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Genetic Analysis of Faropenem-resistant *Enterococcus faecalis* in Urinary Isolates

Noriyuki Hiraga, Tetsuro Muratani, Seiji Naito, Tetsuro Matsumoto

Abstract We isolated faropenem-resistant *Enterococcus faecalis* in urine specimens and studied the mechanisms of resistance to faropenem in these isolates. Three mechanisms of penicillin resistance have been reported in *E. faecalis*; (1) beta-lactamase production, (2) overproduction of penicillin-binding protein (PBP) 4 or PBP5, and (3) decreasing affinities of penicillins for PBP4 by the occurrence of point mutations of the penicillin-binding domain. None of the *E. faecalis* isolates examined produced beta-lactamase or overproduced any PBPs, but the affinities of faropenem for PBP4 were decreased in faropenem-insensitive and -resistant strains. We found single amino acid substitutions at positions 475, 520 or 605 in PBP4 in the insensitive strains and two amino acid substitutions at positions 520 and 605 in PBP4 in the resistant strains by sequencing the entire *pbp4* gene from each isolate. We conclude that development of resistance to faropenem in *E. faecalis* is due to decreasing affinities for PBP4 that are the result of the occurrence of one or two point mutations.

Keywords *Enterococcus faecalis*, faropenem, penicillin-binding proteins, urinary tract infections

Introduction

Faropenem is a penem antibiotic, a discrete class of beta-lactams commonly used in the treatment of a wide range of infections including urinary tract infections (UTIs).

Faropenem is stable to beta-lactamases and has potent antimicrobial activity against Gram-positive pathogens including enterococci and Gram-negative pathogens with the exception of *Pseudomonas aeruginosa* [1, 2]. Accordingly, faropenem shows potent activity against most of the causative isolates of UTIs, especially against *E. faecalis* which is intrinsically resistant to cephems [3, 4]. Faropenem is often used for oral therapy in patients with UTIs in Japan [5].

Enterococci including *E. faecalis* and *E. faecium* are natural inhabitants of the gastrointestinal tract of humans. They are a common cause of UTIs and wound infections. Enterococci are the causative isolates in 3.2 to 16% of the UTIs in adults and 4.2 to 16.8% of those in children [6~9]. They can disseminate from the gastrointestinal tract to cause cholangitis, peritonitis and intra-abdominal abscess. Bacteremia and endocarditis are other well-recognized clinical manifestations [10, 11]. For these reasons, among causative pathogens of UTIs, enterococci are one of the most important pathogens. Among UTI-causing enterococci, multi-drug resistant *E. faecalis* such as vancomycin-resistant strains (VRE) have been reported increasingly in many countries, and in Japan the isolation frequency is similar and has increased alarmingly [12, 13]. Most isolates of *E. faecium* are already resistant to penicillins, cephems and penems [1, 2], but *E. faecalis* is susceptible to and can be treated with penicillins including imipenem and faropenem [1, 2, 14].

Penicillin-resistant *E. faecalis* strains have been isolated infrequently, so there have been only a few reports about

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penicillin-resistant *E. faecalis*. Three mechanisms of resistance to penicillins in *E. faecalis* have been reported: (1) the production of beta-lactamase, which hydrolyse penicillins enzymatically [15~19]; (2) overproduction of penicillin-binding protein (PBP) 4 and PBP5 [20~22]; and (3) decreasing affinities of PBP4 for penicillins by the occurrence of point mutations in the penicillin-recognition domain [23].

E. faecalis is usually susceptible to faropenem, and we have found few reports of resistance to faropenem in *E. faecalis*; however we have isolated faropenem-resistant *E. faecalis* from the urine of patients with UTIs. In the present study, we examined the mechanisms of resistance to faropenem in these isolates.

