, 0.5 μM each primer, 1.5 mM $MgCl_2$, 10 mM Tris-HCl [pH 8.3], 50 mM KCl, 1 U of *Taq* DNA polymerase, 100 ng of chromosomal DNA) was heated at 94°C for 3 min, followed by 32 cycles of 60 s at 94°C, 60 s at 55°C, and 90 s at 72°C, with a final 10-min extension step at 72°C. The PCR products obtained were purified with Micro Bio-Spin chromatography columns (Bio-Rad), cloned in the pGEMT-Easy vector (Promega), and recovered as *Eco*RI fragments. The *Eco*RI fragment containing the *smeT* gene without its promoter was cloned under control of *Ptac* promoter in plasmid pVLT31, rendering plasmid pPS1, and in pBluescript II KS(+) in order to add restriction sites. The *smeT* gene, obtained as a *Kpn*I-*Pst*I fragment from pBluescript II KS(+), was cloned in the appropriate orientation in plasmid pQE-31 (Qiagen, Inc.), rendering plasmid pPS6, to generate an SmeT protein tagged at the N terminus with a six-histidine tag. Confirmation of the construction took place by sequencing with primer PQE30 (5′-ATGAGAGGA TCGCATCACCATC-3′). Plasmid pPS6 was introduced in *E. coli* M15, which contains plasmid pREP4, which carries the *lacI* gene encoding the *lac* repressor, by transformation. Plasmids pPS4 and pPS5 are pALTER-Ex2 (Promega) derivatives that carry the *Eco*RI fragment from pGEMT-Easy containing either the wild-type *smeT* or the Leu166Gln mutant and their promoter sequences. Plasmids pPS1, pPS4, and pPS5 were introduced into *E. coli* TG1 by transformation as described previously (33). The orientations of the inserts were confirmed by sequencing with primer *Ptac* (5′-GACAATTAATCATCGGCTCG-3′) for pPS1 and reverse M13 primer (33) for pPS4 and pPS5.

Primers *sme* 46 (5′-GGGTGTGGGTACGAGTGC-3′) and *sme* 47 (5′GACG GAAAGGCTCTTGGAG-3′) were used for PCR amplification of the intergenic *smeT-smeD* sequence under the same conditions described above. After purification this PCR product was cloned in pGEMT-Easy vector (Promega); recovered as an *Eco*RI restriction fragment; cloned (yielding plasmid pPS3) in the *Eco*RI site of promoter-reporter plasmid pUJ8, which carries a promoterless *lacZ* gene downstream of its multiple cloning site; and introduced in *E. coli* TG1

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by transformation. Sequencing with primer *PUJ* 8 (5′-TTGTACTGAGAGTGC ACC-3′) confirmed the orientation of the *PsmeDEF*::*lacZ* fusion in plasmid pPS3.

Conjugation of *S. maltophilia*. Introduction of pPS1 in *S. maltophilia* strains D457 and D457R was done by conjugation by triparental mating, as described before (15), by using plasmid pRK600 as the helper for transfer functions. Exconjugants were selected in LB supplemented with 20 μg of imipenem per ml and tetracycline at 30 μg/ml for *S. maltophilia* D457 and 60 μg/ml for D457R. *Eco*RI digestion of plasmid minipreps confirmed the presence of pPS1 in both *S. maltophilia* D457 and *S. maltophilia* D457R.

Protein analysis. Whole-cell lysates from *S. maltophilia* D457 and D457R with and without plasmid pPS1 were obtained from overnight and early- logarithmic-phase (optical density at 600 nm, 0.3) cultures, analyzed on sodium dodecyl sulfate–10% (wt/vol) polyacrylamide gels with the Bio-Rad Protean minigel system, and stained with GelCode Blue (Pierce). The protein concentration was determined by the bicinchoninic acid assay (Pierce). Prestained molecular markers were from Bio-Rad. For Western blot analysis, proteins were transferred to a polyvinylidene difluoride membrane (Millipore), stained with Ponceau S (31) to confirm that equal amounts of protein had been loaded in each lane, and analyzed with polyclonal antibody raised against SmeF (2) at a final dilution of 1:5,000. Horseradish peroxidase-conjugated protein A (Sigma) was used at a final concentration of 0.25 μg/ml, and detection of immunoreactive bands was performed by chemiluminescence as described previously (31).

