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Original Article

Nosocomial Outbreak of Ceftazidime-Resistant *Serratia marcescens* Strains That Produce a Chromosomal AmpC Variant with N235K Substitution

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SUMMARY: *Serratia marcescens* is a Gram-negative bacterium that is often associated with nosocomial infections. Here we analyzed the resistance mechanism of the ceftazidime-resistant *S. marcescens* nosocomial strains. The five *S. marcescens* urinary tract infection-associated isolates were positive for chromosomal *ampC* and bla_{TEM-1}. Four of the five strains, ES11, ES31, ES42, and ES46, were single clone and ceftazidime resistant. The fifth strain, ES71, was susceptible to ceftazidime. Analysis of the deduced amino acid sequence revealed a Glu-235-Lys substitution in the third amino acid of the third motif of AmpC from both ES46 and ES71, and a site-directed mutagenesis experiment confirmed that this substitution is involved in the ceftazidime resistance phenotype. However, the resistance phenotypes of strains ES46 and ES71 to ceftazidime were quite different from one another, indicating that another mechanism, in addition to the AmpC mutation, is also involved in the determination of the resistance phenotype of these strains. Basal AmpC activity was more than two times higher in strain ES46 than in ES71, which could result in the differing resistance phenotypes of these two strains. The clinical significance and prevalence of extended-spectrum cephalosporin-resistant *S. marcescens* strains harboring the mutated chromosomal *ampC* gene are unclear in Japan and remain to be elucidated.

INTRODUCTION

Serratia marcescens is a saprophytic, water-borne Gram-negative rod (1) that is often associated with nosocomial infections (2,3) as well as human diseases (4-6). For many Gram-negative bacteria, including *S. marcescens*, production of chromosomal Ambler Class C β-lactamase, or AmpC cephalosporinase, which is encoded by the *ampC* gene, is the intrinsic mechanism of resistance to β-lactam antibiotics (7). *S. marcescens* generally has inducible expression of this enzyme and is resistant to the narrower spectrum cephalosporins but susceptible to expanded-spectrum cephalosporins such as cefotaxime, ceftazidime, cefepime, and cefpirome (8,9).

S. marcescens strains with different mechanisms of resistance to the expanded-spectrum cephalosporins have been reported. In many Gram-negative bacteria including *Enterobacteriaceae*, a mutation affecting the regulatory gene systems of AmpC expression causes constitutive overproduction of AmpC, resulting in resistance to expanded-spectrum cephalosporins (10), and it was also demonstrated that a *S. marcescens* clinical isolate overproducing AmpC was highly resistant to cefotaxime, ceftazidime, and aztreonam (11). Acquisition of Ambler Class A extended-spectrum β-lactamases (ESBLs) (12-14) or Class B metallo-β-lactamases (15) also confers resistance to the expanded-spectrum cephalosporins. Mutation of the chromosomal *ampC* gene is another mechanism conferring an expansion of the substrate specificity of AmpC, resulting in an increase in resistance to expanded-spectrum cephalosporins (16-19). Aside from the case of Ambler class A enzymes, however, only a few clinical strains producing such an extended-spectrum AmpC β-lactamase, as designated by Morosini et al. (18), have been described: *Enterobacter cloacae* GC1 (19), *S. marcescens* GN16694 (17), and *S. marcescens* HD (16). The detailed resistance mechanism of *S. marcescens* producing mutated-AmpC is therefore still not thoroughly understood.

We have isolated ceftazidime-resistant *S. marcescens* strains from inpatients on a cerebral surgical ward over a 14-month period in one hospital of Akita Prefecture, Japan. In this study, we examined the resistance mechanism of the ceftazidime-resistant *S. marcescens* strains.

