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Characterization of an LysR Family Protein, SmeR from *Serratia marcescens* S6, Its Effect on Expression of the Carbapenem-Hydrolyzing β-Lactamase Sme-1, and Comparison of This Regulator with Other β-Lactamase Regulators

THIERRY NAAS,[1] DAVID M. LIVERMORE,[2] AND PATRICE NORDMANN[3]*

Abteilung Mikrobiologie, Biozentrum der Universität Basel, 4056 Basel, Switzerland[1]; Department of Medical Microbiology, The London Hospital Medical College, London E1 2AD, United Kingdom[2]; and Service de Bactériologie-Virologie, Hôpital Antoine Béclère, Faculté de Médecine Paris-Sud, Université Paris XI, 92141 Clamart, France[3]

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***Serratia marcescens* S6 produces a chromosomally encoded carbapenem-hydrolyzing class A β-lactamase, Sme-1 (T. Naas, L. Vandel, W. Sougakoff, D. M. Livermore, and P. Nordmann, Antimicrob. Agents Chemother. 38:1262–1270, 1994). Upstream from *smeA* we identified a second open reading frame (EMBL accession number Z30237). This encodes a 33.1-kDa protein, SmeR, which has a high degree of homology with NmcR, the LysR regulatory protein of the only other sequenced carbapenem-hydrolyzing class A β-lactamase, NmcA from *Enterobacter cloacae* NOR-1. It is weakly related to AmpR of the chromosomal cephalosporinase regulatory systems described in *E. cloacae*, *Yersinia enterocolitica*, *Citrobacter freundii*, and *Pseudomonas aeruginosa* and is very weakly related to other LysR-type regulators of class A β-lactamases. SmeR is a weakly positive regulator for Sme-1 expression in the absence of or in the presence of β-lactam inducers. The −35 and −10 regions of *smeR* are in the opposite orientations and are face-to-face relative to the *smeA* promoter. SmeR acts similarly to NmcR and not as the AmpR regulators described for class C β-lactamase systems. SmeR is a weak inducer in the absence or presence of β-lactams. As was found for the AmpC-AmpR and NmcA-NmcR systems, a putative SmeR-binding site was present upstream from the β-lactamase gene promoter regions. β-Galactosidase activity from a *smeR-lacZ* translational fusion was expressed constitutively and decreased in the presence of SmeR from a coresident plasmid, suggesting that SmeR is autogenously controlled. Finally, β-lactams did not affect the expression of SmeR, which is the second regulator of a class A carbapenem-hydrolyzing β-lactamase to be identified.**

Inducible biosynthesis of AmpC β-lactamases belonging to molecular class C has been well described in *Enterobacter cloacae*, *Citrobacter freundii*, *Yersinia enterocolitica*, and *Pseudomonas aeruginosa* (1, 14, 16–18, 32). Their induction is mediated, in part, by DNA-binding proteins, called AmpR, which belong to the LysR family. These act as negative regulators in the absence of β-lactam inducers and as positive regulators in the presence of inducers such as cefoxitin or imipenem (4). Hyperproduction of AmpC cephalosporinases via induction or mutational derepression confers resistance to many potent β-lactams, including extended-spectrum cephalosporins such as ceftazidime, ceftriaxone, and cefotaxime but not to carbapenems such as imipenem.

β-Lactamase-mediated carbapenem resistance remains very rare among members of the family *Enterobacteriaceae*, having been described in only four clinical isolates, namely, *Serratia marcescens* TN9106, S6, and S8 and *E. cloacae* NOR-1 (23, 25, 38). We have recently sequenced the gene encoding the chromosomally encoded carbapenem-hydrolyzing β-lactamase Sme-1 from *S. marcescens* S6 (23). The enzyme was found to be a class A type related to the only other class A carbapenem-hydrolyzing β-lactamase, NmcA from *E. cloacae* NOR-1, and was found to be distantly related to the inducible AmpC enzyme typically found in enterobacterial species.

* Corresponding author. Mailing address: Service de Bactériologie-Virologie, Hôpital Antoine Béclère, 157 rue de la Porte-de-Trivaux, 92141 Clamart, France. Phone: 33 1 45 37 42 85. Fax: 33 1 46 32 67 96.

In the course of determining the DNA sequence of the cloned *smeA* region, a second open reading frame (ORF), divergent in transcription, also was found. In this report, we identify this ORF as one encoding a novel LysR family protein. We compared this protein with other LysR proteins, especially β-lactamase regulators, and analyzed its function.

MATERIALS AND METHODS

Bacterial strains and plasmids. The strains and plasmids used in the study are listed in Table 1. The *S. marcescens* clinical isolates PNER 1 to PNER 7 were obtained from hospitalized patients with urinary tract infections at the Hôpital Raymond Poincaré, Garches, France, in 1993.

Media, chemicals, and antibiotics. Luria-Bertani (LB) broth and agar and tryptic soy (TS) broth were from Diagnostics Pasteur (Marnes-la-Coquette, France). Reverse transcriptase was from Promega (Madison, Wis.). Restriction enzymes and T4 DNA ligase were from New England Biolabs (Beverly, Mass.). [α-^{32}P]ATP, [γ-^{32}P]dATP, and [α-^{35}S]dATP were from Amersham (Buckinghamshire, England), and the random primer DNA labelling kit was from Bio-Rad (Richmond, Calif.). Routine chemicals were from Merck (Darmstadt, Germany). The following antimicrobial agents were obtained as standard laboratory powders from the indicated suppliers and were used immediately after dilution in sterile water: ampicillin, and cephaloridine, SmithKline Beecham; cefoxitin and imipenem, Merck Sharp & Dohme; chloramphenicol and kanamycin, Sigma Chemical Co.

Bacterial culture and β-lactamase preparation. Cultures for β-lactamase studies were prepared by diluting overnight broth cultures 1:15 into prewarmed TS broth to a final volume of 500 ml. These fresh cultures were grown for 5 h at 37°C in the presence of potential inducers (imipenem at 25 or 50 μg/ml or cefoxitin at 5 μg/ml). The cells were then harvested (centrifugation at 3,000 × *g* for 10 min), washed once, and resuspended in 2 ml of 50 mM phosphate buffer (pH 7.0) and then disrupted by sonication. The debris was removed by centrifugation to obtain clear extracts.

