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Characterization of SFO-1, a Plasmid-Mediated Inducible Class A β-Lactamase from *Enterobacter cloacae*

YOSHIMI MATSUMOTO[1,2]* AND MATSUHISA INOUE[1]

Department of Microbiology, Kitasato University School of Medicine, Sagamihara,[1] and Medicinal Biology Research Laboratories, Fujisawa Pharmaceutical Co., Ltd., Osaka,[2] Japan

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***Enterobacter cloacae* 8009 produced an inducible class A β-lactamase which hydrolyzed cefotaxime efficiently. It also hydrolyzed other β-lactams except cephamycins and carbapenems. The activity was inhibited by clavulanic acid and imipenem. The *bla* gene was transferable to *Escherichia coli* by electroporation of plasmid DNA. The molecular mass of the β-lactamase was 29 kDa and its pI was 7.3. All of these phenotypic characteristics of the enzyme except for inducible production resemble those of some extended-spectrum class A β-lactamases like FEC-1. The gene encoding this β-lactamase was cloned and sequenced. The deduced amino acid sequence of the β-lactamase was homologous to the AmpA sequences of the *Serratia fonticola* chromosomal enzyme (96%), MEN-1 (78%), *Klebsiella oxytoca* chromosomal enzymes (77%), TOHO-1 (75%), and FEC-1 (72%). The conserved sequences of class A β-lactamases, including the S-X(T)-X(S)-K motif, in the active site were all conserved in this enzyme. On the basis of the high degree of homology to the β-lactamase of *S. fonticola*, the enzyme was named SFO-1. The *ampR* gene was located upstream of the *ampA* gene, and the AmpR sequence of SFO-1 had homology with the AmpR sequences of the chromosomal β-lactamases from *Citrobacter diversus* (80%), *Proteus vulgaris* (68%), and *Pseudomonas aeruginosa* (60%). SFO-1 was also inducible in *E. coli*. However, a transformant harboring plasmid without intact *ampR* produced a small amount of β-lactamase constitutively, suggesting that AmpR works as an activator of *ampA* of SFO-1. This is the first report from Japan describing an inducible plasmid-mediated class A β-lactamase in gram-negative bacteria.**

Since the introduction of extended-spectrum cephalosporins into clinical use, gram-negative bacteria have reacted to them mainly by producing various new types of plasmid-mediated extended-spectrum β-lactamases, in addition to overproducing chromosomal β-lactamases. Bush's classification of β-lactamases was updated in 1995 (5). A total of 178 kinds of different β-lactamases were included in that classification. Recently, additional extended-spectrum β-lactamases which hydrolyze oxyimino-cephalosporins have been isolated all over the world (5, 7, 12, 13, 14, 21, 27, 28). Most of them are related to the TEM and SHV families of β-lactamases. However, in Japan, extended-spectrum β-lactamases derived from these families have never been isolated. On the other hand, different types of extended-spectrum β-lactamases classified as 2be and 2e (class A) (10, 17, 20, 31), 1 (class C) (8), and 4 (class B) (11, 30) have been isolated. The differences in enzyme populations are

TABLE 1. Strains used in this study

Host	Plasmid	Phenotype	Restriction enzyme insert (length [kb])	Vector	Marker[a]	Nickname
E. cloacae 8009	pFCX300L	AmpA AmpR		Cryptic	CTX	
E. cloacae 8009	pFCX300M	AmpA AmpR		Cryptic	CTX	
E. cloacae 8009	pFCX300S			Cryptic		
E. coli DH10B						
E. coli DH10B	pFCX310	AmpA	*Sau*3AI (2.3)	pHSG396	CP, CTX	TF2-2
E. coli DH10B	pFCX320	AmpA	*Sau*3AI (2.3)	pHSG396	CP, CTX	TF2-3
E. coli DH10B	pFCX300L	AmpA AmpR			CTX	TF-L
E. coli DH10B	pFCX300M	AmpA AmpR			CTX	TF-M
E. coli DH10B	pFCX301	AmpA AmpR	*Eco*RI (10)	pHSG396	CP, CTX	TF-L7
E. coli DH10B	pFCX302	AmpA AmpR	*Eco*RI (10)	pHSG396	CP, CTX	TF-M4
E. cloacae 199S		AmpC$^-$				
E. cloacae 199S	pFCX310	AmpA	*Sau*3AI (2.3)	pHSG396	CP, CTX	
E. cloacae 199S	pFCX320	AmpA	*Sau*3AI (2.3)	pHSG396	CP, CTX	
E. cloacae 199		AmpC (inducible)				
E. cloacae 199C		AmpC (constitutive)				

[a] CTX, cefotaxime; CP, chloramphenicol; Rif, rifampin.

* Corresponding author. Mailing address: Medicinal Biology Research Laboratories, Fujisawa Pharmaceutical Co., Ltd., 1-6, 2-Chome, Kashima, Yodogawa-ku, Osaka, 532 Japan. Phone: (06) 390-1146. Fax: (06) 304-1192. E-mail: yoshimi_matsumoto@po.fujisawa.co.jp.