MATERIALS AND METHODS

Bacterial strains and plasmids: The bacterial strains employed in this study included one environmental and five clinical isolates of *S. marcescens*, five *Escherichia coli* transformants harboring the *ampC* gene cloned from the *S. marcescens* isolates, and *E. coli* AS226-51 (19), an *ampD* mutant of *E. coli* C600, which has a deletion mutation in *ampC* (Table 1). The cloning vector plasmid pBC SK+ was purchased from Stratagene (La Jolla, Calif., USA) (Table 1). pBC SK+ containing *S. marcescens ampC* genes, with or without artificial mutation, pJYES46, pJYES71, pJYSm4, pJYES46(DAES), and pJYSm4(DAKS) were constructed as described below.

In vitro susceptibility testing: The minimum inhibitory concentrations (MICs) of the *S. marcescens* isolates and *E. coli* transformants were determined by the broth microdilution method according to the guidelines of the National Committee for Clinical Laboratory Standards (20,21). MICs of

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Table 1. Bacterial strains and plasmids employed in this study

Strain or plasmid	Type of isolate	Source or description	Date of isolation
S. marcescens ES11	Clinical isolate	Indwelling catheter urine from an inpatient on a cerebral surgical ward.	Apr. 16, 2002
S. marcescens ES31	Clinical isolate	Indwelling catheter urine from an inpatient on a cerebral surgical ward.	Mar. 3, 2003
S. marcescens ES42	Clinical isolate	Urine from an inpatient on a cerebral surgical ward.	Apr. 11, 2003
S. marcescens ES46	Clinical isolate	Indwelling catheter urine from an inpatient on a cerebral surgical ward.	May 12, 2003
S. marcescens ES71	Clinical isolate	Indwelling catheter urine from an inpatient on a urological ward.	Aug. 19, 2003
S. marcescens Sm4	Environmental isolate	Syringe in a hospital	Feb. 18, 2002
E. coli ampC ES46	Transformant	*E. coli* AS226-51 transfomed with pJYES46	
E. coli ampC ES71	Transformant	*E. coli* AS226-51 transfomed with pJYES71	
E. coli ampC Sm4	Transformant	*E. coli* AS226-51 transfomed with pJYSm4	
E. coli ampC ES46(DAES)	Transformant	*E. coli* AS226-51 transfomed with pJYES46(DAES)	
E. coli ampC Sm4(DAKS)	Transformant	*E. coli* AS226-51 transfomed with pJYSm4(DAKS)	
E. coli AS226-51		An *ampD* mutant of *E. coli* C600 that also has a deletion mutation in *ampC* (19)	
pBC SK+		Cloning vector plasmid digested with *EcoR*I and *Hind*III	
pJYES46		*ampC* of *S. marcescens* ES46 inserted into pBC SK+	
pJYES71		*ampC* of *S. marcescens* ES71 inserted into pBC SK+	
pJYSm4		*ampC* of *S. marcescens* Sm4 inserted into pBC SK+	
pJYES46(DAES)		*ampC* of *S. marcescens* ES46 with DAKS to DAES substitution in third motif inserted into pBC SK+	
pJYSm4(DAKS)		*ampC* of *S. marcescens* Sm4 with DAES to DAKS substitution in third motif inserted into pBC SK+	

aztreonam, imipenem, meropenem, gentamicin, minocycline, and levofloxacin were determined using a commercially available plate, Dryplate "Eiken" DP-21 (Eiken Kagaku Co., Tokyo, Japan), and those of ampicillin, cefazolin, cefotiam, ceftazidime, cefotaxime, and cefepime were determined using panels prepared in house. *E. coli* ATCC 25922 was used as a reference strain for in vitro susceptibility testing. The sources of the antimicrobial agents were as follows: ampicillin, Meiji Seika Kaisha, Ltd., Tokyo, Japan; cefazolin, Sigma Chemical. Co., St. Louis, Mo., USA; cefotiam, Takeda Chemical Industries, Ltd., Osaka, Japan; ceftazidime, Glaxo Smith Kline, Brentford, Middlesex, UK; cefotaxime, Aventis Pharma Japan Co., Tokyo, Japan; and cefepime, Bristol-Myers Squibb Co., Tokyo, Japan.

