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Mechanisms of Resistance to Imipenem and Ampicillin in *Enterococcus faecalis*

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We found ampicillin- and imipenem-resistant isolates of *vanA*-possessing *Enterococcus faecalis* with MICs of 8 to 16 μg/ml and 4 to 32 μg/ml, respectively. There have been few reports about penicillin- and imipenem-resistant *E. faecalis*. Two mechanisms of beta-lactam resistance in *E. faecalis*, the production of beta-lactamase and the overproduction of penicillin-binding proteins (PBPs), have been reported. The resistant isolates in the current study did not produce any beta-lactamases and analysis of the PBPs showed no overproduction. However, the affinities of PBP4 for beta-lactams in the resistant strains were lower than those of susceptible strains but the affinities of other PBPs for beta-lactams did not change. Accordingly, whole *pbp4* fragments from these resistant isolates were sequenced. Two amino acid substitutions at positions 520 and 605 were observed in the highly resistant strains compared to the susceptible ones, Pro520Ser and Tyr605His, and a single Tyr605His amino acid substitution was found in the low-resistance strains. These two point mutations exist in the region between the active-site-defining motifs SDN and KTG of the penicillin-binding domain, the main target of beta-lactams. A strong correlation was seen between these substitutions and decreasing affinities of PBP4 to beta-lactams. In *E. faecalis*, resistance due to mutations in PBPs has not been reported, though it has in *Enterococcus faecium*. Our results suggest that development of high-level resistance to penicillins and imipenem depends on point mutations of PBP4 at positions 520 and 605.

It has been reported that the frequency of nosocomial enterococcal infections has increased markedly in the past two decades in the United States (25). Enterococci account for over 10% of nosocomial bloodstream infections and have been associated with an attributable mortality of over 30% in the United States (5). The impact of multidrug-resistant pathogens has been augmented by the emergence of vancomycin-resistant enterococci (VRE). VRE were first isolated in Europe in 1986 (16, 36) and then in the United States in 1987 (33). The prevalence of nosocomial VRE in the United States increased from 0.3% in 1989 to 7.9% in 1993 (3). In the United States, VRE currently account for 20 to 25% of all enterococcal isolates in intensive care units, according to the National Nosocomial Infections Surveillance System, 1989 to 1998 (25). In contrast to the findings in the United States, clinical isolates of VRE in European hospitals have been rather infrequent (39). In Japan, a few cases have been reported since the first reported case of VRE infection in 1997 (13). Nosocomial VRE infections of 36 inpatients in Kitakyusyu City in Japan were reported in 2002. Most VRE are *Enterococcus faecium* in the United States but *Enterococcus faecalis* is isolated more than *E. faecium* among VRE in Japan (22).

Enterococci are intrinsically resistant to cephems (2, 9, 18) and most enterococcal strains such as those of *E. faecium* and *E. avium*, with the exception of *E. faecalis*, are also resistant to penicillins and imipenem. However, there are few reports about penicillin- and imipenem-resistant *E. faecalis*. Two mechanisms for beta-lactam resistance in *E. faecalis* have been reported. One is production of beta-lactamase to hydrolyze beta-lactams (24) and the other is the overproduction of penicillin-binding proteins (PBPs) that bind the agents (10).

We found ampicillin- and imipenem-resistant isolates of *E. faecalis* carrying *vanA* among clinical specimens. We studied the mechanisms of resistance to penicillin and imipenem in these isolates.

MATERIALS AND METHODS

Bacterial strains. The *E. faecalis* strains used in this study are listed in Table 1. These seven strains were isolated from different inpatients at three hospitals between 1998 and 2000 in Kitakyusyu, Japan. All isolates were identified as *E. faecalis* using the Vitek system (bioMerieux, France). The vancomycin resistance genes, *vanA* and *vanB*, were detected by PCR using specific primers (7, 15). SEF 96, ATCC 29212, SVR 250, and SVR 251 were ampicillin- and imipenem-susceptible strains. SVR 34 and SVR 138 were ampicillin- and imipenem-insensitive strains. SVR 1110 and SVR 1119 were ampicillin- and imipenem-resistant strains.

SVR 1119S, without *vanA*, was selected from SVR 1119 using a serial passage method in vancomycin-free BHI broth (Eiken Chemical Co., Ltd., Japan) and then a replica planting method on BHI agar (17, 31).

Antimicrobial agents and MIC determination. Antimicrobial agents were obtained as follows: ampicillin and vancomycin, Nacalai Tesque, Inc.; imipenem, Banyu; and cefotaxime, Sigma Chemical Co. MICs were determined on Mueller-Hinton agar by a serial dilution method (29).