Materials and Methods

Strains Isolated from Clinical Urine Specimens

The *E. faecalis* strains isolated in this study are listed in Table 1. In this study we used 11 strains, which showed various susceptibilities to faropenem, isolated from urine of different patients with UTIs seen at 5 hospitals in Kitakyushu, Japan between 1999 and 2004. Among the strains, *E. faecalis* Aef7 was isolated from the urine of an outpatient, but the others were isolated from urine of inpatients. We identified all the isolates used in this study as *E. faecalis* using the Vitek system (bioMerieux, France). The vancomycin resistance genes, *vanA* and *vanB*, were detected by PCR using specific primers [24, 25].

Antimicrobial Agents and MIC Determination

Antimicrobial agents were obtained as follows: faropenem, Daiichi Suntory Pharma Co., Ltd. Tokyo Japan; ampicillin, Nacalai Tesque, Inc. Kyoto Japan; imipenem, Banyu Tokyo Japan. We determined MICs on Mueller-Hinton agar (Difco) by a serial agar dilution method [26, 27].

Beta-lactamase Activity

Beta-lactamase activities of all the *E. faecalis* strains used in this study were examined following the method of O'Callaghan *et al.* with some modifications [28]. For hydrolysis studies, *E. faecalis* isolates were grown overnight at 37°C in BHI broth (Eiken Chemical Co., Ltd., Tokyo Japan) harvested and washed once in 200 ml of 50 mM sodium phosphate buffer (pH 7.0), and resuspended them in 20 ml of sodium phosphate buffer. The suspension was sonically disrupted using an Ultrasonic disruptor UD-201 (TOMY, Tokyo, Japan) and debris was removed by centrifugation. We tested the supernatant for beta-lactamase activity with nitrocefin (Oxoid, Ltd., United Kingdom). We

used *Escherichia coli* ATCC 35218 which was a positive control strain for beta-lactamase activity [15].

Isolation of Cytoplasmic Membranes

Bacterial cytoplasmic membranes were prepared following the method of Spratt *et al.* [29]. In brief, cells were grown in BHI broth at 37°C overnight, collected in late exponential growth phase, and disrupted by sonication on ice. Residual cells were removed by centrifugation; protein concentration in the remaining supernatant was estimated using Lowry's method [30]. Then, membranes were stored at -80°C in 50 mM sodium phosphate, pH 7.0, containing 10 mM $MgCl_2$.

PBP Profiles

We used Bocillin FL® (Molecular Probes, Inc., Eugene, Oregon), a commercially available fluorescent penicillin, to detect PBPs in bacterial membrane preparations following the method of Ono *et al.* [23, 31]. In brief, 20 μl of a 20 mg/ml membrane protein suspension was incubated with 15 μl of 50 μM Bocillin FL for 10 minutes at 30°C. Proteins were then solubilized with sarcosyl, 180 mg/ml penicillin G was added, and the membrane fraction was obtained by centrifugation. Labeled membrane proteins were resolved by gel electrophoresis and visualized with UV light (ATTO AB1500, wavelength 312 nm).

Affinities of Antimicrobial Agents for PBPs

We also used a competition assay to analyze the PBPs of these isolates. Membrane proteins (20 mg/ml) were incubated with increasing concentration of faropenem, ampicillin or imipenem (from 0.0625 to 32 μl/ml) for 10 minutes at 30°C. Samples were then incubated with 50 μM Bocillin FL for another 10 minutes at 30°C and analyzed as described above. The 50% inhibitory concentration (IC_{50}) was taken to be the minimum concentration of unlabeled antibiotic which reduced binding of the Bocillin FL to a PBP by more than 50%.

Sequencing

The entire *pbp4* genes of the *E. faecalis* strains were amplified by PCR using specific primers [23]. The PCR products were directly sequenced by the dideoxy chain termination method with an ABI PRISM 3130 DNA sequencer (PE Biosystems).