β-Lactamase assays. The activities of the crude enzyme extracts were mea-

TABLE 1. Bacterial strains and plasmids used in the study

Strain or plasmid	Relevant genotype or phenotype	Source or reference
Strains		
E. coli JM109	*endA1 gyrA96 hsdR17* Δ (*lac proA recAB1*) *relA supE44 thi* F′ [*lacI*^{q} *lacZ*ΔM15 *proAB*$^+$ *traD36*]	39
S. marcescens S6	Clinical isolate producing the Sme-1 (pI 9.7) carbapenem-hydrolyzing β-lactamase and a pI 8.2 cephalosporinase	38
S. marcescens S8	Clinical isolate producing SmeA carbapenem-hydrolyzing β-lactamase, a pI 8.2 cephalosporinase, and TEM-1 β-lactamase	38
S. marcescens 103235T	Reference strain, Institut Pasteur, Paris, France	IPSC[a]
S. marcescens PNER 1 to PNER 7	Clinical isolates, Hôpital Raymond Poincaré, Garches, France	This work
E. cloacae NOR-1	Clinical isolate producing NmcA carbapenem-hydrolyzing β-lactamase	25
Plasmids		
pACYC184	*cat tet*	7
pK19	*kan*	27
pNM480	*amp*	21
pPTN102	4-kb *Hin*dII fragment from pPTN101 (containing a 11.3-kb insert from *S. marcescens* S6) cloned into pACYC184	23
pPTN103	2.5-kb *Hin*dIII fragment from pPTN102 cloned into pK19	23
pPTN104	1.9-kb *Nde*I-*Hin*dIII fragment from pPTN103 into pK19	This work
pPTN105	1.8-kb *Sna*BI-*Hin*dIII fragment from pPTN103 into pK19	This work
pPTN106	1.6-kb *Nru*I-*Hin*dIII fragment from pPTN103 into pK19	This work
pPTN107	1.2-kb *Nsi*I-*Hin*dIII fragment from pPTN103 into pK19	This work
pPTN108	2.5-kb *Hin*dIII fragment from pPTN102 cloned into *Hin*dIII-*Eco*RV-digested pACYC184	This work
pPTN109	1.4-kb *Pvu*II-*Hin*dIII fragment from pPTN102 cloned into *Hin*dIII-*Eco*RV-digested pACYC184	This work
pPTN480	5-kb *Eco*RI-*Stu*I fragment from pNM480 cloned into 1.6-kb *Bsp*HI fragment of pK19	This work
pPTN110	1.2-kb *Nci*I-*Pvu*II fragment from pPTN103 cloned into *Sma*I-*Pst*I-digested pPTN480	This work

[a] IPSC, Institut Pasteur Strain Collection.

sured against 100 μM cephaloridine or imipenem at 262 and 299 nm, respectively, with a UV double-beam spectrophotometer (38). One β-lactamase unit is defined as the quantity of enzyme that hydrolyzes 1 μmol of antibiotic per min at pH 7.0 and 30°C. The protein contents of the extracts were assayed colorimetrically (Bio-rad Protein Kit II) according to the manufacturer's recommendations, and specific activities were expressed as units per milligram of protein.

Cloning, DNA sequencing, and protein analysis. The recombinant plasmid pPTN102, which contained a 4-kb fragment from *S. marcescens* S6, has been already described (23). This insert includes the gene for the carbapenem-hydrolyzing β-lactamase Sme-1 and the second ORF described here. A second recombinant plasmid, pPTN103, was constructed by cloning a 2.5-kb *Hin*dIII fragment from pPTN102 into the multicopy vector pK19 (27). From pPTN103, deletions were performed by standard molecular techniques to obtain plasmids pPTN104, pPTN105, pPTN106, and pPTN107 (Table 1 and Fig. 1). A 2.4-kb *Hin*dIII-*Hin*cII fragment from pPTN103 was cloned into *Hin*dIII-*Eco*RV-digested vector pA CYC184, giving rise to plasmid pPTN108. A 1.4-kb *Hin*dIII-*Pvu*II fragment was similarly cloned from pPTN103 into pACYC184 to give pPTN109.

For β-galactosidase linkage experiments, pPTN480 was constructed by cloning a 5-kb *Eco*RI-*Stu*I fragment from pNM480 (21) into the 1.6-kb *Bsp*HI fragment of pK19 (27). The resulting vector conferred kanamcyin resistance and shared all the properties of pNM480. The *smeR-lacZ* translational fusion plasmid pPTN110 was constructed by ligating the *Nsi*I-*Pvu*II fragment from pPTN103, which encodes the first nine N-terminal amino acids of *smeR* and the entire *smeA* gene, into *Sma*I-*Pst*I-digested pPTN480 (Fig. 1).

The nucleotide sequence of the second ORF, designed *smeR*, was determined from both strands of pPTN103 with double-stranded plasmid DNA and by the dideoxynucleotide chain termination procedure (30). A Sequenase version II sequencing kit (United States Biochemical Corp., Cleveland, Ohio) was used. Custom 18-mer oligonucleotide primers were synthesized by Genset (Paris, France). The nucleotide sequences and the deduced protein sequences were analyzed with the GCG software (Biotechnology Center, University of Wisconsin, Madison) on a VAX computer from Digital Corp. (9). The hydrophobicity profile of the deduced protein was predicted with the GCG program Pepplot, which uses the algorithm of Kyte and Doolittle (15). The DNA and protein sequences of other LysR family proteins were from the European Molecular Biology Laboratory and the Swiss-Prot databases (24). Multiple alignment of deduced peptide sequences was carried out with the GCG program Pileup, which is a simplification of the progressive alignment method of Feng and Doolittle (10).

The deduced SmeR protein was compared with those of other β-lactamase regulators of the LysR family, namely, (i) AmpR proteins of the class C cephalosporinase systems of *E. cloacae* (14), *C. freundii* (16), *Y. enterocolitica* (32), and *P. aeruginosa* (18), (ii) AmpR for the class A β-lactamase from the gram-negative bacterium *Rhodobacter capsulata* (6), (iii) AmpR for the class A β-lactamase from the gram-positive actinomycetale *Streptomyces cacaoi* (36), (iv) the NmcR regulator of the class A carbapenem-hydrolyzing β-lactamase of *E. cloacae* NOR-1 (22), and (v) the type protein of the LysR family, LysR, for the lysine biosynthesis pathway of *Escherichia coli* (34). A dendrogram was derived to show the relatedness of these LysR family proteins by using the phylogeny package PAUP (Phylogenetic Analysis Using Parsimony), version 3.1 (35).

β-Galactosidase assays. Plasmids pPTN480 and pPTN110 were used to transform *E. coli* JM109. β-Galactosidase assays on cultures of the transformants were performed by the method of Miller (20), except that samples were centrifuged for 15 min in an Eppendorf minicentrifuge before the A_{420} was measured in a Kontron Uvikon spectrophotometer. β-Galactosidase activities were calculated as described by Raleigh and Kleckner (28).