Beta-lactamase activity. For hydrolysis studies, *E. faecalis* isolates were grown overnight in BHI broth, harvested, washed once in 50 mM sodium phosphate buffer (pH 7.0), and resuspended in 0.1 volume of sodium phosphate buffer. The suspension was sonically disrupted using an Ultrasonic disruptor UD-201 (TOMY, Tokyo, Japan) and debris was removed by centrifugation. The supernatant was tested for beta-lactamase activity with nitrocefin (Oxoid, Ltd., United Kingdom). *Escherichia coli* ATCC 35218 was used as a positive control for beta-lactamase activity (27).

Isolation of cytoplasmic membranes. Bacterial cytoplasmic membranes were prepared following the method of Spratt et al. (35). Briefly, cells were grown in BHI broth at 37°C overnight, collected in late exponential growth phase, and

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TABLE 1. Bacterial strains and MIC values of selected agents

Strain	Date of isolation (day.mo.yr)	Specimen and relevant characteristics	MIC (μg/ml)[a]				Beta-lactamase produced
			AMP	IPM	CTX	VCM	
Enterococcus faecalis							
SEF96	21.04.2000	Clinical isolate from urine	1	1	>256	1	−
ATCC 29212		Type strain	1	1	256	4	−
SVR 34	15.02.2000	Clinical isolate with *vanB* from urine	8	4	>256	32	−
SVR 138	27.05.2002	Clinical isolate with *vanA* from stool	8	8	>256	>512	−
SVR 250	19.07.2002	Clinical isolate with *vanA* from urine	1	0.5	64	>512	−
SVR 251	19.07.2002	Clinical isolate with *vanA* from urine	1	0.5	64	>512	−
SVR 1110	02.09.2000	Clinical isolate with *vanA* from urine	16	32	>256	>512	−
SVR 1119	01.12.1998	Clinical isolate with *vanA* from urine	16	32	>256	>512	−
SVR 1119S		Δ*vanA*; spontaneous mutant derived from SVR 1119	16	32	>256	1	
Escherichia coli							
ATCC 35218		Beta-lactamase-positive strain					+

[a] Abbreviations: AMP, ampicillin; IPM, imipenem; CTX, cefotaxime; VCM, vancomycin.

disrupted by sonication on ice. Residual cells were removed by centrifugation; protein concentration in the remaining supernatant was estimated using Lowry's method (20). Membranes were then stored at −80°C in 50 mM sodium phosphate, pH 7.0, containing 10 mM $MgCl_2$.

Analysis of PBPs. Bocillin FL (Molecular Probes, Inc., Eugene, Oregon), a commercially available fluorescent penicillin, was used to detect PBPs in bacterial membrane preparations following the method of Zhao et al. (40) with some modifications. Briefly, 20 μl of a 20 mg/ml membrane protein suspension was incubated with 15 μl of 0.05 mM Bocillin FL for 10 min at 30°C. The 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).

PBP competition assay. A competition assay was also used to analyze the PBPs from these isolates. Membrane proteins (20 mg/ml) were incubated with increasing concentrations of ampicillin or imipenem (from 0.0625 to 32 μl/ml) for 10 min at 30°C. Samples were then incubated with 0.05 mM Bocillin FL for another 10 min 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%.

Amplification and sequence analysis of the *pbp4* gene. The whole *pbp4* gene of *E. faecalis* used was amplified by PCR using specific primers (Table 2). The PCR products were subjected to direct sequencing reactions by the dideoxy chain termination method with an ABI PRISM 377 DNA sequencer (PE Biosystems).

Nucleotide sequence accession numbers. The NCBI accession numbers for the nucleotide sequences of the *pbp4* genes of *E. faecalis* ATCC 29212, SVR 251, SVR 1110, and SVR 1119 are AY571368, AY571365, AY571366, and AY571367, respectively.

RESULTS

Susceptibility to beta-lactam antibiotics. Table 1 shows the susceptibility of the strains used in this study to ampicillin, imipenem, cefotaxime, and vancomycin. The MICs of ampicillin and imipenem against *E. faecalis* SEF96, ATCC 29212, SVR 250, and SVR 251, which were ampicillin-susceptible strains, were 1 μg/ml and 0.5 to 1 μg/ml, respectively. The MICs of ampicillin and imipenem against *E. faecalis* SVR 34 and SVR 138, which were ampicillin-insensitive strains, were 8 and 4 to 8 μg/ml, respectively. The MICs of ampicillin and imipenem against *E. faecalis* SVR 1110 and SVR 1119, which were ampicillin-resistant strains, were 16 and 32 μg/ml, respectively.