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thought to arise from the various clinical uses of antibacterial agents. We reported on plasmid-mediated oxyimino-cephalosporin-hydrolyzing class A β-lactamase FEC-1 in 1988 (20). Although it was not from a clinical isolate, the possibility of the appearance of such an enzyme was suggested. Since then, a similar type of enzyme has been identified in Japan (31).

Enterobacter cloacae is known to produce inducible chromosomal β-lactamases. Overproduction or constitutive production of this enzyme causes resistance to most β-lactams except carbapenems (16, 29) without the loss of any porin. On the other hand, an amino acid insertion into the omega loop region of the class C β-lactamase causes substrate specificity extension to extended-spectrum β-lactams (24). We found 1 ceftizoxime-susceptible strain among 12 cefotaxime-resistant *E. cloacae* strains isolated at Teikyo University in 1988. The strain produced an inducible enzyme which hydrolyzed cefotaxime efficiently. The phenotypic characteristics of this enzyme resembled those of FEC-1 except for the inducible production. Since induction of plasmid-mediated β-lactamase is known to be rare in gram-negative bacteria, the origin of this enzyme is of great interest. In this study the gene was cloned and was further analyzed.

MATERIALS AND METHODS

Bacterial strains. *E. cloacae* 8009 was isolated clinically in 1988 and was kindly provided by K. Ubukata of Teikyo University (Tokyo, Japan). Competent *Escherichia coli* DH10B was obtained commercially from GIBCO-BRL. The constructed strains and plasmids are described in Table 1.

Plasmids. pHSG396 was from Takara Shuzo (Otsu, Japan).

Antibiotics. The antibiotics used in this study were commercially available. Imipenem-cilastatin (Banyu, Tokyo, Japan), ceftazidime (Glaxo Japan, Tokyo, Japan), cefoperazone (Toyama, Tokyo, Japan), moxalactam (Shionogi, Osaka, Japan), aztreonam (Eisai, Tokyo, Japan), cefpiramide (Sumitomo, Osaka, Japan), ceftizoxime (Fujisawa, Osaka, Japan), cefotaxime (Chugai, Tokyo, Japan), cefmenoxime (Takeda, Osaka, Japan), ceftriaxone (Roche, Tokyo, Japan), cefuroxime (Glaxo), cefotiam (Takeda), cefoxitin (Banyu), cefamandole (Shionogi), cefazolin (Fujisawa), cephalothin (Shionogi), cephaloridine (Shionogi), and ampicillin (Fujisawa) were used. Cefoselis (FK037) (22), clavulanic acid, and nitrocefin were synthesized in our laboratories.

Susceptibility testing. MICs were determined with serial dilutions of antibiotics in Mueller-Hinton medium with inoculum sizes of 10^4 and 10^6 CFU per spot. The MICs were read as the lowest concentration of antibiotic that inhibited visible growth after 18 h of incubation at 37°C.

Preparation of β-lactamase. Exponentially growing cells of the test strains in Trypticase soy broth (BBL, Becton Dickinson Microbiology Systems, Cockeysville, Md.) were harvested, washed once, and resuspended in a 1/20 volume of 50 mM potassium phosphate buffer (pH 7.0). The suspension was sonically disrupted and the debris was removed by centrifugation (15,000 × *g*, 30 min). β-Lactamase was partially purified by ion-exchange chromatography with POROS 20HS and a BioCAD work station (PerSeptive Biosystems, Tokyo, Japan).

Assay of β-lactamase activity. β-Lactamase activity was determined spectrophotometrically (UV-2200; Shimadzu) at 37°C in 0.067 M potassium phosphate buffer (pH 7.0).

β-Lactamase inhibitor susceptibility. Susceptibility to β-lactamase inhibitors was determined as mentioned previously (20).

Analytical isoelectric focusing. Isoelectric focusing was performed with crude extracts with an LKB Ampholine PAG plate (pH 3.5 to 9.5) and an analytical electrofocusing system (LKB Stockholm, Bromma, Sweden). β-Lactamase activity was detected by overlaying the gel with filter paper containing nitrocefin (0.5 mg/ml).

Inducibility of β-lactamase. Imipenem (0.1 μg/ml) was added to a mid-logarithmic-phase culture, and the culture was incubated for 2 more h. The cells were harvested by centrifugation (10,000 × *g*, 10 min), resuspended in a 1/10 volume of 0.067 M potassium phosphate buffer (pH 7.0), and disrupted by sonication. The β-lactamase activity and protein concentration were measured, and specific activities were compared.

Transformation study. Plasmid DNA was isolated from *E. cloacae* 8009 and was used to transform *E. coli* DH10B by electroporation. Ampicillin-resistant colonies with different plasmids were selected and named TF-L (*E. coli* DH10B/pFCX300L) and TF-M (*E. coli* DH10B/pFCX300M). *E. cloacae* 199S (a chromosomal β-lactamase-deficient strain) was also used as a recipient.

Cloning of β-lactamase gene. Plasmid DNA from *E. cloacae* 8009 was partially digested with *Sau*3AI and was ligated into the *Bam*HI site of pHSG396 (see Fig. 1). The ligation mixture was used to transform *E. coli* DH10B. Transformants harboring plasmids which have short fragment insertions were selected (TF2-2, *E. coli* DH10B/pFCX310; and TF2-3, *E. coli* DH10B/pFCX320).