Analysis of Genotypes by Pulsed-field Gel Electrophoresis (PFGE)

All the genotypes of *E. faecalis* strains used in this study were analyzed by pulsed-field gel electrophoresis (PFGE) following the method of Kaufmann *et al.* [32] with some

Table 1 Bacterial characteristics of urinary isolates and type strains used in this study and their MIC distribution for selected agents

Strain	Characteristics	Date of isolation (day. mo. yr)	Age (yr-old)	Sex	Hospital	MIC (µg/ml) FRPM	AMP	IPM	VCM	LZD	LVFX	ST*	Beta-lactamase produced	Refernce
Enterococcus faecalis														
ATCC29212	Type strain					1	1	1	4	2	1	0.03	−	
SEF96	Clinical specimen	21.04.2000	53	Male	S	1	2	1	1	2	1	0.06	−	Ono, S. et al.[23]
SVR250	Clinical specimen with *vanA*	19.07.2002	69	Female	I	0.5	1	1	>512	2	32	0.03	−	Ono, S. et al.[23]
SVR251	Clinical specimen with *vanA*	19.07.2002	85	Female	I	0.5	2	1	>512	2	32	0.03	−	Ono, S. et al.[23]
SVR9	Clinical specimen with *vanA*	15.11.1999	88	Male	Y	4	4	4	>512	1	32	0.03	−	This study
Aef7	Clinical specimen	23.01.2004	68	Male	K	4	4	4	4	2	32	0.03	−	This study
Aef8	Clinical specimen	20.03.2004	93	Male	I	8	4	4	1	2	64	0.06	−	This study
SVR34	Clinical specimen with *vanB*	01.06.2000	74	Male	D	8	4	8	64	2	256	1	−	Ono, S. et al.[23]
SVR1080	Clinical specimen with *vanA*	26.02.2000	78	Female	I	8	8	8	>512	2	32	0.13	−	This study
SVR1084	Clinical specimen with *vanA*	31.08.1999	51	Female	I	32	16	32	>512	2	16	0.03	−	This study
SVR1110	Clinical specimen with *vanA*	02.09.2000	83	Male	I	32	16	32	>512	2	16	0.06	−	Ono, S. et al.[23]
SVR1119	Clinical specimen with *vanA*	01.12.1998	79	Female	I	32	16	32	>512	2	16	0.03	−	Ono, S. et al.[23]
Escherichia coli														
ATCC35218	Type strain					0.5	>128	0.25					+	

Abbreviations: FRPM, faropenem; AMP, ampicillin; IPM, imipenem; VCM, vancomycin; LZD, linezolid; LVFX, levofloxacin; ST, sulfamethoxazole/trimethoprim; ST, sulfamethoxazole/trimethoprim (19/1).
* MICs represented as amounts of trimethoprim.

modifications. Briefly, the colonies of an overnight culture, grown at 37°C on blood agar, were mixed and suspended in 100 μl of 50 mM EDTA (pH 8.0). This suspension was mixed with 100 μl of 1.2% agarose (chromosomal grade agarose, Bio-Rad Laboratories, USA) and pipetted into small plug molds. The cells suspended in the agarose plugs were lysed by incubation for 16 hours at 37°C in 0.6 ml of 6.0 mM Tris (pH 8.0)-0.1 M EDTA (pH 8.0) buffer containing 0.5% sarcosyl, 0.5% Brij58, 0.2% deoxycholate-Na, 0.8 mg/ml of lysozyme (Wako Pure Chemical Industries, Ltd., Japan), 0.02 mg/ml of lysostaphin (Wako Pure Chemical Industries, Ltd., Japan) and 0.01 mg/ml of ribonuclease (Wako Pure Chemical Industries, Ltd., Japan). The lysis solution was replaced by a 0.5 ml of 0.5 M EDTA containing 1.0% sarcosyl and 0.25 mg/ml of proteinase K (Wako Pure Chemical Industries, Ltd., Japan), which was then incubated at 50°C for more than 16 hours. After being washed with 50 mM EDTA, the plugs were incubated in 50 mM EDTA containing 1.0 mM phenylmethylsulfonyl fluoride (PMSF) at room temperature for 10 minutes. Then the DNA in the plugs was washed twice with 1.0 ml of 50 mM EDTA. The plugs were digested with SmaI (Nippon Gene Co., Japan), and products were separated on a 1.0% agarose gel (pulsed-field certified; Bio-Rad, Hercules, Calif.) by a CHEF Mapper pulsed-field gel electrophoresis system (Nippon Bio-Rad Laboratories, Japan). The electrophoresis conditions were as follows: pulse time ramped linearly from 3 to 45 seconds, run time was 22 hours, temperature was 14°C, and constant voltage was 6 V/cm. PFGE results were interpreted in accordance with the criteria proposed by Tenover *et al.* [33].

