

SME-Type Carbapenem-Hydrolyzing Class A β -Lactamases from Geographically Diverse *Serratia marcescens* Strains

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Three sets of carbapenem-resistant *Serratia marcescens* isolates have been identified in the United States: 1 isolate in Minnesota in 1985 (before approval of carbapenems for clinical use), 5 isolates in Los Angeles (University of California at Los Angeles [UCLA]) in 1992, and 19 isolates in Boston from 1994 to 1999. All isolates tested produced two β -lactamases, an AmpC-type enzyme with pI values of 8.6 to 9.0 and one with a pI value of approximately 9.5. The enzyme with the higher pI in each strain hydrolyzed carbapenems and was not inhibited by EDTA, similar to the chromosomal class A SME-1 β -lactamase isolated from the 1982 London strain *S. marcescens* S6. The genes encoding the carbapenem-hydrolyzing enzymes were cloned in *Escherichia coli* and sequenced. The enzyme from the Minnesota isolate had an amino acid sequence identical to that of SME-1. The isolates from Boston and UCLA produced SME-2, an enzyme with a single amino acid change relative to SME-1, a substitution from valine to glutamine at position 207. Purified SME enzymes from the U.S. isolates had β -lactam hydrolysis profiles similar to that of the London SME-1 enzyme. Pulsed-field gel electrophoresis analysis revealed that the isolates showed some similarity but differed by at least three genetic events. In conclusion, a family of rare class A carbapenem-hydrolyzing β -lactamases first described in London has now been identified in *S. marcescens* isolates across the United States.

Carbapenems are β -lactam antibiotics with broad antibacterial activity, increased stability to hydrolysis, and high rates of penetration through the bacterial outer membrane. Several mechanisms of resistance to carbapenems in gram-negative bacteria have been described and include the loss of outer membrane permeability and the production of β -lactam-hydrolyzing enzymes (2, 11, 16). Most carbapenem-hydrolyzing activity has been due to molecular class B metalloenzymes, such as CcrA in *Bacteriodes fragilis* (23) and IMP-1 in organisms such as *Pseudomonas aeruginosa* and *Serratia marcescens* (9, 20). The metalloenzymes, which contain two zinc atoms at the active site and which are distinguished by EDTA inhibition, were initially identified as chromosomal β -lactamases in Japan, Europe, and the United States (11, 16). In Japan, where carbapenems are used more frequently, IMP-1 has been found on plasmids (9). In Italy, the VIM-1 metallo- β -lactamase is located on an integron (10).

In addition to the zinc-based metalloenzymes, two groups of serine-based β -lactamases capable of carbapenem hydrolysis have emerged. The class D ARI enzyme from *Acinetobacter baumannii* has been found on plasmids in the United Kingdom, Argentina, Turkey, and Spain (7, 19). Three chromosomal class A β -lactamases that cause imipenem resistance have also been described: from *Enterobacter cloacae*, NMC-A in France (14) and IMI-1 in California (18), and from *S. marcescens*, SME-1 in England (12).

SME-1 was isolated from the imipenem-resistant *S. marcescens* strain S6, as well as strain S8, in London in 1982 (12).

This chromosomal enzyme is capable of hydrolyzing penicillin, aztreonam, and cephalosporins in addition to imipenem. Since the discovery of SME-1, three sets of imipenem-resistant *S. marcescens* strains have been isolated from different regions of the United States. In 1985, a single imipenem-resistant isolate was discovered in Minnesota (A. A. Medeiros and R. S. Hare, Abstr. 26th Intersci. Conf. Antimicrob. Agents Chemother., abstr. 116, 1986). Five isolates were collected from the University of California at Los Angeles (UCLA) in 1992 (J. P. Quinn, D. Miyashiro, J. Hindler, C. Holt, and K. Bush, Abstr. 37th Intersci. Conf. Antimicrob. Agents Chemother., abstr. C-99, 1997), and 19 isolates were collected from the Deaconess Hospital (Boston) between 1994 and 1999 (Y. Carmeli, M. Samore, N. Troillet, M. Dube, L. Venkataraman, P. Degirolami, G. Eliopoulos, and K. Bush, Abstr. 37th Intersci. Conf. Antimicrob. Agents Chemother., abstr. C-100, 1997). Isoelectric focusing of extracts from the United States strains revealed that they carried two β -lactamases—an AmpC-type enzyme with a pI of approximately 8.5 and one with a pI of approximately 9.5—similar to the pattern of enzymes seen in the London strain *S. marcescens* S6. Like SME-1 in the London strain S6, the carbapenem-hydrolyzing enzymes in the U.S. strains were assumed to be chromosomal, as no evidence of plasmids was found (12; J. P. Quinn, D. Miyashiro, J. Hindler, C. Holt, and K. Bush, Abstr. 37th Intersci. Conf. Antimicrob. Agents Chemother., abstr. C-99, 1997). The purpose of this study was to compare the SME-type β -lactamases found in the United States to the London SME-1 enzyme.