TABLE 2. Susceptibilities of *E. cloacae* 8009 and various transformants to β-lactams

Strain	Presence of *ampR*	MIC (μg/ml)									
		Cefuroxime	Cefotaxime	Ceftizoxime	Cefoperazone	Ceftazidime	Cefoselis	Cefoxitin	Moxalactam	Aztreonam	Imipenem
E. cloacae 8009	+	>100	100	6.25	>100	3.13	12.5	>100	0.78	12.5	0.78
E. coli DH10B	−	6.25	0.05	≤0.025	0.1	0.2	≤0.025	3.13	0.1	0.1	0.2
E. coli DH10B/pFCX310	−	>100	50	1.56	50	3.13	3.13	3.13	0.2	25	0.39
E. coli DH10B/pFCX320	−	>100	50	1.56	100	3.13	6.25	3.13	0.2	25	0.2
E. coli DH10B/pFCX300L	+	50	0.78	0.05	0.78	0.2	0.1	3.13	0.1	0.78	0.2
E. coli DH10B/pFCX300M	+	>100	6.25	0.2	6.25	1.56	0.78	6.25	0.2	6.25	0.39
E. coli DH10B/pFCX301	+	>100	25	0.78	25	3.13	3.13	6.25	0.39	25	0.2
E. coli DH10B/pFCX302	+	>100	25	1.56	50	3.13	3.13	6.25	0.39	25	0.2
E. cloacae 199S[a]		3.13	0.05	≤0.025	0.2	0.1	≤0.025	0.78	0.05	≤0.025	0.05
E. cloacae 199S/pFCX310		>100	25	0.39	50	1.56	1.56	0.78	0.05	6.25	0.05
E. cloacae 199S/pFCX320		>100	50	1.56	>100	3.13	6.25	1.56	0.1	12.5	0.05
E. cloacae 199[b]		6.25	0.2	0.1	0.39	0.2	0.05	>100	0.1	0.1	0.39
E. cloacae 199C[c]		>100	50	50	25	50	1.56	>100	3.13	25	0.2
E. coli DH5α	−	3.13	≤0.025	≤0.025	≤0.025	0.05	≤0.025	3.13	0.1	≤0.025	0.2
E. coli DH5α/FEC-1	−	>100	>100	6.25	>100	12.5	100	3.13	0.39	50	0.2
E. coli KU3290[d]	+	>100	25	50	50	100	1.56	>100	12.5	25	0.2

[a] β-Lactamase nonproducer.
[b] Inducible β-lactamase producer (original strain).
[c] Constitutive β-lactamase producer.
[d] Transformant producing cloned *P. vulgaris* β-lactamase.

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TABLE 3. Susceptibilities of *E. cloacae* 8009 and various transformants to other antibiotics

Strain	MIC (μg/ml)						
	Gentamicin	Amikacin	Kanamycin	Streptomycin	Chloramphenicol	Ofloxacin	ST[a]
E. cloacae 8009	0.39	1.56	1.56	3.13	3.13	0.05	0.39
E. coli DH10B	0.2	0.78	1.56	>100	3.13	<0.025	0.39
E. coli DH10B/pFCX310	0.1	0.2	0.78	>100	>100	<0.025	0.39
E. coli DH10B/pFCX320	0.2	0.78	1.56	>100	>100	<0.025	0.39
E. coli DH10B/pFCX300L	0.2	1.56	1.56	>100	3.13	<0.025	0.39
E. coli DH10B/pFCX300M	0.2	0.78	1.56	>100	3.13	<0.025	0.39
E. coli DH10B/pFCX301	0.39	0.78	1.56	>100	>100	<0.025	0.39
E. coli DH10B/pFCX302	0.2	0.78	1.56	>100	>100	<0.025	0.39
E. cloacae 199S[b]	0.2	0.78	0.78	1.56	12.5	0.05	1.56
E. cloacae 199S/pFCX310	0.2	0.39	0.78	1.56	>100	0.05	1.56
E. cloacae 199S/pFCX320	0.2	0.39	0.78	1.56	>100	0.05	1.56

[a] ST, sulfamethoxazole-trimethoprim.
[b] β-Lactamase nonproducer.

Alternatively, the DNAs of two plasmids, pFCX300L and pFCX300M, were each digested with *Eco*RI and were cloned into pHSG396 to get TF-L7 (*E. coli* DH10B/pFCX301) and TF-M4 (*E. coli* DH10B/pFCX302).

DNA sequence analysis. DNA sequence analysis was performed by the PCR cycle sequencing method with the Sequthermo kit (Epicenter Technology) and the thermosequenase kit (Amersham) and with the LI-COR 4000LS system. For determination of the sequence of *ampA*, pFCX310 and pFCX320 were used as templates. The AmpR gene and franking region were sequenced by using pFCX301 and pFCX302 and their deletion plasmids as templates and primers synthesized from the sequences of pFCX310 and pFCX320. The ABI PRISM 310 Genetic Analyzer (Applied Biosystems) was also used. Homology was analyzed by the FASTA program of the DNA Data Base of Japan (DDBJ).