DNA-DNA homologies. To search for the gene encoding SmeR among other *S. marcescens* isolates, DNA-DNA hybridizations were performed by the method of Southern by a dot blot technique (19). The strains studied were the *S. marcescens* clinical isolates PNER 1 to PNER 7, *S. marcescens* S6 and S8, *E. coli* JM109(pPTN102), and *E. coli* JM109(pACYC184) (negative control). The DNAs were immobilized onto a Biodyne A Transfer Membrane (PALL, Portsmouth, Hampshire, England) and were probed with an [α-^{32}P]dATP-labelled 800-bp *Nsi*I internal fragment of *smeR* from pPTN103. Hybridization reactions were carried out as described elsewhere (25). Membranes were washed in 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) containing 0.5% sodium dodecyl sulfate (SDS) for 5 min at room temperature, then in 2× SSC containing 0.1% SDS for 15 min, and finally in 0.1× SSC–0.5% SDS for 1 h. This final wash was repeated twice under high-stringency conditions at 68°C (19).

Transcriptional start of the regulator gene. Cultures of *E. coli* JM109 harboring the recombinant plasmid pPTN102 and *S. marcescens* S6 were grown overnight in 5-ml volumes of LB broth containing ampicillin at 100 μg/ml. mRNA was extracted by a modified method (26) of Chomczinski and Saachi (8), and mRNA primer starts were analyzed by the method of Geliebter et al. (11). The probe consisted of an 18-mer oligonucleotide hybridizing over positions 289 to 306 (Fig. 1).

Nucleotide sequence accession number. The nucleotide sequence data reported here will appear in the EMBL Nucleotide Data Base under the accession number Z30237.

RESULTS

Sequence analysis of SmeR from *S. marcescens* S6. A 1,499-bp nucleotide sequence was determined upstream from the β-lactamase structural gene *smeA* within the 2.5-kb genomic

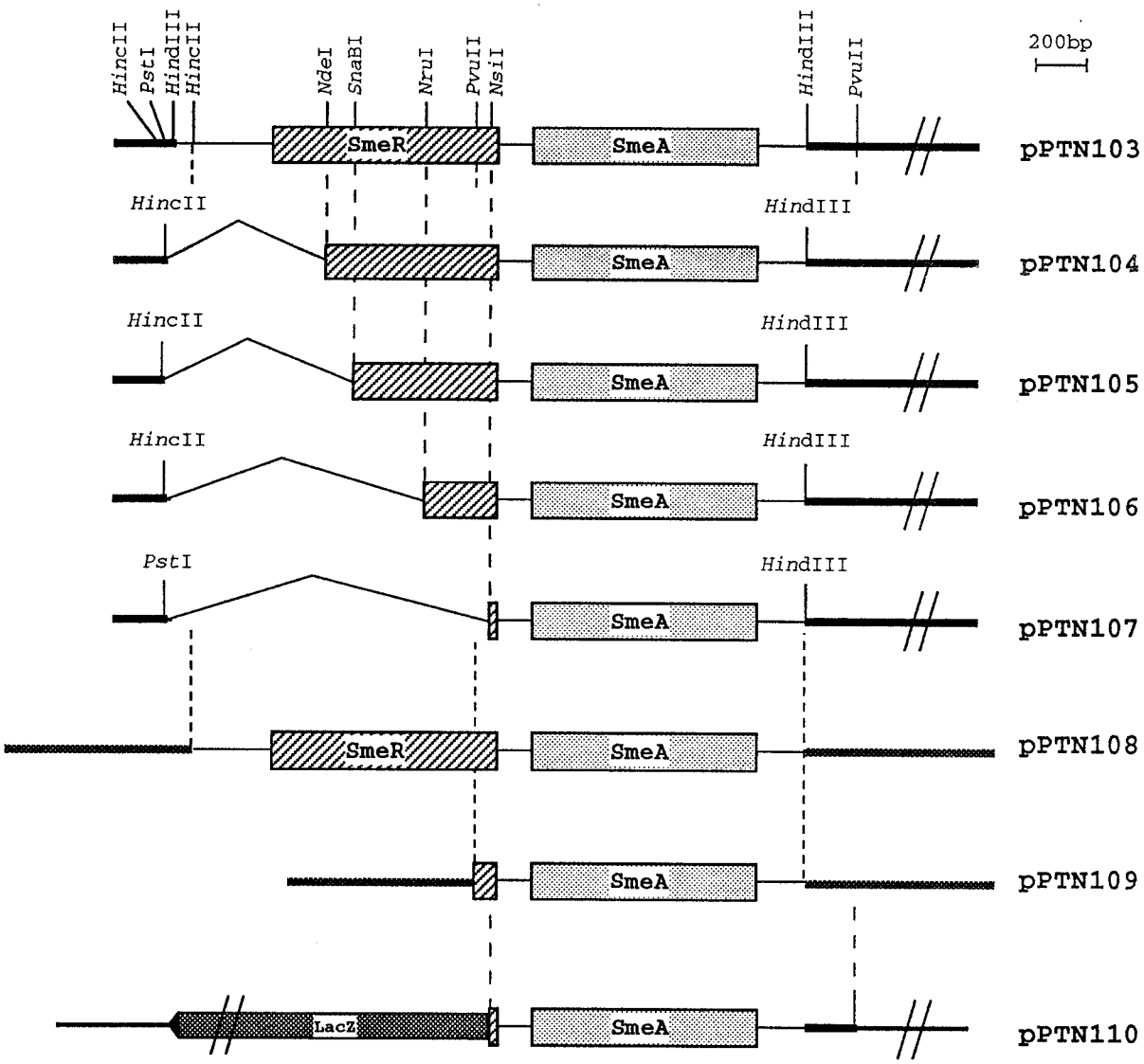

FIG. 1. Restriction endonuclease maps of recombinant plasmids pPTN103, pPTN104, pPTN105, pPTN106, pPTN107, pPTN108, pPTN109, and pPTN110, all of which harbored the *smeA* gene for the carbapenem-hydrolyzing β-lactamase (grey boxes). The cloned inserts from *S. marcescens* S6 are shown as thin lines. *smeR* is shown as hatched boxes. The plasmid vectors are represented by thick dark lines. For plasmids pPTN103 to pPTN107, the plasmid vector was pK19, while for pPTN108 and pPTN109, the vector was pACYC184. In the case of pPTN110, the plasmid vector was pPTN480, which is a hybrid plasmid of pNM480 and pK19. The *lacZ* gene is shown in pPTN110 as a dark box. For pPTN104 to pPTN107, the thin broken lines indicate deletions compared with the sequence of pPTN103. Important restriction sites are indicated.