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TABLE 1. Bacterial strains

Strain	Relevant properties	Reference or source
<i>S. marcescens</i> clinical isolates		
S6	1982 Imipenem-resistant strain from London, producing SME-1	12
4176	1985 Imipenem-resistant strain from Minnesota (MN-2701)	Medeiros and Hare, 26th ICAAC
4126	1992 Imipenem-resistant strain from UCLA (UCLA-1)	Quinn et al., 37th ICAAC
4124	1995 Imipenem-resistant strain from Boston (no. 4)	Carmeli et al., 37th ICAAC
<i>E. coli</i> transformants		
4911	Carries plasmid pIRS1, SME from 4176 cloned into pCR2.1	This work
4912	Carries plasmid pIRS2, SME from 4126 cloned into pCR2.1	This work
4913	Carries plasmid pIRS3, SME from S6 cloned into pCR2.1	This work
4914	Carries plasmid pIRS4, SME from 4124 cloned into pCR2.1	This work
<i>E. coli</i> One Shot	Recipient for <i>sme</i> genes cloned into pCR2.1	Invitrogen
<i>E. coli</i> One Shot/pCR2.1	Vector-only control for MIC testing	Invitrogen

A. A. Medeiros, and K. Bush, Abstr. 39th Intersci. Conf. Antimicrob. Agents Chemother., abstr. 1466, 1999].

MATERIALS AND METHODS

Bacterial strains. The original *S. marcescens* clinical isolates and the *Escherichia coli* transformants harboring the SME-type β -lactamases are shown in Table 1. For locations at which multiple isolates were identified (UCLA and Boston), a representative isolate was chosen for analysis.

Antimicrobial agents. Substrates for the hydrolysis assays were obtained from the following sources: cephaloridine, penicillin G, ampicillin, cefotaxime, and cefoxitin, Sigma Chemical Co. (St. Louis, Mo.); ceftazidime and clavulanic acid, U.S. Pharmacopoeia (Rockville, Md.); imipenem, Merck (Rahway, N.J.); meropenem, AstraZeneca (Wilmington, Del.); and tazobactam, Lederle Laboratories (Pearl River, N.Y.). All substrates were prepared fresh daily as 1-mg/ml stocks in 50 mM phosphate buffer (pH 7.0).

Susceptibility testing. MICs were determined by the National Committee for Clinical Laboratory Standards broth microdilution method (13).

PCR. Two sets of primers were designed to amplify SME-related DNA sequences. Primers of the first set, IRS1 and IRS2 (5' AACGGCTTCATTTTGT TTTAG 3' and 5' GCTTCGCAATAGTTTTATCA 3'), were complementary to bp 151 to 171 and 961 to 981, respectively, of the sequence of SME-1 and flanking DNA (12) and amplified an 830-bp intragenic fragment of SME-1. Primers of the second set, IRS5 and IRS6 (5' AGATAGTAAATTTTATAG 3' and 5' CTCTAACGCTAATAG 3'), complemented bp 5 to 22 and 1128 to 1142 of the same sequence. These primers produced a PCR product that included the putative promoter, the ribosome binding site, and the entire open reading frame. The PCR program for IRS1 and IRS2 consisted of a lysis-denaturation step for 10 min at 95°C; 30 cycles of a 30-s denaturation step at 94°C, a 30-s annealing step at 58°C, and a 30-s extension step with *Taq* polymerase at 72°C; and a final 10-minute extension step at 72°C. The program for IRS5 and IRS6 included an annealing temperature of 50°C and an extension time of 60 s. Genomic DNA was the template for PCR (15).