Purification of SmeT with a six-histidine tag. Purification of the SmeT protein with a six-histidine tag on nickel-nitriloacetic acid (Ni-NTA-Resin) was performed according to the instructions of the manufacturer (Qiagen, Inc.). Briefly, *E. coli* M15 containing pPS6 was grown in LB supplemented with ampicillin (100 μg/ml) and kanamycin (25 μg/ml) until the optical density at 600 nm was 0.9. Protein expression was induced with 2 mM isopropyl-β-D-thiogalactopyranoside. Then the cells were pelleted and resuspended in sonication buffer (50 mM sodium phosphate [pH 8], 300 mM NaCl). After sonication the lysate was centrifuged at 17,000 × *g* for 20 min and the supernatant was collected. Binding of SmeT with the six-histidine tag to Ni-NTA-Resin was done by a batch procedure. After that, a column containing the resin with the bound proteins was prepared. To remove unspecifically bound proteins, the column was washed three times. The first wash consisted of 10 column volumes of sonication buffer, the second one consisted of 15 column volumes of wash buffer (50 mM sodium phosphate [pH 6], 300 mM NaCl, 10% glycerol), and the third one consisted of 15 column volumes of wash buffer supplemented with 20 mM imidazole. SmeT with the six-histidine tag was then eluted with one column volume of 200 mM imidazole in wash buffer and stored in small aliquots at −20°C. The SmeT protein with the six-histidine tag purified in this way was at least 90% pure, as determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis analysis.

Electrophoretic mobility shift assay. *S. maltophilia* D457 and D457R protein extracts were obtained as described before (19) and assayed for their ability to bind to a labeled 197-bp DNA fragment covering the intergenic region between *smeT* and *smeDEF*. The binding conditions were the same for protein extracts and for the purified SmeT protein with the six-histidine tag. First, the 223-bp *smeT-smeD* intergenic region was amplified by PCR with primers *sme* 46 (see above) and *sme* 47 (see above) and 100 ng of cosmid pAS5 as the template. Then, the PCR product was digested with *Hin*fI (Fermentas), producing a 197-bp fragment, which was end labeled with [γ-^{32}P]dATP. The end-labeled DNA (18 nM) was incubated with increasing amounts of cell extracts in binding buffer (10 mM Tris-HCl, 50 mM KCl, 1 mM EDTA [pH 8.0], 50 mg of bovine serum albumin per ml, 1 mM dithiothreitol, 5% [vol/vol] glycerol, 10 mg of salmon sperm DNA per ml) for 30 min at room temperature. For competition assays, increasing concentrations of a cold 197-bp *Hin*fI DNA fragment were added. A total of 300 ng of the purified SmeT protein with the six-histidine tag was used in the binding assay. The samples were immediately loaded on a 6% (wt/vol) nondenaturing polyacrylamide gel. The gels were run with 1× TBE (Tris-borate-EDTA) buffer (33) for 3 h at 180 V and room temperature and dried prior to autoradiography.

RNA techniques. Total RNA from *S. maltophilia* D457 and from its isogenic multiresistant mutant, D457R, was obtained with the guanidine thiocyanate-based Tri Reagent-LS (Molecular Research Center Inc.), according to the instructions of the manufacturer. Residual DNA was removed by treatment with RNase-free DNase I (Boehringer Mannheim), followed by phenol extraction (9). The concentration and purity of the RNA were estimated by measuring the UV absorption at A_{260} and A_{280} (33).