Nucleotide sequence accession number. The nucleotide sequence data reported in this paper will appear in the DDBJ, EMBL, and GenBank nucleotide sequence databases under accession no. AB003148.

RESULTS

Characteristics of β-lactamase from *E. cloacae* 8009. *E. cloacae* 8009 was resistant to various β-lactam antibiotics including extended-spectrum cephalosporins such as cefotaxime, cefoperazone, cefoselis, and monobactam (aztreonam) but excluding carbapenems (Table 2). However, strain 8009 was not as resistant to such oxyimino-cephalosporins (ceftizoxime and ceftazidime) as the high-level producers of chromosomal β-lactamase (Table 2). Also, *E. cloacae* 8009 was not resistant to the other antibiotics tested (Table 3). The β-lactamase was partially purified from *E. cloacae* 8009 by ion-exchange chromatography. The molecular mass of this enzyme was 29 kDa by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and its pI was 7.3. Values of kinetic parameters were determined (Table 4). The enzyme hydrolyzed oxyimino-cephalosporins, in addition to cephaloridine and ampicillin. Ceftazidime was the most stable among the cephalosporins tested. The hydrolysis of cephamycin (cefoxitin), oxacephem (moxalactam), and carbapenem (imipenem) by this enzyme was not detectable (data not shown). The enzyme activity was inhibited by clavulanic acid and imipenem (Table 5) but was not inhibited by EDTA and *P*-chloromercuribenzoate (data not shown). These characteristics resembled those of FEC-1 (20) and some other class A enzymes (5) reported previously.

TABLE 4. Kinetic parameters of SFO-1 and FEC-1 for β-lactam antibiotics

Substrate	SFO-1			FEC-1		
	K_m (μM)	Relative V_{max}[a]	Relative V_{max}/K_m[a]	K_m (μM)	Relative V_{max}[a]	Relative V_{max}/K_m[a]
Cephaloridine	110	100	100	152	100	100
Cephalothin	60	234	428	134	198	225
Cefamandole	171	241	154	122	125	156
Cefotiam	27	50	203	38	43	170
Cefoperazone	6.6	15	246	2.8	2.6	139
Cefpiramide	26	71	297	33	36	167
Cefuroxime	13	23	195	27	32	179
Ceftizoxime	217	12	5.8	821	12	2.3
Cefotaxime	2.2	16	790	61	23	59
Cefmenoxime	21	20	108	84	61	110
Ceftriaxone	19	15	84	27	14	80
Ceftazidime	785	0.47	0.065	393	0.13	0.05
Ampicillin	40	19	51	30	17	89

[a] Values relative to that for hydrolysis of cephaloridine, which was set equal to 100.

Effect of inducer on the β-lactamase production in *E. cloacae* 8009. Imipenem induced cefotaxime-hydrolyzing β-lactamase production in *E. cloacae* 8009, and the specific cefotaxime-hydrolyzing activity was about 10 times higher when it was induced (Table 6).

Transformation of *E. coli*. *E. cloacae* 8009 had plasmids of three different sizes (Fig. 1). *E. coli* DH10B was transformed by electroporation with total plasmid DNA isolated from *E. cloacae* 8009 and was selected on plates containing cefotaxime at 1 μg/ml. The largest plasmid, pFCX300L, was detected in every transformant (TF-L) except one (TF-M), in which middle-size plasmid pFCX300M was detected (Fig. 1). Transconjugation from *E. cloacae* 8009 to *E. coli* was unsuccessful, suggesting that the gene is on a nonconjugable R plasmid.

The antibiotic susceptibility patterns of these transformants

TABLE 5. Susceptibility of SFO-1 to β-lactamase inhibitors

Enzyme	Bush type	IC_{50} (μg/ml)[a]	
		Clavulanic acid	Imipenem
SFO-1	2e	0.1	0.35
FEC-1	2e	0.0022	0.13
Chromosomal β-lactamase of *P. vulgaris*	2e	0.6	0.62
TEM-1	2b	1.0	14
Chromosomal β-lactamase of *E. cloacae*	1	12	0.24
Chromosomal β-lactamase of *E. coli*	1	7.8	1.4

[a] IC_{50}, concentration that inhibits 50% of activity after 10 min of incubation at 37°C; nitrocefin was used as the substrate.

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TABLE 6. β-Lactamase inducibility in *E. cloacae* 8009 and various transformants

Expt. no. and strain	Phenotype	Inducer[a]	β-Lactamase activity[b]		Inducibility and inducibility ratio[c]
			μM/min · mg of protein	Ratio[d]	
Expt 1					
E. cloacae 8009	AmpA AmpR	−	340	1.0	Inducible
		+	2,400	7.1	7.1
E. coli DH10B		−	<1	<0.003	Constitutive
		+	<1	<0.003	1.0[e]
E. coli DH10B/pFCX300L (TF-L)	AmpA AmpR	−	22	0.064	Inducible
		+	210	0.64	9.6
E. coli DH10B/pFCX300M (TF-M)	AmpA AmpR	−	119	0.35	Inducible
		+	1,720	5.1	14.5
E. coli DH10B/pFCX301 (TF-L7)	AmpA AmpR	−	700	2.1	Inducible
		+	6,500	19	9.2
E. coli DH10B/pFCX302 (TF-M4)	AmpA AmpR	−	620	1.9	Inducible
		+	7,400	22	11.9
Expt 2					
E. cloacae 8009	AmpA AmpR	−	260	1.0	Inducible
		+	2,500	9.6	9.3
E. coli DH10B/pFCX310 (TF2-2)	AmpA	−	280	1.1	Constitutive
		+	280	1.1	1.0
E. coli DH10B/pFCX320 (TF2-3)	AmpA	−	350	1.3	Constitutive
		+	350	1.3	1.0