DNA fragment of *S. marcescens* S6 cloned into pPTN103 (Fig. 1 and 2). This sequencing revealed an ORF on the opposite strand from *smeA*. Assuming that the ATG at nucleotide positions 243 to 245 is the initiation codon, this putative gene encodes a 33.1-kDa (293-amino-acid) protein which we named SmeR (Fig. 2). mRNA primer extension analysis located a typical enterobacterial promoter with a −35 sequence (TTGACA) at positions 89 to 94 (Fig. 2) and a −10 sequence (TGTCAT) at positions 112 to 117 (Fig. 2). The former sequence is identical to the −35 consensus sequence of *E. coli*, but the latter sequence has two mismatches compared with the *E. coli* consensus sequence. The SmeR transcriptional start site was mapped to be either of the adenines at position 125 or 126 (Fig. 2 and 3). No typical ribosome-binding site could be detected upstream of the *smeR* ATG (33).

Analysis of regions downstream of *smeR* revealed a palindromic sequence (Fig. 2) which might form a hairpin loop in the mRNA, possibly acting as a Rho-independent transcription terminator (29). The ΔG value for this putative terminator was calculated to be −84.2 kJ/mol at 25°C. The G+C content (40%) and the codon usage of *smeR* were similar to those of *smeA* and were typical of enterobacterial genes (37). Comparison with the organization of other β-lactamase regulatory systems from other gram-negative bacteria revealed that *smeA-smeR* resembles the *ampC-ampR* system of the class C cephalosporinases of *E. cloacae*, *C. freundii*, *P. aeruginosa*, and *Y. enterocolitica* and also the *nmcA-nmcR* system of *E. cloacae* NOR-1 (2) (Fig. 4). In each of these cases, the β-lactamase structural gene and its regulator gene were in divergent orientations. However, unlike *smeA-smeR*, these genes have overlapping promoters. In *smeA-smeR*, the divergent promoters are face-to-face with no overlap of their −35 and −10 regions (Fig. 4).

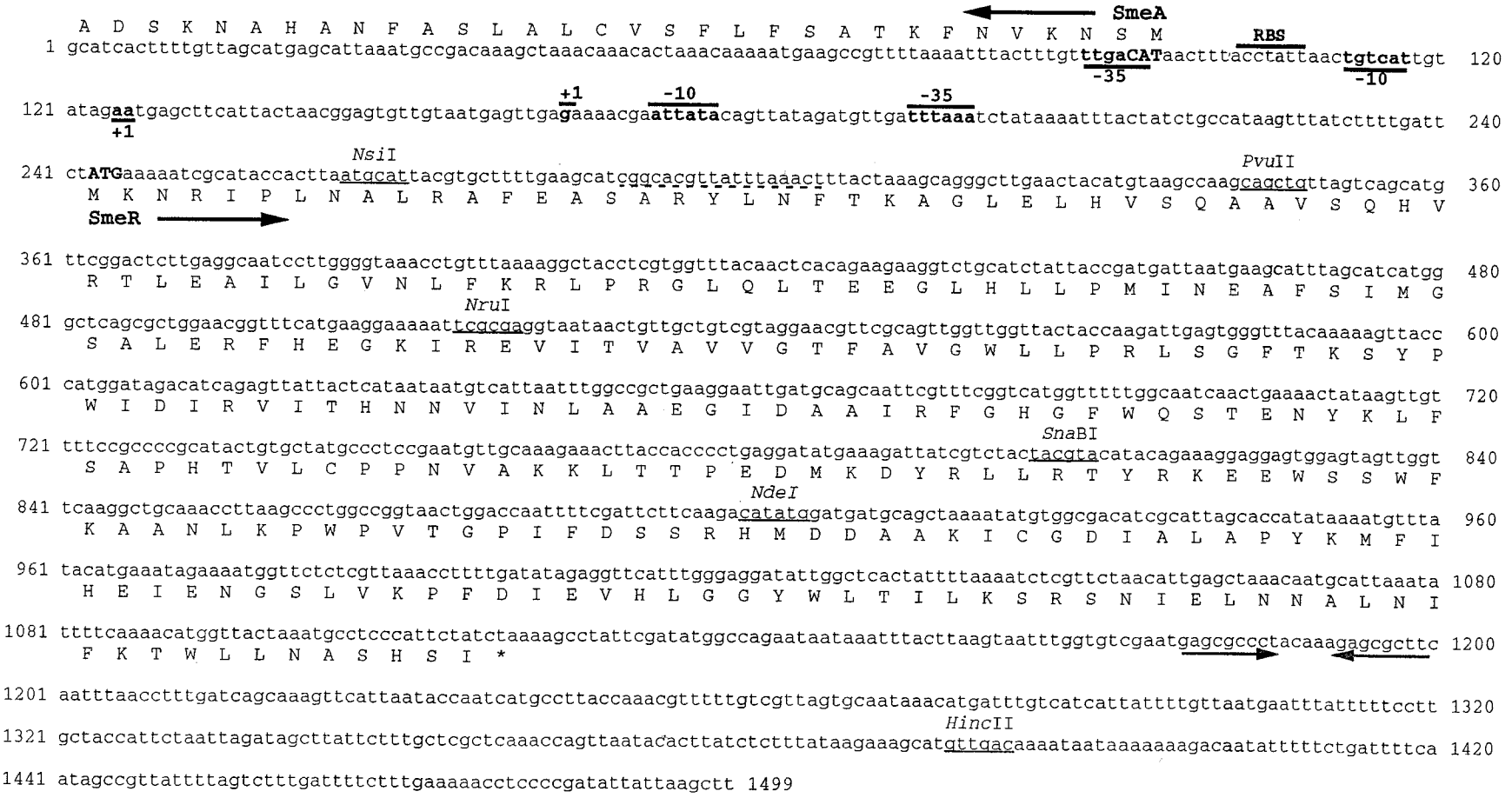

FIG. 2. Nucleotide sequence of the 1,499-bp fragment from pPTN103 containing the *smeA-smeR*-coding regions. The deduced amino acid sequences are designated in single-letter code below the nucleotide sequence for SmeR and above the nucleotide sequence for SmeA. The *smeR* transcription start in *S. marcescens* S6 and *E. coli* JM109 is indicated by +1 below the nucleotide sequence and *smeA* transcription start is indicated by +1 above the nucleotide sequence. The promoter sequences are represented by −10 and −35 regions below and above the nucleotide sequences for *smeR* and *smeA*, respectively. Several relevant restriction endonuclease recognition sites are underlined. The ATG codons for *smeR* and *smeA* are in boldface type. Reversed arrows indicate inverted repeat sequences acting as possible Rho-independent terminators. The *smeR* stop codon is indicated with a single asterisk. The nucleotide sequence with a dashed underline (5′-AGTTTAAATAACGTGCCG-3′) corresponds to the primer-annealing region for mRNA primer extension analysis. RBS indicate the *smeA* ribosome-binding site.