The susceptibility of SVR 1119S, which was selected as a *vanA* deletion mutant from SVR 1119, was the same as the susceptibility of SVR 1119 to antibiotics except vancomycin.

Beta-lactamase activity. None of the strains used in this study hydrolyzed nitrocefin with the exception of a positive control strain, *Escherichia coli* ATCC 35218.

PBP expression. We used a fluorescent penicillin (Bocillin FL) to detect PBP in membrane fractions prepared from all of the isolates. The PBPs of all strains were expressed as the same five bands that corresponded to proteins of approximately 107 kDa, 81 kDa, 76 kDa, 72 kDa, and 67 kDa. The amount of the corresponding PBP of each strain was almost the same. So, overproduction of any PBPs by any strain was not seen.

PBP competition assay. We performed competition assays to measure the affinity of each PBP for ampicillin or imipenem. The IC_{50} (μg/ml) is expressed as the minimum concentration of antibiotic required to reduce Bocillin FL binding to the PBP by more than 50%. There were obvious differences in PBP4 saturation for ampicillin or imipenem between the susceptible strain, SVR 250, and the resistant strain, SVR 1110. The IC_{50} for PBP4 of the susceptible strains for both antibiotics was obviously lower than that of the resistant strains (Table 3).

TABLE 2. Primers designed for sequencing of PBP4 genes

Primer	Sequence	Reference
EF PBP4 140′F	5′CAACGAAAGCCTGATGAA ATGG3′	This study
EF PBP4 1043F	5′CGATTGACAGTGTACAAC AACAAGC3′	This study
EF PBP4 1132R	5′AATCGCCTTTTTGAGGATC GG3′	This study
EF PBP4 2130R	5′CGCTTCATTGTAGCACACT TTCCTTTTTC3′	4

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TABLE 3. Inhibition of binding of Bocillin FL to PBPs by two beta-lactams

Drug	*E. faecalis* strain	IC_{50}[a] (μg/ml)				MIC (μg/ml)
		PBP1	PBP3	PBP4	PBP5	
Ampicillin	SEF96	1	0.5	0.25	>16	1
	ATCC 29212	1	0.5	0.25	>16	1
	SVR 250	2	0.5	1	>8	1
	SVR 251	2	0.5	0.25	>8	1
	SVR 34	2	0.5	4	>32	8
	SVR 138	2	0.5	4	>32	8
	SVR 1110	1	0.5	8	>32	16
	SVR 1119	1	0.25	8	>32	16
Imipenem	SEF96	0.5	0.25	0.25	>16	1
	ATCC 29212	0.5	0.25	0.125	>16	1
	SVR 250	1	0.5	0.25	>8	0.5
	SVR 251	0.5	0.25	0.5	>8	0.5
	SVR 34	0.5	0.25	4	>32	4
	SVR 138	0.5	0.25	4	>32	4
	SVR 1110	1	0.5	32	>32	32
	SVR 1119	1	0.25	16	>32	32

[a] IC_{50}, concentration of unlabeled antibiotic which reduces binding of Bocillin FL to a PBP by more than 50%.

TABLE 4. Amino acid alterations in strains in this study compared with *E. faecalis* SEF96

Strain	MICs (μg/ml), ampicillin/ imipenem	IC_{50} (μg/ml), ampicillin/ imipenem	Alteration at position:			
			50	369	520	605
SEF96	1/1	0.25/0.25	Tyr	Val	Pro	Tyr
ATCC 29212	1/1	0.25/0.125		Ala		
SVR 250	1/0.5	1/0.25	Ile			
SVR 251	1/0.5	0.25/0.5	Ile			
SVR 34	8/4	4/4				His
SVR 138	8/4	4/4				His
SVR 1110	16/32	8/32			Ser	His
SVR 1119	16/32	8/32			Ser	His

The IC_{50} of ampicillin against PBP4 of the resistant strains (*E. faecalis* SVR 1110 and SVR 1119) was 8 μg/ml, 8 to 32 times higher than those against susceptible strains (SEF96, ATCC 29212, SVR 250, and SVR 251). The IC_{50}s of ampicillin against the insensitive strains were 4 to 16 times higher than those against susceptible strains.