[a] The inducer was imipenem-cilastatin at 0.1 μg/ml.
[b] Cefotaxime (100 μM) was used as a substrate.
[c] Induced activity/uninduced activity for each set.
[d] Activity of extract/uninduced activity of *E. cloacae* 8009.
[e] Nitrocefin was used as the substrate for calculation of the inducibility ratio for this strain.

were similar to those of FEC-1-producing *E. coli* DH5α except for cefoselis susceptibility, although TF-M was more susceptible than *E. coli* DH5α/FEC-1 (Table 2). The MICs differed for TF-M and TF-L, perhaps due to a difference in copy numbers.

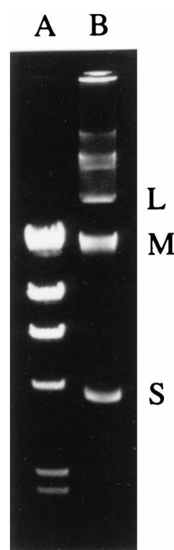

FIG. 1. Agarose gel electrophoresis of plasmid DNAs from *E. cloacae* 8009. Lane A, *Hin*dIII-digested bacteriophage λ DNA; lane B, *E. cloacae* 8009. L, large plasmid; M, medium-size plasmid; S, small plasmid.

Cloning and characterization of the β-lactamase gene. The plasmid-mediated β-lactamase gene of *E. cloacae* 8009 was cloned into vector plasmid pHSG396 and was used to transform *E. coli* DH10B (Fig. 2). Two transformants that have plasmids (pFCX310 and pFCX320) recombined with a partially digested *Sau*3AI fragment and that had an insertion of nearly 2.2 kb were selected. The sequence obtained was analyzed by a search of the GenBank database (GENETYX-MAC/CD) for a homologous sequence, and the search revealed the existence of a β-lactamase gene similar to that for a *Klebsiella oxytoca* chromosomal enzyme (2). The initiation codon and stop codon were designated on the basis of the homologies of their sequences with those for the initiation codon and stop codon of the *K. oxytoca* β-lactamase gene. Following a search for a homologous amino acid sequence, the highest degree of homology was seen with the β-lactamase from *Serratia fonticola*. We named the enzyme SFO-1. The alignments of the deduced amino acid sequence of SFO-1 with those of homologous enzymes are shown in Fig. 3. Sequence identities were 96, 78, 77, and 75% with *S. fonticola* β-lactamase (P80545), MEN-1 (3), *K. oxytoca* chromosomal enzymes (2), and TOHO-1 (11), respectively. The homology between SFO-1 and FEC-1 was 72%. The conserved sequences of class A β-lactamases including the 70S-X(T)-X(S)-K motif in the active site were all conserved in this enzyme (1). It is noteworthy that there was an *ampR*-related sequence that inversely followed the upstream region of *ampA*. However, the *ampR*-like sequence was not intact in pFCX310 or pFCX320. The

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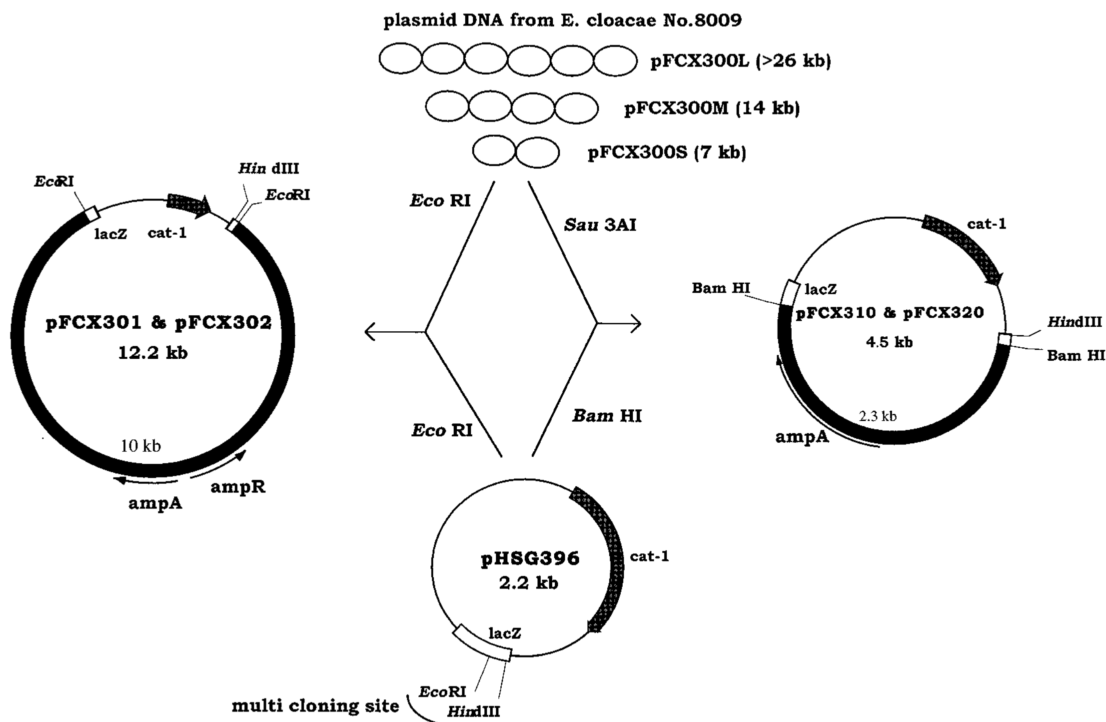