S1 nuclease reactions were performed as described previously (9) with 40 μg of total RNA and an excess of a ^{32}P-labeled single-stranded DNA (ssDNA) probe that hybridized to the 5′region of the mRNA to be analyzed. The DNA template used for the generation of the ssDNA probe was obtained by PCR under the same conditions described above with primers *sme* 48 (5′-CCGTGT TCATGGAAGCAGGC-3′) and *sme* 49 (5′-GACCACGGTGACGTCACCC-3′), which amplify 83 bp of *smeT*, the entire *smeT-smeD* intergenic region, and 117 bp of *smeD*. ssDNA probes for mapping of the transcription start sites of *smeT* and *smeD* were then generated by linear PCR under the same conditions described above, but with only 10 pmol of ^{32}P-labeled primers (primer *sme* 48 for *smeT* and primer *sme* 49 for *smeD*) and 500 ng of purified *sme* 48-*sme* 49 DNA template. Each primer was labeled at the 5′ end with 16 U of polynucleotide kinase (New England Biolabs) and 100 μCi of [γ-^{32}P]ATP (Amersham) as described previously (33). The probes were then purified from a 6% polyacrylamide–7 M urea denaturing gel by the crush-and-soak method (33), and 5 × 10^4 cpm of the probe was used for each S1 nuclease reaction. The S1 nuclease reactions were run in 6% (wt/vol) polyacrylamide-urea denaturing gels. A G+A ladder, obtained by chemical degradation of the corresponding ssDNA probe (33), was included as a molecular size marker in each case.

β-Galactosidase activity. β-Galactosidase activity was measured as described by Miller (28). Activities were determined in *E. coli* TG1 strains harboring plasmids pPS3 and pALTER-Ex2, plasmids pPS3 and pPS4, or plasmids pPS3 and pPS5. In all cases, bacteria were grown in LB supplemented with ampicillin at 100 μg/ml and tetracycline at 12 μg/ml until the late-log or early stationary phase.

Nucleotide sequence accession number. The sequence of the wild-type *smeT* gene and the *smeT-smeD* intergenic region from strain D457 has been deposited in the EMBL nucleotide sequence database under accession number AJ316010.

RESULTS

Cloning and sequence analysis of *smeT*. The *S. maltophilia* efflux pump *smeDEF* (2) has recently been cloned in our laboratory. We have demonstrated that expression of the pump is repressed in wild-type strain D457 but is expressed at a high level in single-step spontaneous mutant D457R (2). This suggests that expression of *smeDEF* is under the control of a transcriptional regulator and mutations either in this putative regulator or in its operator sequence might derepress *smeDEF* expression in *S. maltophilia* D457R. The regulators of the expression of MDR pumps frequently lie in the upstream regions of the structural operon (17). To clone a potential regulator of *smeDEF* expression, we screened a pLAFR3-based cosmid library of *S. maltophilia* D457R by Southern blotting, using a DNA fragment from the *smeD* gene as a probe. Several clones containing DNA fragments that hybridized with the *smeD* probe were obtained, and one of them (pAS5) was chosen because it contained *Eco*RI and *Hin*dIII restriction fragments that were also present in cosmid pAS1, which contains the whole *smeDEF* operon (2). By using primers designed from the *smeD* sequence and further primer walking, a 1-kbp sequence was found to include part of the *smeD* gene and a new open reading frame (hereafter named *smeT*) located upstream from *smeD* and lying in the opposite orientation with respect to the orientation of the structural operon. *smeD* and *smeT* were separated by a 223-bp intergenic region.

Analysis of the SmeT protein sequence shows that it belongs to the TetR family of transcriptional regulators, with the homology being higher at the HTH motif (Fig. 1). In previous work we characterized *smeDEF*-overexpressing mutant *S. maltophilia* D457R (2, 4). The sequence of the *smeT* gene was obtained from a library of *smeDEF*-overproducing strain D457R. Since overexpression of MDR pumps from gram-negative bacteria is usually associated with mutations in the genes coding for their transcriptional regulators, we have also sequenced the *smeT* gene from wild-type strain D457. Comparison of this sequence with that from mutant strain D457R