DNA cloning and sequencing. PCR of purified genomic DNA from imipenem-resistant *S. marcescens* yielded amplification products that were cloned into plasmid pCR2.1 and transformed into One Shot (competent *Escherichia coli*) using the protocol provided by the manufacturer of the TA cloning kit (Invitrogen, Carlsbad, Calif.). Screening for transformants was done using Luria-Bertani agar plates containing 5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside (X-Gal) (80 μ g/ml) and ampicillin (50 μ g/ml) for the intragenic PCR product obtained by using primers IRS1 and IRS2 or imipenem (10 μ g/ml) for selecting plasmids containing the complete open reading frame (cloned PCR products of IRS5 and IRS6). Sequencing of both strands was performed using primers T7 and M13 Reverse at the Molecular Biology Core Facility of the Dana-Farber Cancer Institute (Boston, Mass.). At least two cloned PCR products were sequenced for each template. The Clustal method (8) was used to perform alignments of deduced amino acid sequences with the MEGALIGN software program (DNASTAR, Inc., Madison, Wis.).

β -Lactamase purification. The SME-type β -lactamases for the kinetic analysis were purified from the transformant *E. coli* strains 4911, 4912, 4913, and 4914, which were shown by isoelectric focusing to contain a TEM-1 β -lactamase from the cloning vector in addition to SME-type enzymes (Queenan et al., 39th ICAAC). Bacteria from a 1-liter overnight culture in Trypticase soy broth supplemented with 100 μ g of ampicillin per ml were harvested by centrifugation and lysed with a freeze-thaw procedure (21). The supernatant was loaded onto a Sephadex G-100 column with 50 mM phosphate buffer (pH 7.0). Protein in peak fractions containing nitrocefin-hydrolyzing activity was precipitated with 90% ammonium sulfate; pellets were resuspended in 50 mM phosphate buffer (pH 7.0) and dialyzed in 1 liter of 25 mM Tris-HCl (pH 7.5) at 4°C with two buffer changes. The plasmid vector-derived TEM-1 (pI, 5.4) enzyme was separated from the SME-type β -lactamase (pI, ~9.5) on a QAE Sephadex A-25 ion-

exchange column with 25 mM Tris-HCl (pH 7.5). The protein concentrations of the purified β -lactamases were determined with the Pierce (Rockford, Ill.) bicinchoninic acid protein assay. The purity of the enzymes ranged from 90 to 92%, as determined by spot densitometry of a silver-stained 10% NuPAGE gel.

Kinetic studies. Initial hydrolysis rates were measured on a Shimadzu UV-1601 spectrophotometer at 25°C with 50 mM phosphate buffer (pH 7.0) (21). K_m and V_{max} values were obtained by averaging results from Lineweaver-Burk, Eadie-Hofstee, Hanes-Woolf, and direct linear plot analyses. Substrates were assayed on at least two separate days, with cephaloridine included as a reference each day. Inhibition of hydrolysis was measured after 5 min of preincubation of enzyme with inhibitor in 10 μ l of phosphate buffer (pH 7.0). Cephaloridine (360 μ M) was the substrate for the inhibition studies. Inhibitor concentrations causing 50% inhibition (IC_{50} s) were determined from inhibition graphs of percent control activity versus concentration of inhibitor. K_i values were calculated by the method of Cheng and Prusoff (6).

PFGE. For pulsed-field gel electrophoresis (PFGE), a CHEF-DR11 system (BioRad Laboratories, Hercules, Calif.) was used to analyze *SpeI*-digested DNA from the *S. marcescens* isolates. DNA was prepared with a Bio-Rad CHEF genomic DNA plug kit according to the manufacturer's protocol. Agarose (1.2%) gels were run in 0.5 \times Tris-borate-EDTA for 22 h at 190 V, with pulse times of 5 to 42 s. The imipenem-sensitive *S. marcescens* clinical strain SC 9782, from the Seattle Veterans Affairs Medical Center (3), was used as a control.

Nucleotide sequence accession number. The sequence of *sme-2* was assigned GenBank accession number AF275256.

RESULTS

Antimicrobial susceptibility. The imipenem-resistant *S. marcescens* clinical isolates used in this study were collected from geographically diverse regions of the United States. As shown in Table 2, all of the clinical isolates had decreased susceptibility to imipenem, meropenem, aztreonam, and cefoxitin. The *E. coli* transformant strains in Table 2 contained the *sme* genes from the four clinical isolates inserted into cloning vector pCR2.1. The presence of the *sme* gene on a plasmid elevated the MICs of imipenem, meropenem, ceftazidime, and aztreonam compared to those for the recipient strain alone. A modest increase in MICs was detected for cefoxitin when the SME enzyme was present. The effect on penicillin MICs could not be evaluated due to the TEM-1 enzyme being present on the cloning vector.