Results

Antimicrobial Susceptibility

Table 1 shows the susceptibility of *E. faecalis* strains used in this study to faropenem, ampicillin, imipenem, vancomycin, linezolid, levofloxacin and sulfamethoxazole/trimethoprim. MICs of faropenem against SEF96, ATCC29212, SVR250 and SVR251 were 0.5 to 1.0 μg/ml. These 4 strains were defined as faropenem-susceptible strains in accordance with the faropenem susceptibility breakpoint, 2.0 μg/ml [2, 12]. MICs of faropenem against Aef7, Aef8, SVR9, SVR34 and SVR1080 were 4.0 to 8.0 μg/ml and these 5 strains were defined as faropenem-insensitive strains. MICs of faropenem against SVR1084, SVR1110 and SVR1119 were 32 μg/ml and these 3 strains were defined as faropenem-resistant strains. Faropenem-susceptible strains were also susceptible to ampicillin and imipenem (MICs of ampicillin, 1.0 to 2.0 μg/ml; MICs of

imipenem, 1.0 μg/ml), faropenem-insensitive strains were also insensitive to ampicillin and imipenem (MICs of ampicillin, 4.0 to 8.0 μg/ml; MICs of imipenem, 4.0 to 8.0 μg/ml), and faropenem-resistant strains were also resistant to ampicillin and imipenem (MICs of ampicillin, 16 μg/ml; MICs of imipenem, 32 μg/ml) in accordance with the ampicillin susceptibility breakpoint, 8.0 μg/ml and the imipenem susceptibility breakpoint, 4.0 μg/ml [2, 12]. All the isolates were resistant to levofloxacin (MICs of levofloxacin, 16 to 512 μg/ml) except the type strain ATCC29212 and the clinical isolate SEF96 (MICs of levofloxacin, 1.0 μg/ml). MICs of vancomycin against the strains which had *vanA* (SVR9, SVR250, SVR251, SVR1080, SVR1084, SVR1110 and SVR1119) were more than 512 μg/ml, and the MIC of vancomycin against *E. faecalis* SVR34 which had *vanB* was 64 μg/ml. On the other hand, MICs of vancomycin against the strains having neither *vanA* nor *vanB* (ATCC29212, SEF96, Aef7 and Aef8) were 1.0 to 4.0 μg/ml. MICs of sulfamethoxazole/trimethoprim (19/1) against all the isolates except SVR34 (the MIC of sulfamethoxazole/trimethoprim, 19/1.0 μg/ml) were 0.57/0.03 to 2.47/0.13 μg/ml. MICs of linezolid against all the isolates were 1.0 to 2.0 μg/ml.

Beta-lactamase Activity

E. faecalis strains SVR9, Aef7, Aef8, SVR1080 and SVR1084 did not hydrolyze nitrocefin. In addition, it has already been reported that *E. faecalis* ATCC29212, SEF96, SVR250, SVR251, SVR34, SVR1110 and SVR1119 failed to hydrolyze nitrocefin [23].

PBP Profiles

We used a fluorescent penicillin (Bocillin FL) to detect PBP in membrane fractions prepared from all the *E. faecalis* isolates (the number of experimental trials, n=3). PBPs of the isolates were expressed as the same five bands that corresponded to proteins of approximately 107 kDa, 81 kDa, 76 kDa, 72 kDa, and 67 kDa. Overproduction of PBPs was not seen previously in SVR34, SVR1110 and SVR1119 [23]. Likewise for the 5 new strains (SVR9, Aef7, Aef8, SVR1080 and SVR1084), we found no evidence of overproduction of any PBP.