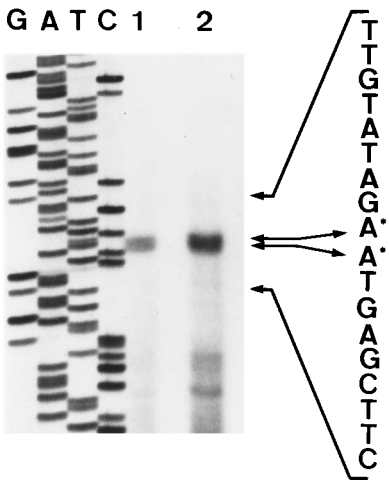

FIG. 3. Mapping of the *smeR* mRNA 5′ end by primer extension analysis by the method of Geliebter et al. (11). The primer extension products were analyzed on a sequencing gel. Lane 1, mRNA extracted and extended from *S. marcescens* S6; lane 2, mRNA extracted and extended from *E. coli*(pPTN102), which contained the cloned *smeR* gene. The nucleotide sequence on the right corresponds to that of the complementary strand which was deduced from the sequencing reaction obtained with the same primer used for mRNA extension and with pPTN102 as the template. The asterisks on the nucleotide sequence indicate the two possible transcriptional start sites, which correspond to nucleotides 125 and 126 in Fig. 2.

Comparison of SmeR with LysR family proteins. The relatedness of the 33.1-kDa protein encoded by *smeR* to LysR family proteins was considered. A helix-turn-helix motif was seen in the same region (amino acids 22 to 42 in Fig. 5) that it is seen in other LysR transcriptional regulators (13, 31). This region possesses 16 of the 20 amino acids that are known to form the helix-turn-helix consensus sequence (data not shown). These motifs have been shown to be involved in the DNA-binding properties of LysR proteins (31). However, the overall homology of SmeR to the LysR family proteins was very weak (less than 20%; data not shown), and detailed amino acid comparisons were undertaken with β-lactamase regulators and the LysR protein itself. SmeR was found to be related to the regulators of class C β-lactamases (AmpR) from gram-negative bacteria and distantly related to the regulators of the class A β-lactamase of *S. cacaoi* and to LysR from *E. coli* (Table 2). It was more closely related to NmcR from *E. cloacae*, which is the regulator of the only other carbapenem-hydrolyzing class A β-lactamase so far described (Table 2). Dendrogram analysis indicated that NmcR and SmeR may form a subgroup among LysR-type β-lactamase LysR regulators (Fig. 6).

SmeR and these LysR proteins are more related within their amino termini than within their carboxy termini (Fig. 5). SmeR possesses 17 lysine and 16 arginine residues, whereas most LysR proteins, including AmpR types, have fewer lysine than arginine residues (31). Hydropathy analysis did not show any long stretch of hydrophobic residues in SmeR, arguing against a transmembrane location (data not shown).

SmeR-regulated Sme-1 expression. When β-lactamase activity from *S. marcescens* S6 was measured against cephaloridine, it reflected the activities of both the AmpC cephalosporinase and the Sme-1 enzyme. However, when activity was measured against imipenem, it reflected only the Sme-1 activity because the cephalosporinase hydrolyzes imipenem extremely poorly. When cefoxitin was added at an inducing concentration of 5 μg/ml, it did not increase carbapenem-hydrolyzing activity in *S. marcescens* S6, but it did increase its cephalosporinase activity 10-fold (Table 3). However, imipenem at a concentration of 50 μg/ml significantly induced both carbapenemase and cephalosporinase activities. With *S. marcescens* 103235T, which lacks *smeA* but has *ampC*, no significant carbapenem-hydrolyzing activity was found and a cefoxitin-inducible cephalosporinase activity was detected (Table 3).

Sme-1 activity was not significantly induced by imipenem in *E. coli* JM109(pPTN103) cultures. However, these had basal β-lactamase activities almost 90-fold greater than those in *S. marcescens* S6. *E. coli* JM109 transconjugants with plasmids pPTN104 to pPTN107, which had various deletions to *smeR* (Fig. 1), expressed about half the β-lactamase activity of JM109 (pPTN103), which had the entire gene expressing SmeR. Sim-