Detection of antibiotic resistance-related genes by PCR: Chromosomal DNA was prepared from the *S. marcescens* isolates by using a High Pure PCR Template Preparation Kit (Roche Diagnostics GmbH, Nonnenwald, Penzberg, Germany). The *S. marcescens* isolates were examined by PCR for the presence of the following genes: bla_{TEM} (13), bla_{SHV} (22), $bla_{CTX\text{-}M}$ (23), and plasmid-borne *ampC* genes (24). Primers specific for the *S. marcescens* chromosomal *ampC* gene, Sma ampC F (5′-CGT TGC CTC GAA GCA GAC-3′) and Sma ampC R (5′-CGG GCG TTG GAC TTG ATG-3′), were used to amplify a 552-bp fragment; these primers were designed by comparing the chromosomal *ampC* gene sequences from *S. marcescens* (GenBank accession Nos. AJ271368, AF384203, and X52964), *Acinetobacter baumannii*, *Citrobacter freundii*, *E. coli*, *Morganella morganii*, *Pseudomonas aeruginosa*, and *Yersinia enterocolitica* (accession Nos. ABA9979, AF349570, AB108683, AY235804, X54719, and X63149, respectively).

Pulsed-field gel electrophoresis (PFGE): Agarose plugs with embedded chromosomal DNA were prepared for PFGE as described previously (25). PFGE was performed as described by Shi et al. (26) using *Spe*I (New England Biolabs Inc., Beverly, Mass., USA).

Sequencing of the chromosomal *ampC* gene and bla_{TEM} gene from *S. marcescens* isolates: A 1,174-bp fragment containing the chromosomal *ampC* gene was amplified from the *S. marcescens* isolates by PCR using the primers ampCseq5 (5′-TCG GAA TTC TAA GAG GAT CTA TCA TGA CG-3′) and ampCseq3 (5′-TTA AAG CTT GAG CGT CAG TGC TTC TCC-3′), and a 1,192-bp fragment containing the bla_{TEM} gene was amplified by PCR using MAb/F and MAb/R primers (13). The PCR-amplified fragments were purified and sequenced using the same primers, as described previously (27).

Cloning of the *S. marcescens* chromosomal *ampC* gene and construction of *ampC*-harboring *E. coli* transformants: The chromosomal *ampC* gene was amplified from *S. marcescens* strains ES46, ES71, and Sm4 by PCR using the primers ampCExp5ER (5′-TCG GAA TTC TAT GAC GAA AAT GAA CCG GCT G-3′) and ampCExp3HND (5′-TTA AAG CTT CTT CAG CGC TTC TCC AGC GCC TGG-3′). *EcoR*I and *Hind*III recognition sites were added to the 5′ end of ampCExp5ER and ampCExp3HND, respectively. These primers were designed to clone the *ampC* gene in-frame with the *lacZ* gene on the pBC SK+ plasmid to construct transformants able to express the *ampC* fusion protein. Because ampCExp5ER and ampCExp3HND prime at the beginning and the end of the *ampC* coding region, respectively, all regulatory signals from the original *S. marcescens* gene were eliminated. The 1,158-bp fragments containing the *ampC* genes from strains ES46, ES71, and Sm4 were purified using a QIAquick PCR Purification Kit (QIAGEN Sciences, Valencia, Calif., USA), digested with *EcoR*I and *Hind*III, and cloned into pBC SK+ to construct pJYES46, pJYES71, and pJYSm4, respectively. In order to avoid interference with the host AmpC enzyme, the *E. coli* strain AS226-51 (19) was used as a recipient for the hybrid plasmid. Transformants expressing cephalosporin resistance were selected on L agar plates containing 30 μg/ml chloramphenicol, 3 μg/ml cefazolin, and 20 mM IPTG. Cefazolin was added to the plates at this low concentration to avoid any unnecessary mutations that might take place during the process of selecting AmpC-expressing transformants. The transformants were confirmed to be positive for the *S. marcescens* chromosomal *ampC* gene using PCR with a set of Sma ampC F and Sma ampC R primers, and for the 1,158-bp fragment containing the *ampC* gene using PCR with a set of ampCExp5ER and ampCExp3HND primers.