Sequence analysis. Two protein sequences, initially designated SME-1 and SME-2, containing an isoleucine and a tyrosine, respectively, at position 245, have been determined for the same carbapenem-hydrolyzing enzyme from the London strain *S. marcescens* S6 (12; B. A. Rasmussen, D. Keeney, and C. Cohen, GenBank accession number U60295). Recently, this discrepancy has been resolved, and it has been determined that tyrosine is the correct amino acid for this position (P. Nordmann, personal communication). We now refer to the enzyme from the London strain S6 as SME-1.

The cloned *sme* genes from the U.S. isolates were sequenced from both strands of the plasmids from the *E. coli* transformants. The Minnesota strain MN-2701 produced an enzyme

TABLE 2. Antimicrobial susceptibility of *S. marcescens* isolates and *E. coli* transformants with SME enzymes

Location	Strain	β -Lactamase(s)	MIC ($\mu\text{g/ml}$) of ^a :				
			IPM	MEM	ATM	FOX	CAZ
London	<i>S. marcescens</i> S6	SME-1, AmpC	512	64	128	32	<0.25
	<i>E. coli</i> 4913	SME-1, TEM-1	256	32	>256	16	8
Minnesota	<i>S. marcescens</i> 4176	SME-1, AmpC	512	128	64	128	2
	<i>E. coli</i> 4911	SME-1, TEM-1	256	32	>256	32	8
UCLA	<i>S. marcescens</i> 4126	SME-2, AmpC	512	128	128	128	0.5
	<i>E. coli</i> 4912	SME-2, TEM-1	256	64	>256	32	8
Boston	<i>S. marcescens</i> 4124	SME-2, AmpC	512	128	>256	64	<0.25
	<i>E. coli</i> 4914	SME-2, TEM-1	256	32	>256	16	16
Controls	<i>E. coli</i> recipient		<0.5	<0.5	<0.25	4	<0.25
	<i>E. coli</i> recipient/pCR2.1	TEM-1	<0.5	<0.5	<0.25	8	<0.25

^a IPM, imipenem; MEM, meropenem; ATM, aztreonam; FOX, ceftaxitin; CAZ, ceftazidime.

identical to the SME-1 enzyme from strain S6 (B. A. Rasmussen, D. Keeney, and C. Cohen, GenBank Accession number U60295). A modified enzyme was found in the Boston and UCLA strains. This new member of the SME family contained three nucleotide substitutions in the gene relative to SME-1—A for G at 258, A for T at 620, and A for G at 714—resulting in a single amino acid change, Glu for Val at position 207, according to the Ambler numbering system (1). As a result of the sequence data, we have designated the Minnesota enzyme SME-1 and the Boston and UCLA enzymes SME-2. The promoter region for the *sme-2* gene was identical to the published sequence for the *sme-1* gene (data not shown).

Biochemical characterization. The SME β -lactamases were purified chromatographically from the *E. coli* transformants. As expected from the sequence analysis, all four of the cloned *sme* genes produced proteins of approximately 30 kDa when examined on a polyacrylamide gel, in accord with the 29.3-kDa protein predicted from the sequence (data not shown).

Kinetic data for the SME-1 and SME-2 β -lactamases are summarized in Table 3. K_m and k_{cat} values for each substrate were similar for both enzymes, indicating that the single amino

acid change in SME-2 did not affect the substrate binding or hydrolysis rates. SME-1 and SME-2 hydrolyzed a variety of β -lactams from the penicillin, cephalosporin, monobactam, and carbapenem groups. The highest k_{cat} values were obtained for cephaloridine, followed by ampicillin, aztreonam, and imipenem. Meropenem had a k_{cat} value 10 times lower than that of imipenem.

The SME enzymes had the highest affinity, or the lowest K_m , for penicillin and meropenem. Ceftaxitin, cefotaxime, and ceftazidime were poor substrates, with hydrolysis rates too slow to obtain accurate K_m values under these assay conditions. Taking the binding affinity into account, k_{cat}/K_m values showed that the catalytic efficiencies of both enzymes varied approximately threefold for all the hydrolyzable substrates.