Affinities of the Antibiotics for PBPs

We performed competition assays to measure the affinity of faropenem, ampicillin and imipenem for each PBP (n=3). IC_{50} (μg/ml) is expressed as the minimum concentration of antibiotics required to reduce Bocillin FL binding to the PBP by more than 50%. There were no differences in IC_{50}s of faropenem against any PBPs of all strains except for PBP4. There were obvious differences in PBP4 saturation

Table 2 Inhibition of binding Bocillin FL to PBPs by antimicrobial agents used in this study

Drug	E. faecalis strain	IC$_{50}$ (μg/ml)				MIC (μg/ml)
		PBP1	PBP3	PBP4	PBP5	
FRPM	ATCC29212	8	0.5	1	>32	1
	SEF96	16	0.5	1	>32	1
	SVR250	8	1	1	>16	0.5
	SVR251	4	0.5	0.5	>32	0.5
	SVR9	8	1	2	>32	4
	Aef7	8	1	2	>32	4
	Aef8	8	0.5	2	>32	8
	SVR34	8	1	2	>32	8
	SVR1080	8	0.5	2	>32	8
	SVR1084	4	0.5	32	>32	32
	SVR1110	8	1	32	>32	32
	SVR1119	8	0.5	16	>32	32
AMP	ATCC29212	1	0.5	0.25	>16	1
	SEF96	1	0.5	0.25	>16	2
	SVR250	2	0.5	1	>8	1
	SVR251	2	0.5	0.25	>8	2
	SVR9	2	0.5	4	>32	4
	Aef7	2	0.5	4	>32	4
	Aef8	2	0.5	4	>32	4
	SVR34	2	0.5	4	>32	4
	SVR1080	2	0.5	4	>32	8
	SVR1084	1	1	8	>32	16
	SVR1110	1	0.5	8	>32	16
	SVR1119	1	0.5	8	>32	16
IPM	ATCC29212	0.5	0.25	0.125	>16	1
	SEF96	0.5	0.25	0.25	>16	1
	SVR250	1	0.5	0.25	>32	1
	SVR251	0.5	0.25	0.5	>32	1
	SVR9	0.5	0.5	4	>32	4
	Aef7	1	0.5	4	>32	4
	Aef8	1	0.25	4	>32	4
	SVR34	0.5	0.25	4	>32	8
	SVR1080	0.5	0.25	4	>32	8
	SVR1084	0.5	0.5	16	>32	32
	SVR1110	1	0.5	32	>32	32
	SVR1119	1	0.25	16	>32	32

Abbreviations: FRPM, faropenem; AMP, ampicillin; IPM, imipenem.
IC$_{50}$, Minimum concentration of unlabeled antibiotic which reduces binding of Bocillin FL to a PBP by more than 50%.

for faropenem between faropenem-susceptible strains and faropenem-resistant strains. MICs and IC$_{50}$s of faropenem against PBP4 of faropenem-resistant strains were obviously higher than those of faropenem-susceptible strains. Similar to MICs and IC$_{50}$s of faropenem, MICs and IC$_{50}$s of ampicillin or imipenem against PBP4 of faropenem-resistant strains were obviously higher than those of faropenem-susceptible strains (Tables 2, 3).

The IC$_{50}$s of faropenem, ampicillin and imipenem against PBP4 of all strains had a positive correlation with

Table 3 Substitutions in amino acid sequences of *pbp4* of *E. faecalis* strains used in this study compared with *E. faecalis* SEF96, MICs and IC$_{50}$s of three antimicrobial agents for the strains