The IC_{50}s of imipenem against PBP4 of the resistant strains (*E. faecalis* SVR 1110 and SVR 1119) were 16 and 32 μg/ml, respectively, 32 to 256 times higher, respectively, than those against susceptible strains (SEF96, ATCC 29212, SVR 250, and SVR 251). Those of imipenem against insensitive strains were 8 to 32 times higher than those against susceptible strains.

The IC_{50}s of ampicillin and imipenem against PBP4 of all strains had a positive correlation with the respective MICs. There were no differences between the IC_{50}s of ampicillin and imipenem against any of the other PBPs of susceptible or resistant strains.

Sequencing of PBP4. We amplified and sequenced the entire *pbp4* gene, corresponding to amino acids 1 to 680, to identify mutations that may account for the differences in the affinity of this protein between the susceptible and resistant strains for beta-lactams. The beta-lactam-resistant strains (SVR 1110 and SVR 1119) had two amino acid substitutions encoded by the *pbp4* gene compared to the reference strain (SEF96), Pro520Ser and Tyr605His (Table 4). The insensitive strains (SVR 34 and SVR 138) had a single Tyr605His amino acid substitution encoded by *pbp4*. All substitutions were due to point mutations. Point mutations were also seen in the susceptible strains ATCC 29212 (Val359Ala) and SVR 250 and SVR 251 (Tyr50Ile) compared with SEF96, but these point mutations had no effect on the MIC of beta-lactams for these strains.

DISCUSSION

We found ampicillin- and imipenem-resistant isolates of *E. faecalis* carrying *vanA*. There have been few reports about ampicillin- and imipenem-resistant *E. faecalis*. MICs for ampicillin and imipenem against both SVR 1119 and SVR 1119S, which was cured of a *vanA*-carrying plasmid from SVR 1119, were identical. Therefore, *vanA* was not involved in the development of penicillin resistance in these strains.

Two mechanisms of beta-lactam resistance have been reported in enterococci. Beta-lactamase production is one of these resistance mechanisms. Murray et al. have reported beta-lactamase-mediated penicillin resistance in *E. faecalis* (26–28). However, with the exception of a few reports (14, 23), enterococci do not produce beta-lactamases. In fact, the penicillin- and imipenem-resistant *E. faecalis* isolates in this study did not produce any beta-lactamases.

The other mechanism of resistance is a change in the affinity of penicillin-binding proteins for beta-lactams or overproduction of specific penicillin-binding proteins. When penicillin binds to a specific PBP at a certain fixed ratio, cell growth stops or cells die; these PBPs are called low-affinity PBPs. Alteration of the affinity of a PBP could have an important role in the mechanism of resistance to beta-lactams. Williamson et al. showed that at least five PBPs have been found in *E. faecalis* by labeling membranes with radioactive penicillin (38), and five PBP genes were found in the genome of *E. faecalis* V583 (GenBank NC004668). We also identified five PBPs in the *E. faecalis* strains used in this study when membranes were labeled with Bocillin FL. The roles of PBPs in penicillin resistance are partially known in some *Enterococcus* species, such as *E. faecium*, *E. hirae*, and *E. faecalis*.

Fontana et al. have reported that PBP 1 and -2 were not detected when *E. faecalis* was cultured at 32°C. These two PBPs were not essential for survival or cell growth of *E. faecalis* (11). Mutants of *E. faecium* lacking PBP5 were hypersensitive to penicillin (8). Overproduction of low-affinity PBPs has also been associated with development of penicillin resistance in enterococcal species (*E. faecium*, *E. faecalis*, *E. avium*, etc.) by studies of in vitro-selected penicillin-resistant mutants or penicillin-resistant clinical isolates (1, 12, 37, 41).

In *E. faecium*, ampicillin resistance has been shown to arise from not only overproduction but also amino acid substitutions of PBP5 (19, 42). Ligozzi et al. showed that in *E. faecium*, four amino acid changes in PBP5 occurred between an ampicillin-sensitive strain, *E. faecium* 28S (0.5 μg/ml), and an ampicillin-resistant strain, *E. faecium* 9439 (128 μg/ml) (19). Zorzi et al. showed that development of intermediate resistance (benzylpenicillin MICs of 6 to 64 μg/ml) in some *E. faecium* strains

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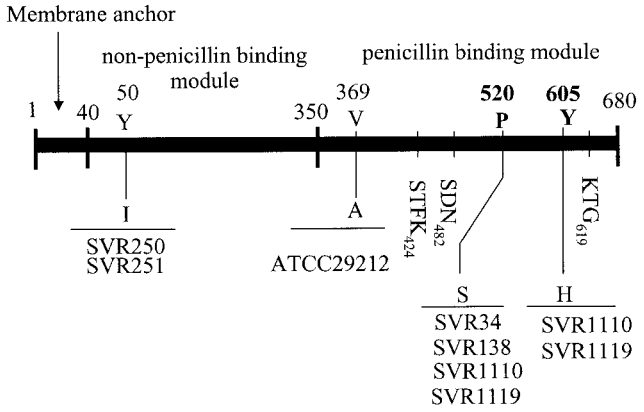