Site-directed mutagenesis: PCR-based site-directed mutagenesis of the *ampC* gene was performed with an LA

PCR in vitro Mutagenesis KIT (TAKARA Bio Inc., Ohtsu, Japan). The sequence of the MUT primer targeting the *Eco*RI site of the pBC SK+ plasmid and the 5′ portion of the cloned *ampC* gene was 5′-GGC TGC AGG AGT TCA ATG ACG-3′. An R1 (DAES to DAKS) primer was used to introduce a point mutation leading to substitution of the third motif of the *ampC* gene on pJYSm4 from DAES to DAKS; the sequence of the primer was 5′-GCC GTA AGA CTT GGC ATC CAG CGG-3′. An R1 (DAKS to DAES) primer was used to introduce a mutation leading to substitution of the third motif of the *ampC* gene on pJYES46 from DAKS to DAES; the sequence of the primer was 5′-GCC GTA AGA CTC GGC GTC CAG CGG-3′. The substitution was confirmed by sequencing. The pJYSm4 plasmid containing the DAKS third motif and the pJYES46 plasmid containing the DAES third motif constructed by the site-directed mutagenesis were designated pJYSm4(DAKS) and pJYES46(DAES), respectively.

AmpC β-lactamase induction and enzyme assay: AmpC β-lactamase induction and preparation of a crude enzyme solution from whole cells were performed as described by Okamoto et al. (28) with slight modifications as follows: Overnight cultures of *S. marcescens* strains ES46 or ES71 were diluted 1:20 into 10 ml of fresh BHI broth. After incubation for 3 h on a shaker at 35°C, cefoxitin (6 μg/ml) was added as an inducer, and incubation was continued for 2 h. Cells were harvested, washed once, and suspended in 4 ml of 50 mM phosphate buffer (pH 7.0). The cells were sonicated with an ultrasonic disrupter (Branson Sonifier model W-200P; Branson Ultrasonic Corp., Applied Technologies Group, Danbury, Conn., USA.), and the cellular debris was removed by centrifugation at 13,000 rpm for 10 min at 4°C. AmpC β-lactamase activity was determined by spectrophotometry (model 220A; Hitachi, Ltd., Tokyo, Japan) at 30°C in 50 mM phosphate buffer (pH 7.0) (29). Protein content was determined with a BCA protein assay reagent (Pierce, Rockford, Ill., USA.). One unit of AmpC β-lactamase activity was defined as the amount of AmpC β-lactamase that hydrolyzed 1 μmol of cephalothin in 1 min at 30°C (29).

DNA sequence analysis: DNA sequence data were analyzed with DNASIS software (Hitachi Software Engineering Co., Tokyo, Japan). Similarity searches against sequence databases were performed using an updated version of the Basic Local Alignment Search Tool (BLAST) program at the National Center for Biotechnology Information server (http://www.ncbi.nlm.nih.gov/).

Nucleotide sequence accession numbers: The partial nucleotide sequences of the chromosomal *ampC* genes of the *S. marcescens* strains ES46, ES71, and Sm4 were deposited in the GenBank database under the accession numbers AY538703, AY538704, and AY954692, respectively.

RESULTS

Antibiotic susceptibility of the *S. marcescens* isolates: As shown in Table 2, the cerebral surgical ward strains ES11, ES31, ES42, and ES46 showed very similar susceptibility patterns. They were resistant to ampicillin, cefazolin, cefotiam, and ceftazidime; had reduced susceptibility to cefotaxime; and were susceptible to cefepime, aztreonam, imipenem, meropenem, amikacin, minocycline, and levofloxacin. The urological ward strain ES71, in contrast, was resistant to ampicillin, cefazolin, and cefotiam, but susceptible to all other antibiotics examined. The environmental strain Sm4 was resistant to cefazolin only.