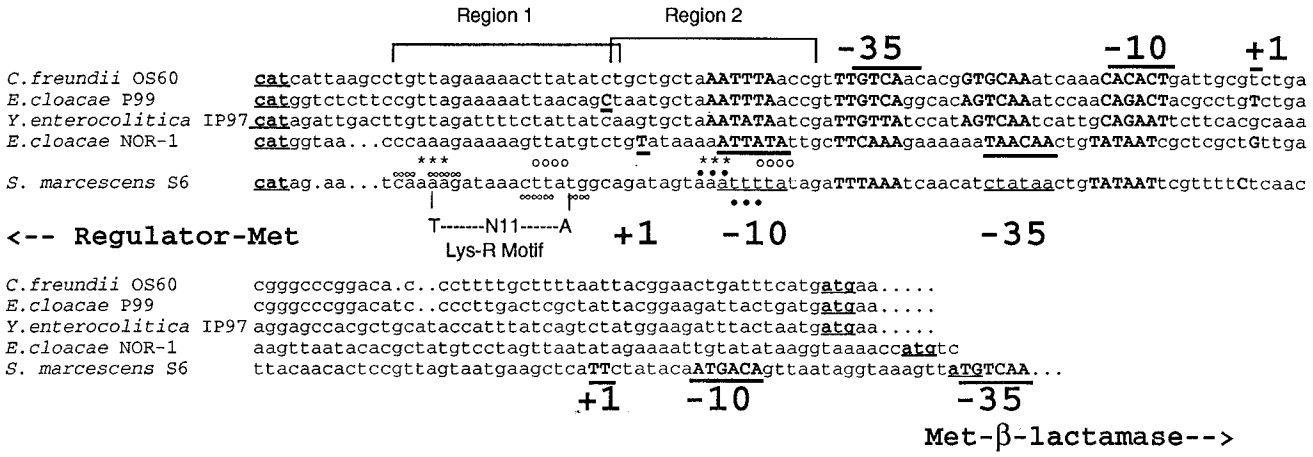

FIG. 4. Alignment of the intercistronic region of *smeA-smeR* from *S. marcescens* S6 with the *ampC-ampR* regions from *E. cloacae*, *Y. enterocolitica*, and *C. freundii* and *nmcA-nmcR* from *E. cloacae* NOR-1. The start codons and the −10 and −35 regions of the promoters are shown below the DNA sequences for *smeR*, *ampR*, and *nmcR* and above the DNA sequences for *smeA*, *ampC*, and *nmcA*. For *smeR*, the promoter used in vivo is underlined with a heavy line, and the other putative −35 and −10 promoter regions are underscored with thin lines. The +1 signs indicate the mRNA transcription starts when they were known. The sequences marked region 1 and region 2 correspond to those conserved among *ampC-ampR* intercistronic regions and were also found in *smeA-smeR* and *nmcA-nmcR*. Regions 1 and 2 are the two components of the putative SmeR-binding site. Palindromic sequences in regions 1 and 2 are indicated either by an infinity symbol or a solid circle. Sequences of homology between regions 1 and 2 are shown by either circles or stars. Only region 1 contained a LysR motif (T-N_{11}-A).

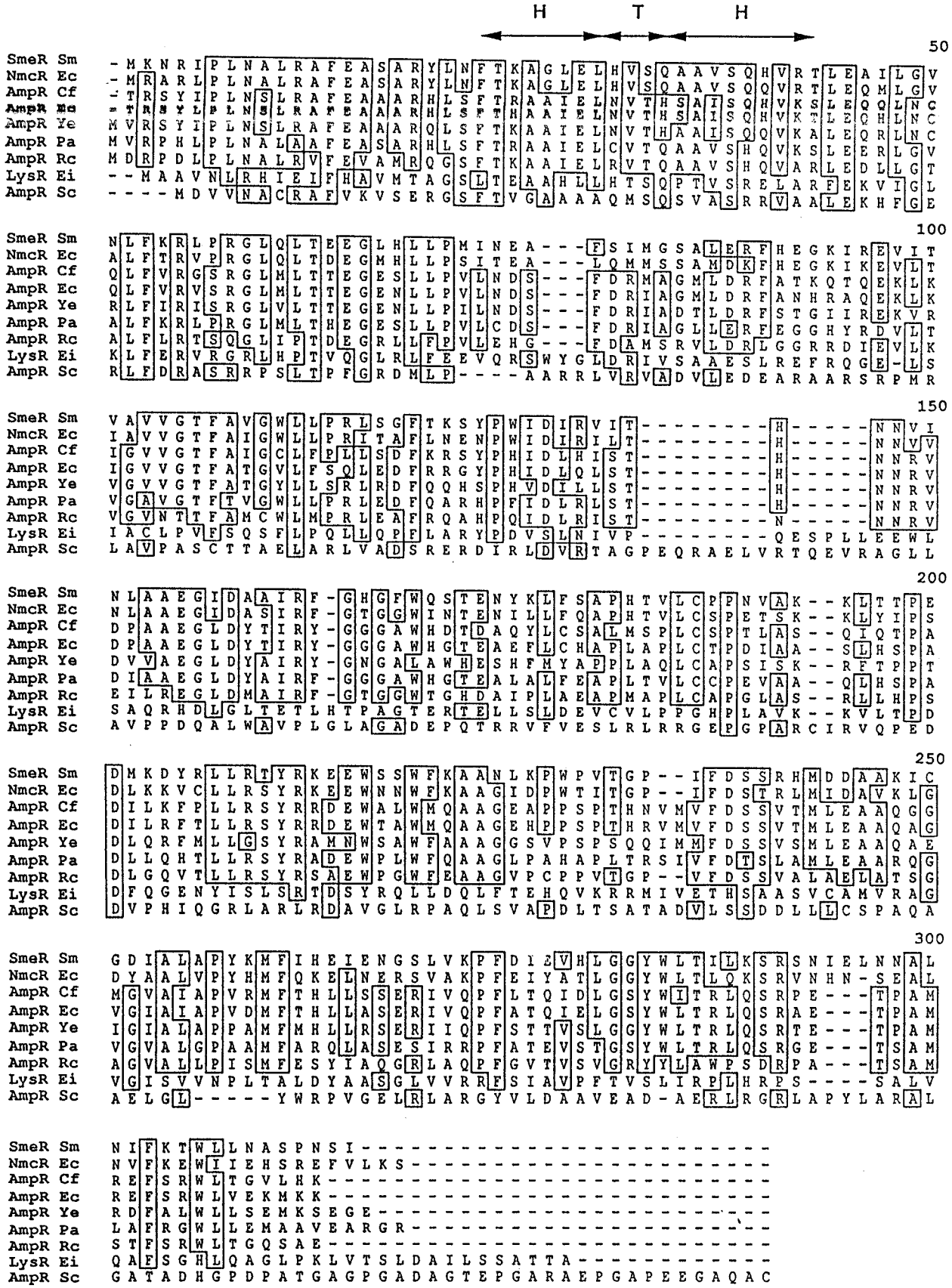

FIG. 5. Alignment of the amino acid sequences of SmeR (this study) with those of the other LysR family β-lactamase regulators: AmpR for AmpC regulation in *C. freundii* (AmpR Cf), *E. cloacae* (AmpR Ec), *Y. enterocolitica* (AmpR Ye), and *P. aeruginosa* (AmpR Pa); AmpR for class A β-lactamase regulation in *Rhodopseudomonas capsulata* (AmpR Rc) and *S. cacaoi* (AmpR Sc); and regulatory protein NmcR for the carbapenem-hydrolyzing β-lactamase NmcA from *E. cloacae* NOR-1 (NmcR Ec) and the LysR-type protein from *E. coli* (LysR Ei). Dashes indicate gaps inserted to optimize the alignment. Identical amino acids are boxed. The predicted helix-turn-helix DNA-binding motif of LysR family proteins is shown (H-T-H).

TABLE 2. Pairwise homologies between the amino acid sequences of some LysR proteins[a]

LysR protein	% Pairwise homology							
	NmcR Ec	AmpR Cf	AmpR Ec	AmpR Ye	AmpR Pa	AmpR Rc	LysR Ei	AmpR Sc
SmeR Sm	69	50	48	50	53	47	25	22
NmcR Ec		46	48	46	51	49	24	22
AmpR Cf			83	66	61	51	26	23
AmpR Ec				70	66	53	25	24
AmpR Ye					60	51	25	23
AmpR Pa						56	26	25
AmpR Rc							27	24
LysR Ei								18

[a] The abbreviations are defined in the legend to Fig. 5. Percentage identities were calculated for entire proteins according to the progressive alignment method of Feng and Doolittle (10) and represent the number of perfect matches divided by the length of the shorter sequence, excluding gaps.

ilarly, *E. coli* JM109 harboring pPTN108, which contained *smeA* and *smeR*, had about a twofold higher β-lactamase activity than did *E. coli* JM109(pPTN109), in which only *smeA* was cloned into the same vector, pACYC184. Taken together, these data show that SmeR acts as a weakly positive activator for SmeA expression.