Clavulanic acid inhibited the SME-1 and SME-2 β -lactamases, with K_i values of 0.19 and 0.18 μM , respectively (Table 3). Tazobactam was a slightly better inhibitor for these enzymes, with K_i values of 0.11 and 0.12 μM for the SME-1 and SME-2 enzymes respectively (Table 3). No inhibition was observed when the enzymes were preincubated with 2.5 μM EDTA at pH 7.0, as expected for serine-based β -lactamases.

TABLE 3. Kinetic parameters of SME β -lactamases

Enzyme ^a	k_{cat} (s^{-1}) for:		Relative k_{cat} for:		K_m (μM) for:		k_{cat}/K_m for:	
	SME-1	SME-2	SME-1	SME-2	SME-1	SME-2	SME-1	SME-2
Substrate								
LOR	980 \pm 18	1081 \pm 5	100	100	770 \pm 30	859 \pm 45	1.27	1.26
PEN	19.3 \pm 1.1	21.3 \pm 0.4	2.0	1.9	16.7 \pm 6	17.7 \pm 0.4	1.16	1.20
IPM	104 \pm 4.2	136 \pm 21	11	13	202 \pm 38	313 \pm 57	0.52	0.44
MEM	8.9 \pm 0.6	7.3 \pm 4.9	0.9	0.7	13.4 \pm 3.2	9.6 \pm 8.9	0.66	0.76
AMP	181 \pm 13	204 \pm 18	18	19	488 \pm 47	609 \pm 124	0.37	0.34
ATM	108 \pm 21	140 \pm 5	11	13	259 \pm 23	277 \pm 8.5	0.42	0.51
FOX	<0.15	<0.17	<0.02	<0.02	ND ^b	ND	ND	ND
CTX	<0.98	<1.0	<0.10	<0.09	ND	ND	ND	ND
CAZ	<0.07	<0.09	<0.01	<0.01	ND	ND	ND	ND
Inhibitor								
CLA	NA ^c	NA	NA	NA	0.19 ^d	0.18 ^d	NA	NA
TZB	NA	NA	NA	NA	0.11 ^d	0.12 ^d	NA	NA

^a LOR, cephaloridine; PEN, penicillin; IPM, imipenem; MEM, meropenem; AMP, ampicillin; ATM, aztreonam; FOX, ceftaxitin; CTX, cefotaxime; CAZ, ceftazidime; CLA, clavulanic acid; TZB, tazobactam.

^b ND, not determined; hydrolysis was too slow.

^c NA, not available.

^d K_i , IC_{50} s for SME-1 and SME-2, respectively: CLA, 0.28 and 0.25 μM ; TZB, 0.16 and 0.17 μM .

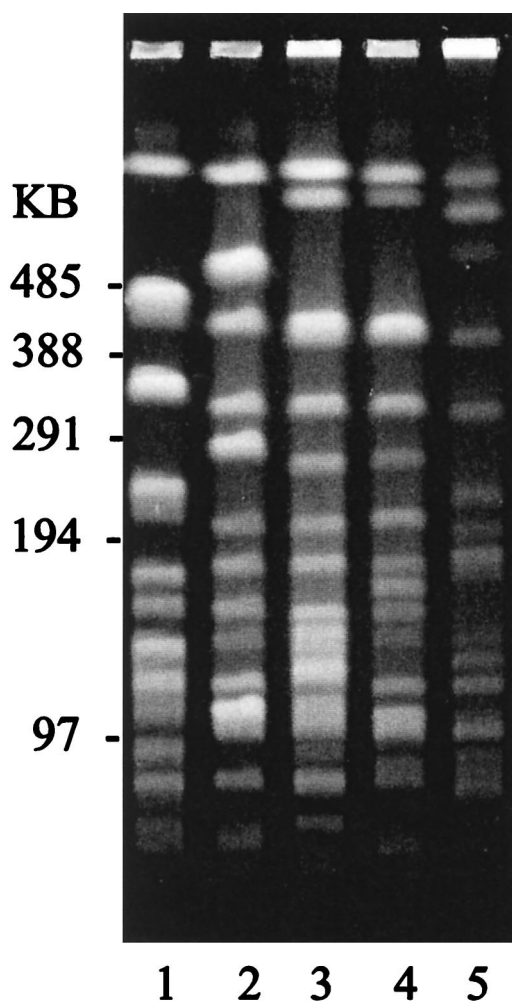


FIG. 1. PFGE of *SpeI*-digested DNA from *S. marcescens* isolates. Lane 1, imipenem-sensitive strain SC 9782. Lanes 2 to 5, imipenem-resistant strains S6 (London), 4176 (Minnesota), 4124 (Boston), and 4126 (California), respectively. Markers are from a Bio-Rad lambda ladder.