Antibiotic resistance-related genes and PFGE patterns of the *S. marcescens* isolates: The strains ES11, ES31, ES42, ES46, and ES71 were positive for the *ampC* gene and $bla_{\text{TEM-1}}$ gene, indicating that these isolates are not TEM-type ESBL producers. The environmental strain Sm4 was positive for the *ampC* gene only. None of these isolates were positive for bla_{SHV}, $bla_{\text{CTX-M}}$, or plasmid-borne *ampC* genes (data not shown). As shown in Figure 1, the cerebral surgical ward isolates ES11, ES31, ES42, and ES46 showed identical *Spe*I PFGE patterns, indicating that they are identical clones. The strain ES71, isolated from an inpatient on the urological ward, showed a distinctive *Spe*I PFGE pattern, indicating that this strain is a distinctive clone from the cerebral surgical ward isolates.

Analyses of *ampC* genes and deduced amino acid sequences from *S. marcescens* strains ES46, ES71, and Sm4: The deduced amino acid sequences of the *ampC* genes from strains ES46, a representative strain of the cerebral surgical ward isolates belonging to the identical clone, ES71, and Sm4 were compared with those of *S. marcescens* strains SLS73 (accession No. AJ271368), SR50 (accession No. X52964),

Table 2. MICs for *S. marcescens* isolates and *E. coli* transformants

Strain	MIC (μg/ml)												
	AMP	CFZ	CTM	CAZ	CTX	FEP	ATM	IPM	MEM	GEN	AMK	MIN	LVX
S. marcescens													
ES11	>1,280	>1,280	1,280	80	40	5	16	≦0.5	≦0.5	2	16	2	2
ES31	>1,280	>1,280	1,280	80	40	5	16	1	≦0.5	2	16	2	2
ES42	>1,280	>1,280	1,280	80	40	5	16	1	1	2	16	2	2
ES46	>1,280	>1,280	1,280	80	40	5	32	≦0.5	≦0.5	2	16	2	2
ES71	>1,280	>1,280	160	10	2.5	0.31	4	1	≦0.5	1	8	2	2
Sm4	20	1,280	5	0.16	0.16	0.08	≦0.5	≦0.5	≦0.5	≦0.5	≦0.5	2	≦0.5
E. coli Transformants													
ampC ES46	160	320	20	40	20	0.31	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
ampC ES71	80	320	10	20	5	0.63	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
ampC Sm4	10	80	5	0.16	0.31	0.04	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
ampC ES46(DAES)	80	320	40	0.31	2.5	0.16	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
ampC Sm4(DAKS)	10	160	5	5	1.25	0.16	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
AS226-51/pBCSK+	0.31	0.16	0.04	0.08	0.04	0.04	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.

AMP, ampicillin; CFZ, cefazolin; CTM, cefotiam; CAZ, ceftazidime; CTX, cefotaxime; FEP, cefepime; ATM, aztreonam; IPM, imipenem; MEM, meropenem; GEN, gentamicin; AMK, amikacin; MIN, minocycline; LVX, levofloxacin; N.D., not determined.

Serr1 (accession No. AY125470), 520R (accession No. AF327325), and SRT-1 (accession No. AB008454). As shown in Figure 2, the comparison revealed that the deduced amino acid sequences of AmpC from ES46 and ES71 are identical, even though these strains showed distinctive susceptibility patterns. The comparison also identified a Glu-235-Lys amino acid substitution in the third amino acid of the third motif of the deduced AmpC amino acid sequences for both ES46 and ES71. This is the same substitution found in the SRT-1 AmpC amino acid sequence (17).

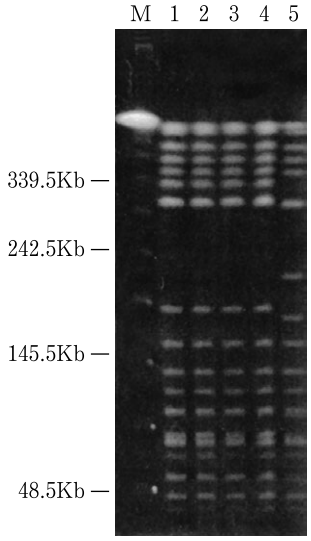

Fig. 1. PFGE patterns of *Spe*I-digested chromosomal DNA fragments from *S. marcescens* clinical isolates. Lanes: M, lambda molecular weight ladder; 1, ES11; 2, ES31; 3, ES42; 4, ES46; 5, ES71.