E. faecalis strain	Alteration at position					MICs (µg/ml) of FRPM/AMP/IPM	IC$_{50}$s (µg/ml) of FRPM/AMP/IPM to PBP4
	50	369	475	520	605		
SEF96	Thr	Val	Leu	Pro	Tyr	1/2/1	1/0.25/0.25
ATCC29212		Ala				1/1/1	1/0.25/0.125
SVR250	Ile					0.5/1/1	1/1/0.25
SVR251	Ile					0.5/2/1	0.5/0.25/0.5
SVR9				Ser		4/4/4	2/4/4
Aef7			Met			4/4/4	2/4/4
Aef8			Met			8/4/4	2/4/4
SVR34				Ser		8/4/8	2/4/4
SVR1080					His	8/8/8	2/4/4
SVR1084				Ser	His	32/16/32	32/8/16
SVR1110				Ser	His	32/16/32	32/8/32
SVR1119				Ser	His	32/16/32	16/8/16

Abbreviations: FRPM, faropenem; AMP, ampicillin; IPM, imipenem.

MICs of the agents, respectively.

Deduced Amino Acid Sequences of PBP4 Based on Sequences of *pbp4* Genes

We amplified the *pbp4* genes of the *E. faecalis* isolates and sequenced the entire *pbp4* genes corresponding to amino acids 1 to 680, to identify mutations that may account for the differences in the affinity of this protein for antibiotics among the faropenem-susceptible, -insensitive and -resistant strains. SVR1084, a faropenem-resistant strain (the MIC of faropenem, 32 µg/ml, the IC$_{50}$ of faropenem against PBP4, 32 µg/ml) had the same two amino acid substitutions (Pro520Ser and Tyr605His) in the *pbp4* as faropenem-resistant strains SVR1110 and SVR1119, the sequences of which had been previously reported, compared to the amino acid sequences of SEF96, a penicillin-susceptible strain enrolled in GenBank [23]. Four faropenem-insensitive strains had one out of three single amino acid substitutions in PBP4, which were Leu475Met, Pro520Ser or Tyr605His (Table 3).

Analysis of Genotypes by Pulsed-field Gel Electrophoresis (PFGE)

In accordance with the criteria proposed by Tenover *et al.* [33], we did not find any indistinguishable genotypes by PFGE, but SVR1080, SVR1084, SVR1110 and SVR1119 appeared to have possibly related genotypes (n=3) (Fig. 1).
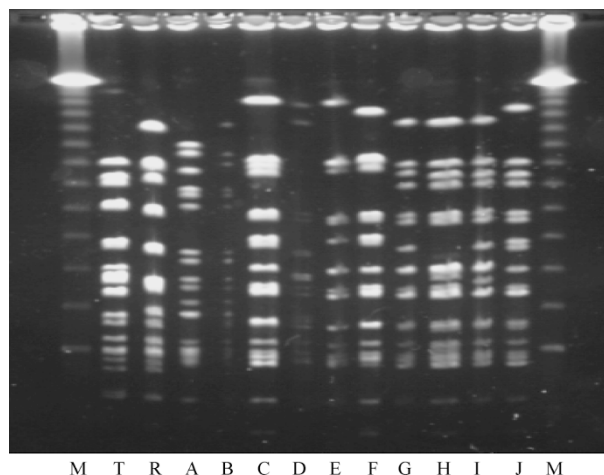
Fig. 1 Pulsed-field gel electrophoresis (PFGE) typing.

Total genetic DNA was digested with Sma I and subjected to PFGE. Lane M, DNA marker; Lane T, type strain ATCC29212; Lane R, reference strain SEF96; Lane A, SEF250; Lane B, SEF251; Lane C, SVR9; Lane D, Aef7; Lane E, Aef8; Lane F, SVR34; Lane G, SVR1080; Lane H, SVR1084; Lane I, SVR1110; Lane J, SVR1119.

Discussion

Faropenem is a member of the penems which form a discrete class of beta-lactams. Faropenem has potent antimicrobial activity against most of the causative isolates

of UTIs, especially against *E. faecalis* which is intrinsically resistant to cephems [3, 4]. *E. faecalis* is susceptible to ampicillin and is also susceptible to imipenem and faropenem, a difference from *E. faecium*. In fact, most of the *E. faecalis* isolates are treatable with these antibiotics [1, 2, 14]. We have found few reports of faropenem-resistant *E. faecalis*, but we isolated faropenem-resistant *E. faecalis* strains in the urine of patients with UTIs. Here, we studied the mechanisms of resistance to faropenem in these isolates.