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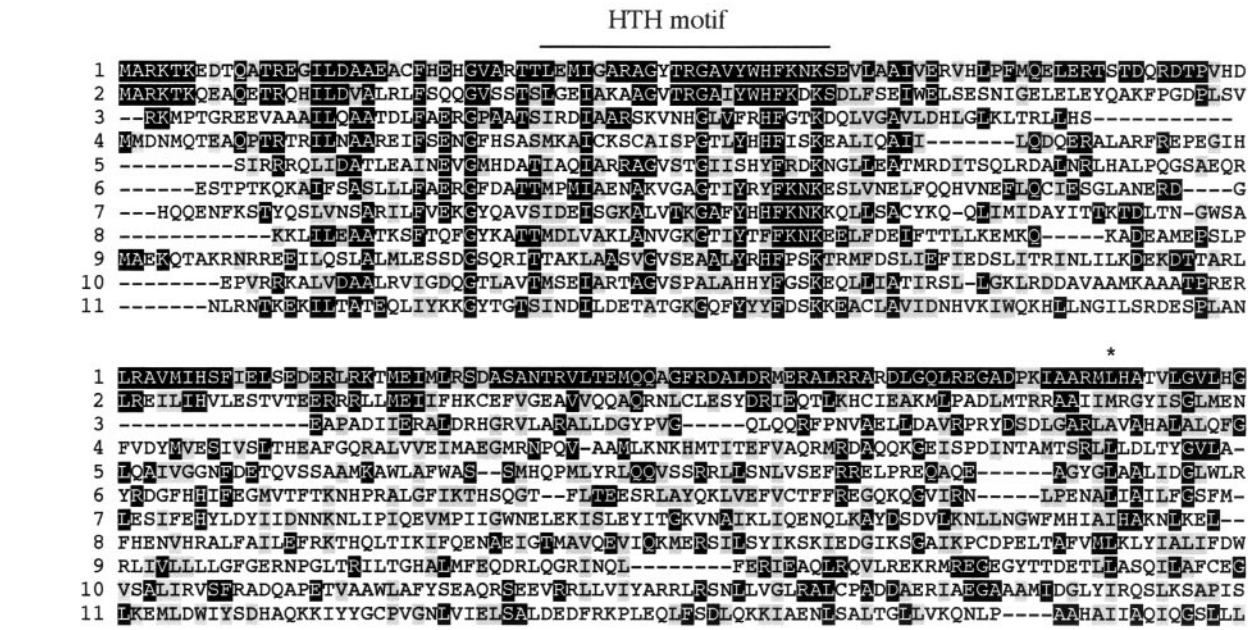

FIG. 1. Homology of SmeT with members of the TetR family of transcriptional regulators. Amino acids identical to those present in SmeT are highlighted with a black background. Homologous amino acids are highlighted with a grey background. The position of the Leu166Gln mutation is indicated with an asterisk. The HTH motif is also indicated. Rows: 1, SmeT; 2, AcrR from *E. coli* (25); 3, hypothetical protein Yy05 from *Mycobacterium tuberculosis* (11); 4, UidR from *E. coli* (8); 5, BetI from *E. coli* (22); 6, Bm3R1 from *Bacillus megaterium* (36); 7, TetC from Tn*10* (35); 8, hypothetical protein YhgD from *B. subtilis* (EMBL accession number P32398); 9, TetK protein from *E. coli* (8); 10, BetI from *Sinorhizobium meliloti* (29); 11, hypothetical Ypb3 protein from *Lactococcus lactis* (20).

demonstrated that the *smeT* gene from D457R contains an A/T mutation at position 498, which results in a Leu166Gln change. No other change was found either in *smeT* or in the intergenic *smeT-smeD* region, consistent with our hypothesis that this point mutation is responsible for the inactivation of *smeT* and further overexpression of *smeDEF*.