Susceptibility of *E. coli* transformants: As shown in Table 2, *E. coli ampC*ES46 and *E. coli ampC*ES71 showed similar susceptibility patterns to the antibiotics examined, and they showed markedly higher ceftazidime and cefotaxime MICs than did *E. coli ampC*Sm4. It is noteworthy that *E. coli ampC*ES46, which produces wild-type AmpC harboring the DAKS third motif, had ceftazidime and cefotaxime MICs of 40 μg/ml and 20 μg/ml, respectively, while *E. coli ampC*ES46(DAES), which produces AmpC harboring the substituted DAES third motif, showed markedly decreased ceftazidime and cefotaxime MICs of 0.31 μg/ml and 2.5 μg/ml, respectively. Consistent with this, substitution of DAES with DAKS in the third motif of AmpC of *E. coli ampC*Sm4 markedly increased the ceftazidime MIC from 0.16 μg/ml to 5 μg/ml, and the cefotaxime MIC from 0.31 μg/ml to 1.25 μg/ml.

AmpC β-lactamase activities: Without induction, strain ES46 produced 4.7 U of AmpC β-lactamase/mg total cell protein (U/mg protein) and strain ES71 produced 2.2 U/mg protein (Table 3). Treatment of the strains with 6 μg/ml cefoxitin induced a marked increase in the amount of AmpC