PFGE analysis. DNAs from the four imipenem-resistant *S. marcescens* strains and an imipenem-sensitive *S. marcescens* clinical strain were compared by PFGE (Fig. 1). There were at least seven band differences between any two of the imipenem-resistant strains. The imipenem-sensitive strain had a minimum of 17 band differences compared to any of the imipenem-resistant strains.

DISCUSSION

Imipenem resistance due to class A serine-based β -lactamases is rare. Only three sets of enzymes have been reported in functional group 2f: NMC-A and IMI-1, both from *E. cloacae*, and SME-1, from *S. marcescens* (12, 14, 17). Each of these enzymes was found either as a single isolate or in a pair of clinical isolates from geographically diverse regions. In the United States, imipenem-resistant *S. marcescens* strains were isolated in Minnesota, California, and Boston over a period of 15 years. Preliminary testing suggested that the imipenem resistance was due to an enzyme similar to SME-1 (Medeiros and Hare, 26th ICAAC; Quinn et al., 37th ICAAC; Carmeli et al., 37th ICAAC).

The molecular relatedness of the β -lactamase genes cloned

from the imipenem-resistant *S. marcescens* strains was investigated. The SME-1 enzyme and a single-amino-acid variant, SME-2, were identified in this study of U.S. isolates. The isolate from Minnesota produced an enzyme identical to the London SME-1 enzyme. SME-2, from the Boston and California strains, was distinguished by a single glutamic acid-for-valine substitution at position 207. This sequence change introduced another identical amino acid between SME-2 and the other molecular class A, functional group 2f enzymes that also contain a glutamic acid at position 207 (4). The SME enzymes shared 70% amino acid identity with NMC-A and IMI-1 (17).

SME-1 and SME-2 hydrolyzed a range of substrates, including penicillins, aztreonam, cephaloridine, and carbapenems. There was no detectable hydrolytic activity against ceftazidime, consistent with low ceftazidime MICs for the clinical isolates. A similar hydrolysis profile is seen for NMC-A and IMI-1. The relative hydrolysis rates for the SME enzymes purified in this study also compared well with those published for the other group 2f enzymes, where relative rates for cephaloridine and ampicillin were higher than those for imipenem (16). In contrast to previously published data for clavulanic acid inhibition of SME-1 (IC_{50} , 14 μ M [5]), the purified SME-1 and SME-2 enzymes in this study had IC_{50} s similar to those published for NMC-A and IMI-1, in the range of 200 to 300 nM. The IC_{50} for tazobactam found in these experiments was 10-fold lower than that previously published for SME-1 (5). These differences may be due to the fact that nitrocefin was used as the reporter substrate in the earlier study (5) and that cephaloridine, at a sub- K_m concentration, was used in this study.

The SME β -lactamases had a broad substrate spectrum, with hydrolysis of penicillins and early cephalosporins, in addition to carbapenems. This property could have been a factor in their emergence in strains isolated before imipenem was approved for clinical use in 1985. The original source of these enzymes remains unknown. It is unlikely that the SME β -lactamases evolved from an enzyme previously existing in the *Serratia* genome, as 16 imipenem-sensitive *S. marcescens* clinical isolates did not hybridize with an *sme* probe (H. S. Gold, unpublished data).

Initially, the rarity and geographically diverse locations of the imipenem-resistant *S. marcescens* strains suggested convergent evolution of the SME β -lactamases. This idea was tested by PFGE analysis of DNA from the four isolates. The results indicated that the four isolates differed by at least three genetic events (22). There was a degree of relatedness among these isolates of imipenem-resistant *S. marcescens*, as they shared more bands with each other than with an *S. marcescens* strain that lacked an SME enzyme. The PFGE results are consistent with global dissemination of a distinct *S. marcescens* subtype. SME-1 and SME-2, since they are not encoded on plasmids, are not readily transmissible, like the TEM and SHV extended-spectrum β -lactamases. The fact that these enzymes are chromosomally encoded is one reason for their rarity, although, as with all resistance genes, the possibility exists that their genes may be acquired by a mobile element. It appears that carbapenem-hydrolyzing β -lactamases have been present in the genetic environment for some time. It is likely that the increased frequency of carbapenem use will increase the frequency with which these β -lactamases are found in a clinical setting.

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