Cellular factors capable of binding the intergenic *smeT-smeD* region produced by *S. maltophilia* D457 and D457R. As stated above, regulation of MDR systems is usually exerted by the binding of repressor proteins to upstream sequences of the structural operon. Derepression of the system in MDR mutants might occur because the repressor is unable to bind to its operator. To test such a possibility, we analyzed whole-cell extracts from wild-type strain D457 and its isogenic MDR mutant, D457R, for the presence of proteins capable of binding to the intergenic *smeT-smeD* region. As shown in Fig. 2a, the extracts from D457 were able to produce a band shift of the intergenic *smeT-smeD* region in a dose-dependent fashion. The band shift was abolished when homologous DNA was added as competitor (Fig. 2b). These results indicate the presence of a protein(s) in the D457 extracts capable of binding in a specific way to the promoter region of *smeDEF*. When the same experiment was performed with extracts from MDR mutant D457R, we did not observe any band shift of the *smeD* promoter region, which indicates the absence of proteins capable of binding to the *smeT-smeD* intergenic region in those extracts.

A recombinant His-tagged SmeT protein is capable of binding to the intergenic *smeT-smeD* region. The *smeT* gene was cloned in the vector pQE31 to obtain a His-tagged SmeT protein. The plasmid encoding the recombinant protein was named pPS6. We then analyzed whole-cell extracts from *E. coli* containing either plasmid pPS6 or plasmid pQE31for the presence of proteins capable of binding to the intergenic *smeT-smeD* region. As shown in Fig. 3, the extracts from the strain containing pPS6 (and thus expressing His-tagged SmeT) were able to produce a band shift of the intergenic *smeT-smeD* region, whereas no band shift was observed with extracts from the *E. coli* strain containing plasmid pQE31 (Fig. 3, lanes 3 and 4). The purified His-tagged SmeT protein also produced a band shift under the same conditions (Fig. 3, lane 2). These results indicate that SmeT is able to bind to the intergenic *smeT-smeD* region.

SmeT displays a repressor activity in both *S. maltophilia* and *E. coli*. To ascertain the possible role of SmeT as a repressor of *smeDEF* transcription, two approaches were used. First, the wild-type *smeT* gene was cloned in low-copy-number vector pVLT31, giving pPS1, and introduced both in wild-type strain *S. maltophilia* D457 and in *smeDEF*-overproducing strain *S. maltophilia* D457R. The expression of the *smeDEF* operon was

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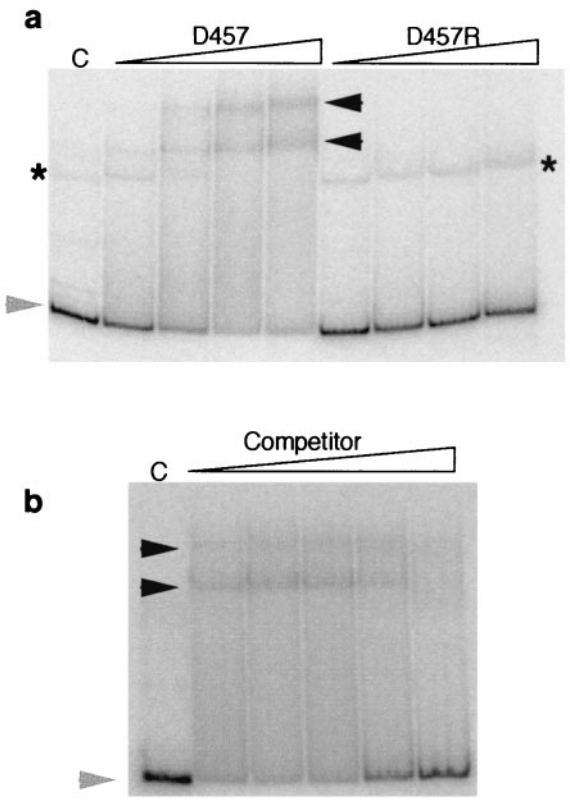

FIG. 2. Interaction of cellular extracts from *S. maltophilia* D457 and D457R with the intergenic *smeT-smeD* region. (a) Increasing concentrations of cellular protein extracts (10, 40, 100, and 300 μg) from *S. maltophilia* D457 and D457R were incubated with labeled intergenic *smeT-smeD* region. Lane C, a control without cellular extracts. The position of the probe is indicated with a grey arrowhead, and the positions of the two retarding complexes are indicated with black arrowheads. The asterisk indicates the position of an unspecific band that is present on the control and the lanes with extracts from D457R but that is shifted with extracts from D457. (b) Effect of unlabeled probe on the formation of the retarding complexes. In all cases, 50-μg cellular protein extracts from D457 were incubated with the labeled probe under the conditions described in Materials and Methods and increasing (0-, 1-, 20-, 250-, and 2,000-fold) amounts of unlabeled probe were added. The grey arrowhead indicates the position of the probe, and the black arrowheads indicate the positions of the retarding complexes.