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SLS73     1 MTKMNRLAAA LIAALILPTA HAAQQQDIDA VIQPLMKKYG VPGMAIAVSV DGKQQIYPYG VASKQTGKPI TEQTLFEVGS LSKTFTATLA VYAQQQGKLS 100
SR50        ------C--- ---------- ---------- ---------- ---------- ---------- ---------- ---------S LSK------- ------S---
Serr1       ---------- ---------- Q--------- ---------- ---------- -----Y--L- ---------- ---------S LSK------- ----------
Sm 4           ------- ---------- ---------- ---------- ---------- ---------- ---------- ---------S LSK------- ----------
520R        ---------- ---------- ---------- ---------- ---------- ---------- ---------- --E------S LSK--I---- ----------
SRT-1       ---------- ---------- Q--------- ---------- ---------- ---------- ---------- ---------S LSK------- ----------
ES46           ------- ---------- Q--------- ---------- ---------- ---------- ---------- --E------S LSK------- ----------
ES71           ------- ---------- Q--------- ---------- ---------- ---------- ---------- --E------S LSK------- ----------
                                                                                                    First motif
SLS73   101 FKDPASRYLP ELRGSAFDGV SLLNLATHTS GLPLFVPDDV TDNAQLMAYY RAWQPKHPAG SYRVYSNLGI GMLGMIAAKS LDQPFIQAME QGMLPALGMS 200
SR50        ------H--- DV-------- ---------- ---------- -N-------- ---------- ----YSN--- ---------- ---------- ----------
Serr1       -N-------- ---------- TV-------- ---------- -ND------- ---------- ----YSN--- ---------- ---------- ----------
Sm 4        ---------- ---------- ---------- ---------- ---------- ---------- ----YSN--- ---------- ---------- ----------
520R        -N-------- ---------- ---------- ---------- -N-------- ---------- ----YSN--- ---------- ---------- ----------
SRT-1       -N-------- ---------- ---------- ---------- -ND------- ---------- ----YSN--- V--------- ---------- ----------
ES46        -N-------- ---------- ---------- ---------- -ND------- ---------- ----YSN--- ---------- ---------- ----------
ES71        -N-------- ---------- ---------- ---------- -ND------- ---------- ----YSN--- ---------- ---------- ----------
                                                                                Second motif
SLS73   201 HTYVQVPAAQ MANYAQGYSK DDKPVRVNPG PLDAESYGIK SNARDLIRYL DANLQQVKVA QPWREALAAT HVGYYKAGAF TQDLMWENYP YPVKLSRLVE 300
SR50        ---------- ---------- ---------- --DAES---- ---------- ---------- SVA-RWPRR- S-ITS----- ---------- --------I-
Serr1       ---------- ---------- ---------- --DAES---- ---------- ---------- ----D----- ---------- ---------- --------I-
Sm 4        ---------- ---------- ---------- --DAES---- ---------- ---------- ---------- ---------- ---------- ----------
520R        ---------- ---------- ---------- --DAES---- ---------- ---------- ----D----- ---------- ---------- --------I-
SRT-1       ---------- ---------- ---------- --DAKS---- ---------- ---------- HA--D----- ---------- ---------- --------I-
ES46        ---------- ---------- ---------- --DAKS---- ---------- ---------- ----D----- ---------- ---------- --------I-
ES71        ---------- ---------- ---------- --DAKS---- ---------- ---------- ----D----- ---------- ---------- --------I-
                                              Third motif
SLS73   301 GNNAGMIMNG TPATAITPPQ PELRAGWYNK TGSTGGFSTY AVFIPAKNIA VVMLANKWFP NDDRVEAAYH IIQALEKH* 378
SR50        ---------- ---------- ---------K TG-------- ---------- -E-------- ---------- -------R
Serr1       ---------- ----T----- ---------K TG-------- ---------- ---------- ---------- -V-----R
Sm 4        ---------- ---------- ---------K TG-------- ---------- ---------- -------
520R        ---------- ---------- ---------K TG-------- ---------- ---------- ---------- -V-----R
SRT-1       ---------- ---------- ---------K TG-------- ---------- ---------- ---------- -V-----R
ES46        ---------- ---------- ---------K TG-------- ---------- ---------- ---------- -V--
ES71        ---------- ---------- ---------K TG-------- ---------- ---------- ---------- -V--
                                             Fourth motif
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Fig. 2. Alignment of deduced amino acid sequences of the *S. marcescens* AmpC enzymes SLS73, SR50, Serr1, 520R, SRT-1, and those from ES46, ES71, and Sm4, as described in the text. Dashes represent residues identical to those of SLS73. The first through fourth motifs are underlined. The amino acid sequence from positions 1 to 22 is assumed to be the signal peptide.

Table 3. AmpC β-lactamase activity of *S. marcescens* strains

Strain	Relative AmpC β-lactamase activity (U/mg protein)[1)]	
	Noninduced	Induced
ES-46	4.7	28.1
ES-71	2.2	154.2

[1)]: mean of duplicated experiments.

produced to 28.1 U/mg protein in strain ES46 and 154.2 U/mg protein in strain ES71. The basal AmpC activity was more than two times higher in strain ES46 than in strain ES71, while the induced AmpC activity was more than five times higher in strain ES71 than in ES46.

DISCUSSION

S. marcescens can cause outbreaks of infections, including urinary tract infections, wound infections, pneumonia, and bacteremia (2,3,30,31). PFGE has been shown to be a highly discriminatory and reproducible method for the epidemiological investigation of nosocomial outbreaks of *S. marcescens* infection (12,26). Our present results show that four *S. marcescens* strains, ES11, ES31, ES42, and ES46, which were isolated from urine specimens collected from inpatients on a cerebral surgical ward, showed an identical *Spe*I PFGE pattern, indicating that a single *S. marcescens* clone had caused nosocomial urinary tract infections over a 14-month period in this ward of the hospital. The four *S. marcescens* strains were resistant to ceftazidime, while another *S. marcescens* strain associated with urinary tract infection, ES71, which was isolated from an inpatient on the urological ward, was susceptible to ceftazidime.