FIG. 1. Amino acid substitutions in *pbp4* of *E. faecalis* strains. The homology boxes (STFK with active-site serine SDN and KTG) are indicated above. The positions at which resistant and low-resistant strains are altered from the sequence of the reference strain *E. faecalis* SEF96 are indicated.

could be explained by the overproduction of unchanged PBP5 and high-level resistance of two *E. faecium* strains, EFM-1 and H80721 (MICs, 90 and 512 μg/ml) was related to different amino acid substitutions yielding low-affinity PBP5s that were synthesized in small quantities (42).

On the other hand, the synthesis of PBP5 in some strains of *E. hirae* and *E. faecium* was reported to be under the control of a repressor-encoding gene, *psr* (for PBP5 synthesis repressor), which is localized immediately upstream of the *pbp5* gene (18, 21, 32). *E. faecalis* JH2-2r (benzylpenicillin MIC, 75 μg/ml) was selected from *E. faecalis* JH2-2 by successive growth on BHI plates containing increasing concentrations of benzylpenicillin. This strain showed overproduction of PBP4, which is regarded as a low-affinity PBP. But, in their study, the cloning and sequencing of that *psr*-like gene from both *E. faecalis* JH2-2 and JH2-2r indicated that they were identical (4). No overproduction of PBPs, including PBP4, of the four imipenem-resistant strains in this study was observed. The beta-lactam affinities of PBP4 of these imipenem-resistant strains were lower than those of imipenem-susceptible strains, but the affinities of other PBPs did not change. The affinities of PBP4 of *E. faecalis* to ampicillin and imipenem were proportionally related to the MICs.

We showed that imipenem resistance in *E. faecalis* depends on low-affinity PBP4. Therefore, we focused on amino acid alterations of PBP4 regardless of a *psr* (or *psr*-like) gene in enterococci. Signoretto et al. have described the domain structure of the *E. faecalis* PBP5 as a membrane protein composed of 679 amino acids with three distinct modules. One is a penicillin-binding module (amino acids 350 to 679) located towards the C terminus, in which the three typical penicillin-binding motifs, SXXK, SDN, and KTG, were identified. The others are a non-penicillin-binding domain (amino acids 40 to 349) and an uncleaved N-terminal segment that acts as a membrane-spanning domain (amino acids 1 to 39) (34) (Fig. 1). Similar PBP arrangements could be seen in *E. faecium* and *E. hirae*, although the number of amino acids differs slightly (6, 19, 41). In *E. faecium* strains, it has been shown in earlier studies (10, 18, 19) that resistance to ampicillin or benzylpenicillin was associated with amino acid substitutions in the region between the active-site-defining motifs SDN and KTG of the penicillin-binding domain.

In our study, 605-Thr was changed to His in the insensitive strains (SVR 34 and SVR 138). Simultaneously, 520-Pro was replaced with Ser, and the same alteration at position 605 as in the insensitive strains occurred in the resistant strains (SVR 1110 and SVR 1119). The simultaneous occurrence of these two point mutations at positions 520 and 605 might be the cause of the high-level resistance to beta-lactam antibiotics. *E. faecalis* SEF96, which is a penicillin-susceptible strain, and JH2-2 have completely identical PBP4 protein sequences. Therefore, *E. faecalis* SEF96 was used as a reference strain.

Substitution of Ala for Val at position 369 in *E. faecalis* ATCC 29212 did not change susceptibility to ampicillin and imipenem. A point mutation between imipenem-susceptible VRE strains (SVR 250 and SVR 251) and *E. faecalis* ATCC 29212 produced almost no resistance to beta-lactams, either. Because all of these amino acid alterations occurred in the non-penicillin-binding module or outside the region between the active-site-defining motifs SDN and KTG, they were not likely to change the affinity of PBP4 and susceptibility of those strains.

We conclude that development of high-level resistance to beta-lactams such as imipenem and ampicillin depends on amino acid alterations of PBP4 at both positions 520 and 605. This is the first report regarding amino acid alterations of PBP4 in *E. faecalis* which are able to lower the affinities for and susceptibilities to beta-lactams.

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