FIG. 2. Construction of recombinant plasmids.

sequence of SFO-1 also had 70% homology with the sequences of the inducible chromosomal β-lactamases of *Citrobacter diversus* (25) and *Proteus vulgaris* (24).

Characterization of *ampR* of SFO-1. To obtain the full length of *ampR* with *ampA* on the same fragment, *Eco*RI-restricted fragments from pFCX300L and pFCX300M were cloned into pHSG396 (Fig. 2). Two kinds of plasmids, pFCX301 from pFCX300M and pFCX302 from pFCX300L, were obtained. They seemed to have the same fragment in different directions from the results of restriction mapping and sequencing of both ends. By using these transformants as a template, the sequence of the flanking region of *ampA* was read as described in Materials and Methods. From the homology with the *ampR* gene of *P. vulgaris* (6), a complete *ampR*

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                         30         40         50         60         70         80
SFO-1          1:QSANAKANIQ QQLSELEKNS GGRLGVALID TADNSQI-LY RGDERFPMCS TSKVMAVSAL   59
S.fonticola    1:QPANAKANIQ QQLSELEKNS GGRLGVALID TADNSQI-LY RGDERFPMCS TSKVMAVSAL   59
MEN-1          1:Q----TADVQ QKLAELERQS GGRLGVALIN TADNSQI-LY RADERFAMCS TSKVMAVAAV   55
TOHO-1         1:Q----ANSVQ QQLEALEKSS GGRLGVALIN TADNSQI-LY RADERFAMCS TSKVMAAAAV   55
                 *        * * *  **  * *********  ******* ** * **** *** ******  *
                         90        100        110        120        130        140
SFO-1         60:LKQSEMDKNL LAKRMEIKQS DLVNYNPIAE KHLDTGMTLA ELSAAAIQYS DNTAMNKILE  119
S.fonticola   60:LKQSETDKNL LAKRMEIKQS DLVNYNPIAE KHLDTGMTLA EFSAATIQYS DNTAMNKILE  119
MEN-1         56:LKKSESEPNL LNQRVEIKKS DLVNYNPIAE KHVDGTMSLA ELSAAALQYS DNVAMNKLIS  115
TOHO-1        56:LKQSESNKHL LNQRVEIKKS DLVNYNPIAE KHVNGTMTLA ELGAAALQYS DNTAMNKLIA  115
                 ** **    * *  * *** * ********** **    * ** *  **  *** ** ****
                        150        160        170        180        190        200
SFO-1        120:HLGGPAKVTE YARTIGDKTF RLDRTEPTLN TAIPSDKRDT TSPLAMAKSL QTLTLGKALG  179
S.fonticola  120:HLGGPAKVTE FARTIGDKTF RLDRTEPTLN TAIPGDKRDT TSPQAMAISL QNLTLGKALA  179
MEN-1        116:HVGGPASVTA FARQLGDETF RLDRTEPTLN TAIPGDPRDT TSPRAMAQTL RNLTLGKALG  175
TOHO-1       116:HLGGPDKVTA FARSLGDETF RLDRTEPTLN TAIPGDPRDT TTPLAMAQTL KNLTLGKALA  175
                 * ***  **   **  ** ** ********** **** * *** * * ***  *   *******
                        210        220        230        240        250        260
SFO-1        180:EPQRAQLVEW MKGNTTGGAS IRAGLPATWV VGDKTGSG-D YGTTNDIAVI WP-ANHAPLV  237
S.fonticola  180:EPQRAQLVEW MKGNTTGGAS IRAGLPTTWV VGDKTGSG-D YGTTNDIAVI WP-ANHAPLV  237
MEN-1        176:DSQRAQLVTW MKGNTTGAAS IQAGLPASWV VGDKTGSG-D YGTTNDIAVI WP-KDRAPLI  233
TOHO-1       176:ETQRAQLVTW LKGNTTGSAS IRAGLPKSWV VGDKTGSG-D YGTTNDIAVI WP-ENHAPLV  233
                   ****** *  ****** ** * ****  ** ******** * ********** **    ***
                        270        280        290
SFO-1        238:LVTYFTQPQQ NAEARKDVLA AAAKIVTEGL                                  267
S.fonticola  238:LVTYFTQPQQ NAEARKDVLA AAAKIVTAGL                                  267