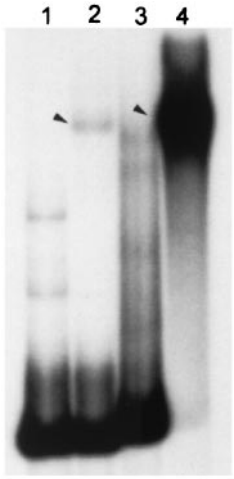

FIG. 3. Interaction of His-tagged SmeT with the intergenic *smeT-smeD*. Lane 1, a control containing the probe in the absence of protein; lane 2, the probe was incubated with purified His-tagged SmeT; lane 3, the probe was incubated with cellular extracts from *E. coli*(pQE31) (which does not express His-tagged SmeT); lane 4, the probe was incubated with cellular extracts from *E. coli*(pPS6) (which expresses His-tagged SmeT).

then monitored by analyzing the amount of the SmeF protein in whole-cell extracts by using an anti-SmeF antibody. As shown in Fig. 4, the introduction of the wild-type *smeT* gene in *S. maltophilia* reduced the level of expression of SmeF both in wild-type strain D457 and in *smeDEF*-overproducing mutant D457R compared with the level of expression in control strains without plasmids. It is noteworthy that expression of the wild-type SmeT protein in D457R did not reduce the amount of SmeF to the levels observed in wild-type strain D457.

As a second approach, three *E. coli* strains were constructed. Two of them contained a *lacZ*-based plasmid reporter system (pPS3) for analysis of *PsmeDEF* expression, and a second plasmid (pPS4 or pPS5), containing either the wild-type *smeT* gene (pPS4) or the Leu166Gln mutant one (pPS5). In both plasmids (pPS4 and pPS5) *smeT* was cloned under the control of *PsmeT*. The third one is a control strain that contains pPS3 and the vector used to clone SmeT, pALTER-Ex2. Replication of pPS3 and pALTER-Ex2 is compatible in *E. coli*, which makes this system suitable for analysis of the effect of SmeT on *PsmeDEF* promoter activity. In the presence of the wild-type SmeT protein, the activity of the *smeDEF* promoter was only 17% compared with the activity of *PsmeDEF* in the absence of SmeT. On the other hand, expression of the mutant SmeT protein did not reduce the activity of the *PsmeD* promoter. This reinforces the idea that SmeT is a repressor and that the Leu166Gln change abolishes the capability of SmeT to repress *PsmeDEF* activity.

Transcriptional origins of *smeT* and *smeDEF*. Expression of the SmeDEF pump is transcriptionally regulated (2; present work). To gain more insight into the transcriptional regulation of the system, we have mapped the transcriptional origins of *smeT* and *smeDEF* by S1 nuclease analysis. As shown in Fig. 5, in both cases only one transcriptional origin was detected. The expression of *smeT* and *smeDEF* was analyzed in wild-type strain D457 and *smeDEF*-overproducing mutant D457R. In both cases, the levels of expression were higher in D457R than

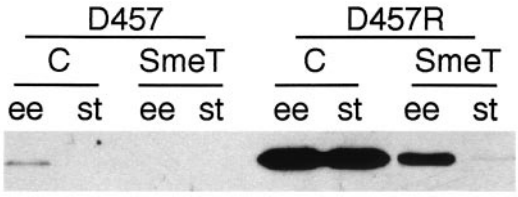

FIG. 4. Expression of SmeF by *S. maltophilia* D457 and D457R carrying a plasmid expressing SmeT. The expression of SmeF was analyzed by Western blotting at two growth phases. Lanes ee, early-exponential-phase cells; lanes st, stationary-phase cells; lanes C, controls containing cellular extracts from strains D457 and D457R with no plasmid; lanes SmeT, cellular extracts from bacteria carrying SmeT-expressing plasmid pPS1.