β-Galactosidase activity. A *smeR-lacZ* translational fusion was constructed to monitor *smeR* transcription and translation to determine the effect of SmeR on *smeR* transcription and to see whether SmeR expression was susceptible to induction by imipenem. pPTN110 has a small 5′ part of *smeR* fused in-frame to *lacZ*, the *smeR-smeA* intercistronic region, and *smeA* (Fig. 1). The β-galactosidase activity of *E. coli* JM109(pPTN110) was low but was significantly greater than that of the vector pTNA480-containing control strain (0.7 ± 0.2 U). Expression of SmeR from pPTN110 in the presence of SmeR expressed in *trans* from pPTN102 resulted in a threefold decrease in β-galactosidase activity. β-Galactosidase specific activity was calculated as described previously (28), where specific activity (U) = $[A_{420}/(\min A_{600}\ \text{ml})] \cdot$ (final volume at assay/initial sample volume). Each value is the arithmetic mean ± standard deviation of four independent assays on each of four clonally independent cultures. For pPTN110 (*smeR-lacZ*), noninduced and induced β-galactosidase activities were 3.9 ± 0.39 and 3.4 ± 0.35 U, respectively. For pPTN110 (*smeR-lacZ*)–pPTN102 (*smeR*), noninduced and induced β-galactosidase activities were 1.3 ± 0.21 and 1.1 ± 0.19 U, respectively. No increase in SmeR, measured as β-galactosidase induction, was noted after challenge with imipenem (50 μg/ml) 45 min before samples were withdrawn. Thus, SmeR appeared to be autoregulated since it had a repressor function on its own transcription and its own synthesis was not affected by the addition of β-lactams.

Distribution of *smeR*. Hybridization was performed with an internal probe for *smeR*, specifically, the 800-bp *Nsi*I fragment from pPTN103. This probe gave a positive signal with *E. coli*(pPTN103), *S. marcescens* S6 and S8 (S8 is an *S. marcescens*

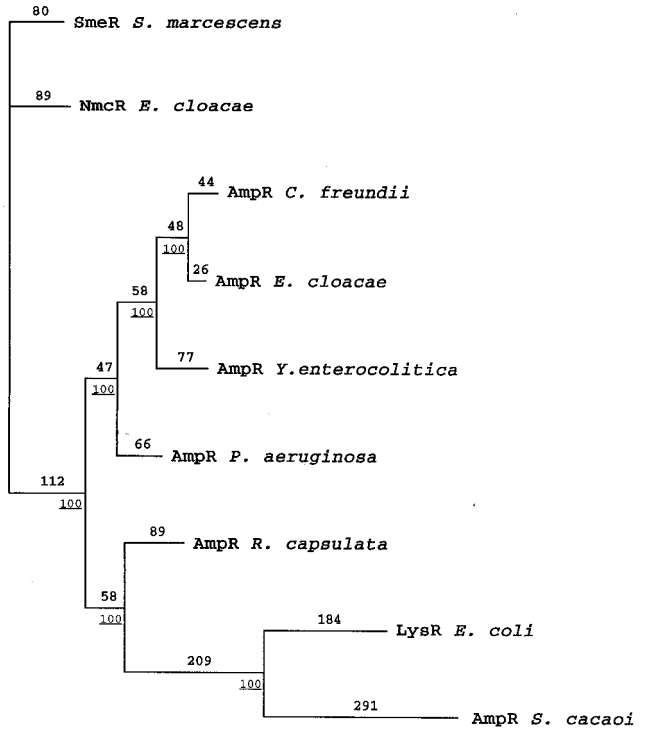

FIG. 6. Dendrogram of known LysR family proteins involved in β-lactamase regulation and the type protein, LysR from *E. coli*. Branch lengths are to scale and are proportional to the numbers of amino acid changes. The percentages at the branch points refer to the number of times a particular nod was found in 100 bootstrap replications (underlined number). The distance along the vertical axis has no significance.

TABLE 3. β-Lactamase activities of bacteria.

Strain and growth conditions[a]	β-Lactamase activity[b]	
	Cephaloridine	Imipenem
S. marcescens 103235T	120 ± 05	0.1 ± 0.1
S. marcescens 103235T + cefoxitin	10,010 ± 50	0.2 ± 0.1
S. marcescens S6	94 ± 10	19 ± 10
S. marcescens S6 + cefoxitin	550 ± 20	25 ± 5
S. marcescens S6 + imipenem	1,180 ± 210	90 ± 25
E. coli JM109(pPTN103)	1,700 ± 110	1,655 ± 120
E. coli JM109(pPTN103) + imipenem	1,820 ± 95	1,730 ± 106
E. coli JM109(pPTN104)	1,000 ± 65	910 ± 35
E. coli JM109(pPTN108)	560 ± 30	450 ± 20
E. coli JM109(pPTN109)	250 ± 40	210 ± 20

[a] The final concentrations of antibiotics added into the cultures were 5 μg/ml for cefoxitin and 50 μg/ml for imipenem. When strains contained plasmids pPTN103 or pPTN104, kanamycin (15 μg/ml) was added to ensure plasmid maintenance. Similarly, for *E. coli* with pPTN108 or pPTN109, chloramphenicol at 20 μg/ml was added.

[b] One unit of β-lactamase activity was defined as the enzymatic activity that hydrolyzed 1 μmol of cephaloridine or imipenem per min and expressed per milligram of protein. Each result is the arithmetic mean ± standard deviation of four independent measurements.

strain which encodes a phenotypically related carbapenem-hydrolyzing β-lactamase [22]) (data not shown). However, total DNAs of the *S. marcescens* reference strain 103235T and of the seven *S. marcescens* clinical strains tested did not hybridize with the *smeR* probe.

DISCUSSION

This report shows that the *smeR* sequence from *S. marcescens* S6 encodes a 31.1-kDa LysR family protein. The gene is located immediately upstream from the previously identified structural gene for the carbapenem-hydrolyzing β-lactamase, *smeA*, and is transcribed divergently from it. This type of organization for β-lactamase genes and their regulators has been identified in other systems, notably in the *ampC-ampR* systems for the cephalosporinases in *E. cloacae*, *C. freundii*, *Y. enterocolitica*, and *P. aeuruginosa* (2), and it seems likely that the AmpC β-lactamase of the *Serratia* spp. also has this type of organization. However, in these cephalosporinase regulatory systems, the AmpR component acts both as a weak repressor in the absence of β-lactam inducers and as an activator in their presence (31). By contrast, in the *smeR-smeA* system, the *S. cacaoi* β-lactamase system (36), and *nmcR-nmcA* from *E. cloacae* (22), the LysR-like proteins act as positive regulators both in the absence of a β-lactam inducer and, more strongly, in the presence of a β-lactam inducer.