MEN-1        234:LVTYFTQPQP KAESRRDVLA SAAKIVTNGL                                  263
TOHO-1       234:LVTYFTQPEQ KAERRRDILA AAAKIVTHGF                                  263
                 ********    ** * * **  ****** *
```

FIG. 3. Alignment of deduced amino acid sequence of SFO-1 (AmpA) with the sequences of homologous enzymes. The amino acid numbering for the class A β-lactamase is used (1). The S-X(T)-X(S)-K motif with the active-site serine is shaded. Asterisks indicate the conserved amino acid residues among these class A sequences.

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```
SFO-1           1:M-RSNPPLNALRAFEASARHLSFTRAALELYVTQAAVSQQVRLLEERLGMVLFKRLPRGL  59
C. diversus     1:M-RSNLPLNALRAFEASARHLSFTRAALELCVTQAAVSQQVRILEDRLNRVLFKRLPRGL  59
P. vulgaris     1:M-RTHLPLNALRAFEASARHLNFTKAALELYVTQGAVSQQVRMLEERLGVILFKRLPRGL  59
P. aeruginosa   1:MVRPHLPLNALAAFEASARHLSFTRAAIELCVTQAAVSHQVKSLEERLGVALFKRLPRGL  60
                  * *   ***** ********* ** ** ** *** *** **  ** **   *********

SFO-1          60:EMTEESRALFAVLTDAFGHIEKAFRQFEGGQFQEVLTVAAVGTFAVGWLLPRLDRFRQEH 119
C. diversus    60:EMTDEAQALFAVLTDAFGQIDTIFRQFEGGEYREVLTVAAVGTFAVGWLLPRIEQFRQAH 119
P. vulgaris    60:EMTDDAQILFSVLTTAFSDIERVFKQFERGEYRDVVSIAAVGTFAVGWLLPRLAEFRQLY 119
P. aeruginosa  61:MLTHEGESLLPVLCDSFDRIAGLLERFEGGHYRDVLTVGAVGTFTVGWLLPRLEDFQARH 120
                    *     *  **   *  *      ** *    *    ***** *******   *

SFO-1         120:PFVELRLRTNNNVVNLAAEGLDFAIRFGTGLWPSTHNEMLFNAPLTVLCTPATAKRLVTP 179
C. diversus   120:PFVELRLRTNNNVVNLAAEGLDFAIRFGNGLWPATHNEMLFEAPLTVLCTPETAQRLRRP 179
P. vulgaris   120:PRIELNLRTNNNVVNLATEGLDFAIRFGEGLWPLTHNKALFSAPLTVLCSSDTAKPLQHP 179
P. aeruginosa 121:PFIDLRLSTHNNRVDIAAEGLDYAIRFGGGAWHGTEALALFEAPLTVLCCPEVAAQLHSP 180
                  *   * * * ** *  * **** ***** * *  *    ** *******    *  *  *

SFO-1         180:ADLLQEDLLRSYRAEEWENWFAAA--GLQAVRVNGAIFDSSRLMIESAIHSGGVALAPAK 237
C. diversus   180:ADLLQENLLRSYRVDEWDNWFAAA--GVTAERINGAVFDSSRLMVETVIHTGGAALVPAV 237
P. vulgaris   180:TDLINETLYRSYREDEWLQWFEKA--NMSPIKITGSIFDPSRLMIESAIYEGGVALAPAK 237
P. aeruginosa 181:ADLLQHTLLRSYRADEWPLWFQAAGLPAHAPLTRSIVFDTSLAMLEAARQGVGVALGPAA 240
                   **    * ****  **  **  *             ** *  * *      * ** **

SFO-1         238:MFVRELTTAQLVRPFATEINMGSYWLTHLKSKAMTPAMEIFSEWLLKEAAEDNT--     291
C. diversus   238:MFARELAAGQLVRPFDIEIQMG-YWLTHLKSKPMTPAMEIFRDWIVKMA-------     285
P. vulgaris   238:MFSREIENGQLVQPFKIEVELGKYWLTYLKSKPMTASMEIFQQWLMNEALKEISE-     292
P. aeruginosa 241:MFARQLASESIRRPFATEVSTGSYWLTRLQSRGETSAMLAFRGWLLEMAAVEARGR     296
                  ** *         **  *   * **** * *   *  *  *  *    *
```

FIG. 4. Alignment of deduced amino acid sequence of AmpR of SFO-1 with homologous AmpR sequences. Asterisks indicate the conserved amino acid residues.

gene was identified upstream from *ampA*. The deduced amino acid sequence of AmpR had 80, 68, 60, and 56% similarities with the AmpR sequences of the chromosomal β-lactamases from *C. diversus* (15), *P. vulgaris* (6), *Pseudomonas aeruginosa* (19), and *S. marcescens* (23), respectively. The alignments of these AmpR sequences are shown in Fig. 4.