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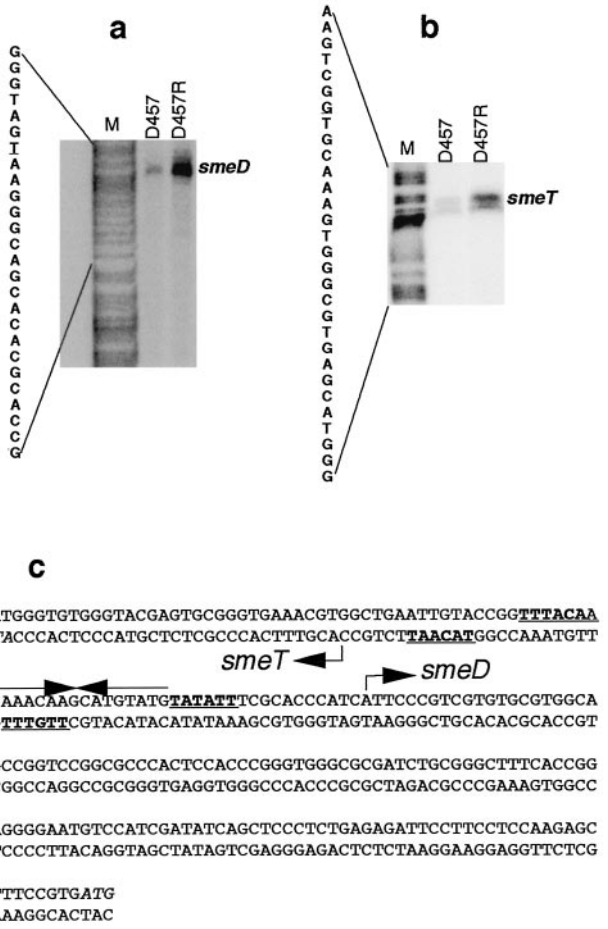

FIG. 5. Mapping of the transcription start sites for *smeD* and *smeT* transcripts. The positions of the transcription start sites for *smeD* (a) and *smeT* (b) were determined by S1 nuclease analysis. (c) Structure of the intergenic *smeT-smeD* region. The ATG codons for both genes are in italics, and the positions of the putative −10 and −35 promoter regions are highlighted in boldface and underlined. The position of the inverted repeat that could have a role in SmeT binding is indicated with two arrows pointing at each other. The positions of the transcription starts are also indicated with arrows.

in D457. This indicates that *smeT* expression is also derepressed in the *smeDEF*-overproducing mutant and strongly suggests that *smeT* expression is down-regulated by its product, SmeT.

DISCUSSION

In the present work we have described that expression of *smeDEF*, the first MDR pump so far characterized in *S. maltophilia* (2), is repressed by a transcriptional regulator, SmeT, encoded by a gene located 223 bp upstream from *smeD* and divergently transcribed. SmeT belongs to the TetR family of transcriptional repressors to which AcrR (25), the repressor of the *E. coli* MDR determinant AcrAB (26), also belongs. It is noteworthy that SmeT showed the highest degree of homology with AcrR, whereas the structural proteins of the SmeDE efflux pump also showed the highest degrees of homology with AcrAB (2). The possibility that bacterial operons and their transcriptional regulators might form patchwork genetic structures in which the regulators have been recruited from an evolutionary branch different from that from which the structural genes of the operons were recruited has been discussed (10, 14, 17). The finding that SmeT and SmeDE are mostly homologous to AcrR and AcrAB, respectively, suggests, however, that both the structural proteins and the regulators of these MDR pumps have a common origin. An ancient origin for the linkage between the AcrR-like regulators to the MDR operons regulated by them is suggested because the degree of homology of the primary amino acid sequences between the two systems is not very high. This ancient origin agrees with the fact that MDR pumps are widely distributed but are also conserved in the genomes of all members of the same bacterial species (32) and supports the notion that they should have a relevant role in bacterial physiology other than antibiotic resistance.