Mutation of the structural gene is the mechanism leading to formation of TEM or SHV ESBLs from the prototype enzymes TEM-1 or SHV-1, which confer resistance to expanded-spectrum cephalosporins (32). However, only a few in vitro selected or clinically isolated strains have been reported to harbor chromosomal AmpC enzymes that are resistant to extended-spectrum cephalosporins due to structural gene mutations (16-19,33). Matsumura et al. characterized AmpC SRT-1 from *S. marcescens* GN16694 and identified a Glu-to-Lys substitution in the third amino acid of the third motif, suggesting that expansion of the substrate specificity in SRT-1 toward oxyimino cephalosporins is attributable to this substitution (17). In the present study, a Glu-235-Lys substitution in the third motif of the AmpC enzyme was identified in both *S. marcescens* strains ES46 and ES71, which coincided with the substitution identified in the SRT-1 AmpC. This substitution was not found in the deduced AmpC amino acid sequence from the environmental *S. marcescens* strain Sm4, which was susceptible to all of the expanded-spectrum cephalosporins examined in this study, nor in the reported AmpC sequence from susceptible *S. marcescens* SR50. The results of our site-directed mutagenesis experiments using *E. coli* transformants harboring cloned *ampC* gene from ceftazidime-resistant strain ES46 or susceptible strain Sm4 confirmed that the Glu-235-Lys substitution in the third amino acid of the third motif of the AmpC enzyme is attributable to ceftazidime resistance phenotype. Constitutive overproduction of chromosomally encoded AmpC confers resistance to expanded-spectrum cephalosporins in many *Enterobacteria*, including *S. marcescens* (10,11). Our results, however, demonstrated that the AmpC activity of ceftazidime-resistant strain ES46 could be induced from 4.7 U/mg protein to 28.1 U/mg protein by cefoxitin treatment, indicating that ES46 is not a constitutive overproducer of AmpC and that constitutive overproduction of AmpC is thus not involved in the ceftazidime resistance mechanism of strain ES46.

The susceptibility patterns of *S. marcescens* strains ES71 and ES46 were quite different, even though the sequences of the *ampC* genes from these two strains were identical. In fact, *E. coli* transformants harboring cloned *ampC* genes from either strain showed comparable resistance patterns, suggesting that mechanism(s) other than structural gene mutation of *ampC* is also involved in determining the resistance phenotype of these two strains. Actually, basal AmpC activity was more than twice as high in strain ES46 as in strain ES71, which could result in the differing resistance phenotypes of these two strains. Mahlen et al. (33) recently proposed that regulation of the *S. marcescens* AmpC expression occurs at multiple levels, e.g., RNA initiation with induction through AmpR, and events downstream of transcriptional initiation including regulation of RNA half life, which might be involved in the mechanism(s) that determines the differing basal and induced AmpC activity of strains ES46 and ES71. Although the data are not shown, we have sequenced the *ampC*/*ampR* intergenic region and confirmed that sequences of the regulatory genes including putative-35 and -10 boxes, stem-loop, and RBS (33) of these two strains are identical. We also determined the AmpR deduced amino acid sequence of these two strains and identified one amino acid substitution (data not shown). The significance of the substitution in the mechanism that determines the resistance phenotypes of the two strains remains unclear, however. The difference in outer membrane permeability to cephalosporins is another possible mechanism that could contribute to the differing resistance phenotypes of these two strains. These possibilities should be examined further.

The process that results in the emergence of these ceftazidime-resistant *S. marcescens* strains in the hospital is unclear, but frequent use of extended-spectrum cephalosporins could result in the emergence of resistant strains with different resistance mechanisms including strains producing a chromosomal AmpC variant with gene mutation(s). In fact, based on in vitro antibiotic selection experiments, Raimondi et al. (34) and Morosini et al. (18) proposed that increased use of extended-spectrum cephalosporins may eventually select mutated AmpC in clinical settings, leading to the emergence of extended-spectrum cephalosporin-resistant strains. The clinical significance and prevalence of extended-spectrum cephalosporin-resistant *S. marcescens* strains harboring the mutated chromosomal *ampC* gene remain unclear in Japan and should be elucidated.

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