Three mechanisms of penicillin resistance have been reported in *E. faecalis*. Beta-lactamase production was first reported in 1983 [15], and there have been several subsequent reports of beta-lactamase-producing penicillin resistance in *E. faecalis* [16~19]; nevertheless, beta-lactamase production is a very rare mechanism in enterococci [3, 4, 34]. *E. faecalis* strains carrying *blaR1* and *bla1*, staphylococcal beta-lactamase regulatory genes, produced small amounts of class A beta-lactamases [17, 35]; these enzymes do not hydrolyze either carbapenems or penems [2, 4, 5]. Thus, even beta-lactamase-producing *E. faecalis* are expected to be susceptible to faropenem and imipenem.

Another mechanism of resistance is overproduction of PBP4 or PBP5 [20~22]. *E. faecalis* JH2-2r (the MIC of benzylpenicillin, 75 μg/ml) was selected from *E. faecalis* JH2-2 by successive growth on BHI plates containing increasing concentrations of benzylpenicillin. JH2-2r showed overproduction of PBP4 compared with JH2-2. In their study, however, the cloning and sequencing of *psr*-like gene, which was a repressor-encoding gene (for PBP5 synthesis repressor), from both Jh2-2 and JH2-2r indicated that they were identical [20]. Clinical isolates of ampicillin-resistant *E. faecalis* (the MIC of ampicillin, 32 μg/ml), which had no beta-lactamases, showed overproduction of PBP5 [21]. MICs for ampicillin in mutant derivatives of *E. faecalis* 56 carrying a plasmid coding for *pbp5* were 64 μg/ml, which were 16 times higher than that of parent strain *E. faecalis* 56 (the MIC of ampicillin, 4.0 μg/ml). The mutant derivatives also showed overproduction of PBP5 compared with the parent strain [22].

The other mechanism of resistance is decreasing affinities of penicillins for PBP4 by the occurrence of point mutations in the penicillin-recognition domain [23]. *E. faecalis* PBP4 is a membrane protein composed of 680 amino acids with three distinct modules. One is a penicillin-binding module (amino acids 350 to 680) located towards the *C*-terminus, in which three typical penicillin-binding motifs (the active-site-defining motifs), STFK, SDN and KTG, were identified as the main target of beta-lactams. The others are a non-penicillin-binding module

(amino acids 40 to 349) and an uncleaved *N*-terminal segment that acts as a membrane-spanning (anchoring) domain (amino acids 1 to 39) [22, 23]. Ono *et al.* reported the mechanisms of resistance to ampicillin and imipenem in vancomycin-resistant *E. faecalis* isolated from clinical specimens [23]. These strains lacked beta-lactamases and did not overproduce PBPs. It was also reported that the MICs of ampicillin and imipenem for *E. faecalis* SVR1119 and *E. faecalis* SVR1119S, which was cured of a *vanA*-carrying plasmid from SVR1119, were identical, suggesting that *vanA* was not involved in the development of resistance to ampicillin and imipenem in the strains.

The MICs of ampicillin or imipenem for ampicillin, imipenem-insensitive strains increased twice or 8 times (ampicillin) and 16 times (imipenem) compared with those for ampicillin, imipenem-susceptible strains (MICs of ampicillin, 1.0 μg/ml; MICs of imipenem, 0.5 to 1.0 μg/ml), respectively. The MICs of ampicillin or imipenem for ampicillin, imipenem-resistant strains increased 32 to 64 times compared with those for ampicillin, imipenem-susceptible strains. On the other hand, the IC$_{50}$s of ampicillin or imipenem against PBP4 for ampicillin, imipenem-insensitive strains also increased twice or 4 to 8 times compared with those for ampicillin, imipenem-susceptible strains, respectively. The IC$_{50}$s of ampicillin or imipenem against PBP4 for ampicillin, imipenem-resistant strains also increased 8 to 32 times or 32 to 256 times compared with those for ampicillin, imipenem-susceptible strains, respectively. Sequencing of *pbp4* for the strains showed that Pro-520 was changed to a Ser residue in ampicillin, imipenem-insensitive strains (MICs of ampicillin, 8.0 μg/ml; MICs of imipenem, 4.0 μg/ml), and Pro-520 was replaced with a Ser residue. The same Pro520Ser alteration was seen at position 520 and Tyr-605 was replaced with a His residue in ampicillin, imipenem-resistant strains (MICs of ampicillin, 16 μg/ml; MICs of imipenem, 32 μg/ml) [23].