Sequencing of *smeT* from wild-type strain D457 and MDR strain D457R has shown that the sequences differ by a single point mutation that produced a Leu166Gln change. It is noteworthy that this leucine is highly conserved within the TetR family of regulators (Fig. 1), which strongly suggests a relevant role of this residue in the activities of those regulators. In fact, cellular extracts containing the wild-type form of SmeT produced a band shift of the intergenic *smeT-smeD* region that contains the promoters of both genes, whereas no band shift was observed when the extracts containing the mutant form of SmeT were evaluated (Fig. 2a). This hypothesis is also supported by the fact that the wild-type SmeT protein can repress the activity of *PsmeDEF* in *E. coli*, whereas the protein from the Leu166Gln mutant is unable to produce any effect.

The fact that the mutant form of SmeT cannot bind to its operator(s) and in these circumstances expression of *smeDEF* is derepressed (Fig. 5) implies that SmeT is probably a repressor. As stated above, experiments with *E. coli* as a heterologous host have shown that this is indeed the case. When these experiments were performed with *S. maltophilia*, we noticed that introduction of the wild-type SmeT protein reduced the levels of expression of SmeF in both wild-type strain D457 and MDR mutant strain D457R (Fig. 4). The fact that expression of SmeF is hyperrepressed upon introduction of increasing amounts of SmeT in the wild-type *S. maltophilia* D457 strain suggests that SmeT is present at limiting concentrations in D457, since increasing concentrations of SmeT also increased the strength of the repression. On the other hand, the introduction of SmeT into MDR mutant D457R did not fully restore the repression observed in the wild-type strain, which indicates that the wild-type form of the SmeT protein is not dominant over SmeT from the Leu166Gln mutant. It has been stated that the transdominance of negative TetR mutants indicates that they retain the ability to form oligomers with the wild-type repressor (7). If this is the case for SmeT, the Leu166Gln mutation should not be relevant for in vivo SmeT dimerization. As stated before, the SmeT protein from the mutant is probably unable to bind to its operator. However, the Leu166Gln mutation lies outside of the HTH region that is supposed to be involved in DNA binding (Fig. 1). Deletion mutagenesis analyses of the TetR protein have shown that the residues needed for the activity of the repressor are distributed all along the protein sequence (6, 7, 18, 37, 41). The N-terminal

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region contains the DNA-binding domain; and the C-terminal region contains the dimerization domain, the region required for the binding of tetracycline, and regions involved in the folding and stability of the protein. We speculate, then, that the Leu166Gln mutation may produce a defect on SmeT folding which makes it inactive.

In a previous work, we stated that *smeDEF* expression is transcriptionally regulated (2). Herein, we have shown that the same occurs for *smeT*, with its expression probably being autoregulated by the SmeT product. In both cases, expression is triggered by a single promoter for each gene. Those promoters support the low level of expression observed in wild-type strain D457 and also the high level of expression detected in MDR mutant strain D457R. These data fit with the idea that a single inducible promoter drives the expression of *smeDEF* and that the same occurs for *smeT*. It is noteworthy that the positions of the transcription start sites of *smeT* and *smeDEF* are separated by just 56 bp. This indicates that the promoters must somehow be overlapping and suggests the possibility of interplay between *PsmeT* and *PsmeDEF*. This type of interplay between the promoters of the structural genes and the transcriptional regulators of MDR pumps has recently been described (34) for the *mexR-mexABOprM* system from *Pseudomonas aeruginosa.*

ACKNOWLEDGMENTS

P. Sánchez and A. Alonso contributed equally to the work.

This research was aided by grants QLRT-2000-01339, BIO2001-1081, CAM 08.2/0020.1/2001, and QLRT-2000-00873. P. Sánchez was the recipient of a fellowship from MEC. A. Alonso was the recipient of a fellowship from Gobierno Vasco.

Thanks are given to Fernando Rojo for criticisms on the manuscript.

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