Role of *ampR* gene. The inducibility of β-lactamase production in each *E. coli* transformant was determined as described in Materials and Methods (Table 6, experiment 2). In *E. coli* strains harboring pFCX310 and pFCX320 with an incomplete *ampR* gene, β-lactamase was not induced by imipenem, and the amount of β-lactamase produced in them stayed low. On the other hand, in *E. coli* strains harboring pFCX301 and pFCX302 with a complete *ampR* gene on the same vector, β-lactamase production was induced by the addition of imipenem. *E. coli* DH10B harboring native pFCX300L and pFCX300M also produced β-lactamase inducibly, although specific activities were lower than those for *E. cloacae* 8009.

Susceptibilities of transformants to various antibiotics. The patterns of susceptibility to various agents for the transformants that were obtained are presented in Tables 2 and 3. The MICs were higher for *E. cloacae* 8009 than those for any of the other transformants tested. Moreover, *E. cloacae* 8009 was also resistant to cefoxitin and moxalactam, suggesting that the strain produces a fair amount of chromosomal cephalosporinase, although it was not detectable by analytical isoelectric focusing. The MICs for transformants harboring pHSG396-derived plasmids were higher than the MICs for transformants harboring low-copy-number original plasmids. The effect of copy number was larger than the effect of the AmpR activator on the SFO-1 producer.

DISCUSSION

The SFO-1 β-lactamase isolated from E. cloacae 8009 seems to be more threatening than the common TEM-1- or TEM-2-type β-lactamase because it has a broad spectrum of activity and hydrolyzes various β-lactam antibiotics but not cephamycin, oxacephem, or carbapenem. However, *E. cloacae* is known to gain resistance to β-lactams easily as a result of mutations of the regulatory genes for chromosomal inducible β-lactamase, making it a high-level β-lactamase producer (4, 6, 16, 18). In fact, the level of resistance of *E. cloacae* 8009 to ceftizoxime, ceftazidime, and moxalactam was lower than those for strains that constitutively produce large amounts of chromosomal β-lactamase (Table 2). If *E. cloacae* 8009 was a high-level producer of chromosomal β-lactamase, it would be almost impossible to predict the presence of a β-lactamase like SFO-1 on the basis of patterns of susceptibility to various β-lactams. Moreover, the strain would have no need to acquire SFO-1 genes for defense. *E. cloacae* 8009 was also resistant to cefoxitin, in contrast to the SFO-1-producing transformant derived from β-lactamase-negative *E. cloacae* 199S. The cefoxitin resistance of *E. cloacae* 8009 is considered to come from a porin change or small amounts of chromosomal β-lactamase produced by the strain, although it was difficult to detect chromosomal β-lactamase by isoelectric focusing. SFO-1 seems to have an important role in the β-lactam resistance of *E. cloacae* 8009. SFO-1 could be an effective weapon that various gram-negative bacteria could use to resist β-lactams.

β-Lactamase is usually produced constitutively in *E. coli*, which has no *ampR* gene (4). SFO-1 was produced constitutively in TF2-2 and TF2-3, as shown in Table 6.

Are there any merits to the coexistence of *ampR* with *ampA*? In gram-negative bacteria, virtually all of the known plasmid-mediated β-lactamases are produced constitutively, although SFO-1 was produced inducibly. The presence of *ampR* seems to be a disadvantage for the host strain, because *E. cloacae* becomes highly resistant to β-lactams when β-lactamase production is changed from inducible to constitutive. However, the amount of β-lactamase was larger in the strain which has both *ampR* and *ampA* on the plasmid, which indicates that AmpR acts as an activator as well as a repressor (4). The coexistence of *ampR* with *ampA* is considered to aid in the progression of resistance. In fact, after induction TF-L7 and TF-M4, which had *ampR*, produced SFO-1 in amounts more than 10 times larger than the amounts produced by TF2-2 and TF2-3, which did not have *ampR*, although they have SFO-1 on

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the same vector (Table 6). However, the MICs of the cephalosporins for the transformants were not dramatically influenced by the presence of *ampR*. The low level of resistance of TF-L was considered to come from the low copy number of plasmid pFCX300L.

Concerning the origin of the plasmid-mediated β-lactamase, it is most possible that the *ampA* of SFO-1 is from *S. fonticola* (8), because the AmpA sequences of both *E. cloacae* 8009 and *S. fonticola* had a high degree of homology (95.5%), although there is no information about the AmpR of *S. fonticola*. The AmpA sequence of *E. cloacae* 8009 also had a high degree of homology with those of the enzyme from *K. oxytoca* and MEN-1, although the latter two enzymes are constitutively produced. *C. diversus* is another possible source because its AmpA and AmpR sequences were 70 and 80% homologous to those of *E. cloacae* 8009, respectively. In the PCR study with primers designed to detect *ampA* and *ampR* of SFO-1, no strains of ordinary gram-negative organisms produced both amplified fragments, although we have not examined *S. fonticola* (data not shown). *S. fonticola*, a kind of flora usually isolated from well waters and springs, is not well known because it is not a pathogenic organism (8). However, because it is a possible source of a plasmid-mediated resistance factor, it is necessary to pay attention to minor species as possible resistance gene donors, even if they are uncommon.

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