SmeR influenced Sme-1 expression much less strongly than NmcR affected NmcA expression (22). Moreover, a major difference between *smeA-smeR* and either *nmcA-nmcR* or *ampC-ampR* is that in the former system the promoters of the β-lactamase gene and the regulator gene, although divergent, sit face-to-face and do not overlap (2, 22). Binding of RNA polymerase to a promoter in vivo deforms the DNA and stresses it torsionally.

LysR-type proteins usually effect a 2- to 80-fold increase in protein expression, and the increase seen for Sme-1 was at the bottom of this range (31). This low level of induction may reflect steric hindrance between RNA polymerase molecules traveling in opposite directions. Therefore, transcription from closely spaced promoters could influence a direct interaction between polymerase molecules which either strengthen or hinder binding or by changes in the topology of the DNA as a consequence of polymerase binding. However, with other face-to-face promoters, transcription is not affected, such as in the artificial construct made of *trp* facing the *lacUV5* promoter (3). In *smeA-smeR*, another putative promoter which might have been used for SmeR expression and which does overlap the *smeA* promoter was found and corresponded exactly to the −35 and −10 regions of the *ampR* and *nmcR* promoters (Fig. 4). However, this promoter is not used in vivo, as was shown by SmeR mRNA primer extension studies with *S. marcescens* S6 and *E. coli* JM109(pPTN103).

Interestingly, as found initially by Bartowsky and Normark (2) for *ampC-ampR* and more recently for *nmcA-nmcR* (22), there is a putative binding site composed of two regions for the LysR-type DNA-binding protein between *smeA* and *smeR* (Fig. 4). Region 1 of this site is within the 5′ half of the putative protected region and contains a partial palindrome separated by a 3- to 5-bp AT-rich region. Region 2 corresponds to the 3′ half of the putative protected region and also contains palindromic sequences but without the AT-rich spacer region. Each half of the palindromic sequence at region 1 shows partial homology with the corresponding half of region 2. In region 1 only, a LysR motif consisting of a T residue (or an A residue) separated by 11 nucleotides from an A residue (or a T residue) around a twofold symmetry axis (12, 31) was found. Comparison of *smeA* and *nmcA* promoters indicated just one base change between the −35 promoter regions (Fig. 4). This base change may explain the relatively high level of SmeA expression even in the absence of SmeR. During evolution, it is possible that such a mutation in the *smeA* −35 region was selected to counteract the long distance between *smeR* and *smeA* promoter regions, therefore leading to a high level of Sme-1 expression in the absence of SmeR. Whatever the explanation, the high level of expression of Sme-1 in the absence of *smeR* suggests an increased risk of spreading carbapenem resistance compared with the risk from NmcA-NmcR expression, in which no carbapenem resistance was conferred when *nmcA* was cloned into a multicopy plasmid in the absence of *nmcR* (25).

As with most LysR-type proteins, including the AmpR proteins of the AmpR-AmpC systems from *C. freundii* and *E. cloacae*, the level of synthesis of SmeR was independent of the inducer and appeared to be autoregulated (14, 16, 17). The low level of SmeR expression may be due to the absence of a corresponding Shine-Dalgarno sequence. In addition to SmeR, Sme-1 induction may be mediated by the other proteins such as AmpD, AmpE, and AmpG which are implicated in AmpC regulation (4) and which, alone or together, may induce conformational changes in SmeR rather than increase its expression.

Dendrogram analysis indicated that SmeR, which is the first LysR family member identified in *Serratia* species, is most closely related to NmcR, which regulates the only other carbapenem-hydrolyzing class A β-lactamase so far described. SmeR and NmcR may together form a special subgroup among LysR β-lactamase regulators. Since SmeA and NmcA are more closely related to each other than to any other β-lactamase, it is likely that *sme-1–smeR* and *nmcA-nmcR* have a common ancestor. Apart from their similarity to each other, SmeR and NmcR are more related to regulators of the class C cephalosporinase described in members of the family *Enterobacteriaceae* than to the regulators of the class A β-lactamase of *S. cacaoi*. This finding may indicate that the relationships among LysR proteins depend more on the organism in which they were found than on the type of β-lactamase they regulate.

A final particular similarity of SmeR and NmcR was that both have Lys/Lys + Arg ratios of 0.51, as does the LysR PhcA from *Pseudomonas solanacearum* (5), whereas most other LysR proteins, including AmpR of the AmpR-AmpC systems, have ratios of <0.25 compared with a general average of 0.46 for *E. coli* proteins. The significance of these data remains unknown.

Detailed analysis of the alignments of various LysR regulators revealed several conserved amino acids. Such homologies were much more apparent in the N-terminal domain than in the C-terminal domain (Fig. 5). The helix-turn-helix motifs shared two conserved amino acids; threonine at position 24 and serine at position 38. Other conserved residues included proline at position 188 and aspartic acid at position 201. Interestingly, the glycine at position 105 (position 102 in the numeration of Bartowsky and Normark [2]) (Fig. 5) may be especially noteworthy. This residue is found in all enterobacterial β-lactamase regulators described so far, and mutagenesis to alanine increases the ability of AmpR to act as a transcriptional activator for AmpC in the absence of β-lactam inducers (2). The conserved C-terminal amino acids may play a role in binding to signal transducers, thus effecting conformational changes as described for other LysR proteins. As with other AmpR proteins, SmeR did not possess the S-X-X-K and KTG motifs that might have indicated that it could itself bind or hydrolyze β-lactams.

The absence of *smeR* from other cephalosporinase-inducible *S. marcescens* isolates indicated that SmeR did not correspond to the putative (not yet identified) AmpR of the AmpR-AmpC regulatory system of *Serratia* species. Nevertheless, in view of its homology to the AmpR proteins from various enterobacterial species, it is possible that SmeR, although different from AmpR of *Serratia* species, might still act as a regulator for AmpC expression in *S. marcescens* S6. Future work will aim at investigating the effects of SmeR on cephalosporinase biosynthesis and to characterize the DNA-binding sites of SmeR in relation to those of AmpR. Site-directed mutagenesis of key positions within SmeR may help to elucidate its mechanism of regulating SmeA.

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