In the present study, none of the isolates used produced any beta-lactamases and none overproduced PBPs. All faropenem-resistant strains had *vanA*; 3 faropenem-insensitive strains had *vanA* and *vanB*; 2 faropenem-susceptible strains had *vanA* and 2 faropenem-insensitive strains did not have either *vanA* or *vanB*. In addition, it was already reported that there were no differences between the susceptibilities of ampicillin and imipenem for SVR1119 and SVR1119S [23]. Therefore, *vanA* and *vanB* were not involved in development of resistance to faropenem, ampicillin and imipenem in these strains (Table 1).

In faropenem-insensitive strains, we found a single point mutation at positions 475, 520 or 605 in the penicillin binding module; these amino acid substitutions produced

similar effects on the MICs and IC$_{50}$s for faropenem. In the faropenem-resistant strains, we found point mutations at both position 520 and 605 in the penicillin binding module; this double substitution also produced similar effects on the MICs and IC$_{50}$s for faropenem against PBP4 (Tables 2, 3). These point mutations occurred in the region between the active-site-defining motifs, STFK, SDN and KTG, in the penicillin-binding module. SVR1080 had a novel single Tyr605His amino acid substitution and both Aef7 and Aef8 also had another novel single Leu475Met amino acid substitution; all three strains were faropenem-insensitive. Two other faropenem-insensitive strains SVR9 and SVR34 had the same single Pro520Ser amino acid substitution [23]. It has been reported that point mutations outside the penicillin-binding domain of PBP4 (*E. faecalis* ATCC29212, SVR250 and SVR251) had no effect on MICs and IC$_{50}$s of ampicillin and imipenem for these strains [23]. In the present study, point mutations outside the penicillin-binding domain of PBP4 also failed to alter MICs and IC$_{50}$s of faropenem (Table 3). Thus, it is considered that MICs and IC$_{50}$s of faropenem as well as ampicillin and imipenem against PBP4 were increased by point mutations that occurred in the penicillin binding module of PBP4.

SVR1080, faropenem-insensitive strain, is similar by PFGE to the resistant strain, SVR1110 (Fig. 1). In the insensitive strains, Pro-520 was replaced with Ser or Tyr-605 was replaced with His, but in the resistant strains both changes (Pro520Ser and Tyr605His) were observed in the region between the active-site-defining motifs, STFK, SDN and KTG. Therefore, it is suggested that an insensitive strain, SVR1080 was transformed to the more resistant strain, SVR1110 by acquiring an additional point mutations.

We conclude that development of resistance to faropenem as well as ampicillin and imipenem depends on decreasing affinities of the antimicrobial agents for PBP4 by the occurrence of one or two point mutations at position 475, 520 or 605 in the penicillin binding module. In this present study, we would like to stress here that one point mutation in the PBP4 of *E. faecalis* shows low resistance to the aforementioned antibiotics, but just two point mutations lead to drastically higher resistance. This study is the first report regarding the mechanisms of resistance to faropenem in *E. faecalis*. Faropenem plays an important role in the treatment of a wide range of infections, especially UTIs in Japan. If faropenem-resistant *E. faecalis* strains continue to increase, these strains will cause serious problems for treatment of the enterococcal infections. Strict control and prevention of nosocomial infections should be required.

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