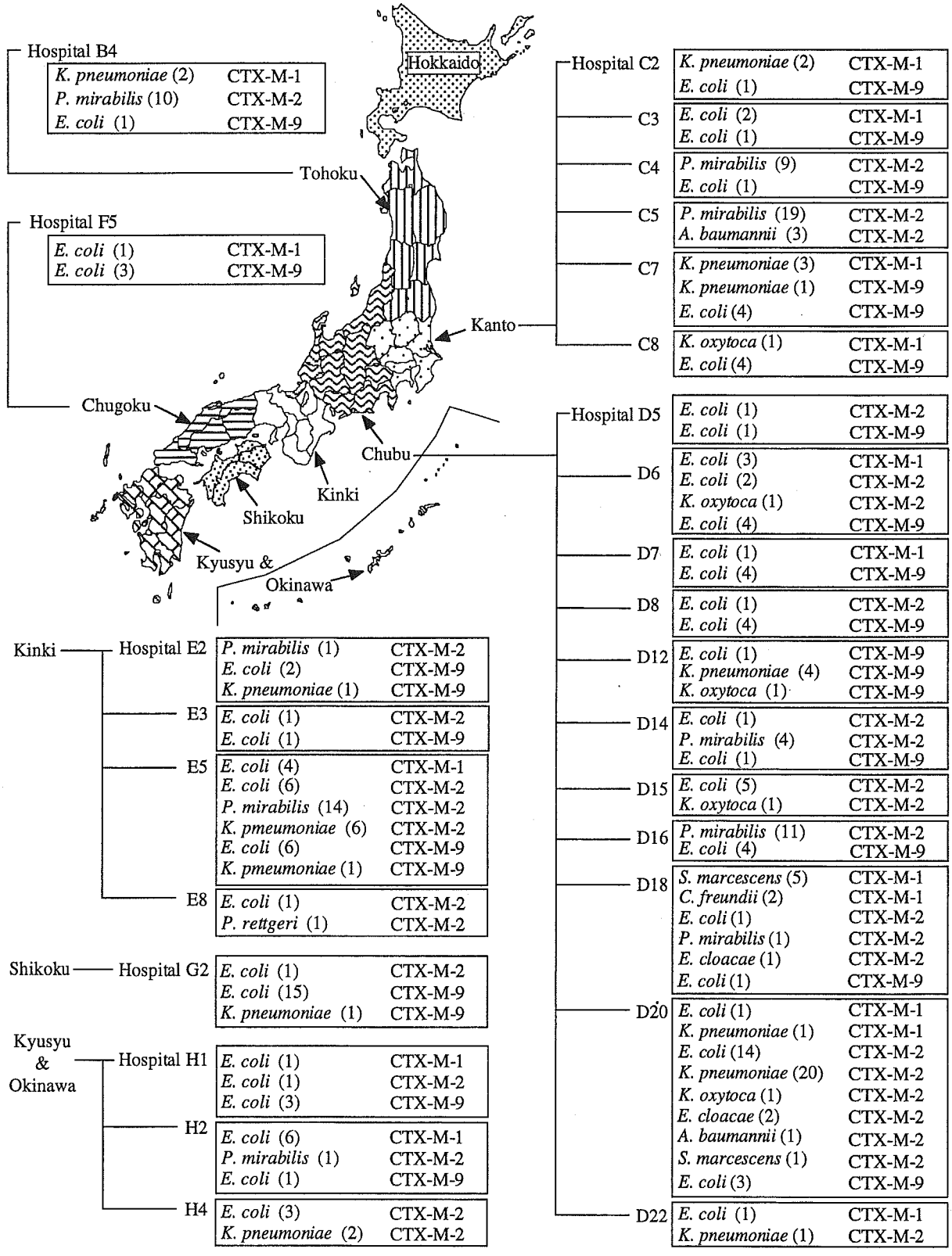

FIG. 1. Clinical facilities where multiple bla_{CTX-M} genes belonging to different genetic clusters were identified. Facilities where multiple bacterial species that bear bla_{CTX-M} genes were isolated are also added. The numbers in parentheses demonstrate the number of clinical isolates of each bacterial species.

all 71 *Proteus mirabilis* strains were identified as CTX-M-2 producers, and they were isolated in widely separate medical facilities located far apart in Japan, implying a close relatedness between CTX-M-2 and *P. mirabilis* in Japanese clinical environments. The plasmids carrying $bla_{CTX\text{-}M\text{-}2}$ may be very adaptive for *P. mirabilis*, which may either serve as a reservoir for plasmids carrying $bla_{CTX\text{-}M\text{-}2}$ gene (16, 17) or have preferentially accepted $bla_{CTX\text{-}M\text{-}2}$ genes from some environmental *Kluyvera* spp. (11, 20). Comparative analyses of plasmids that bear the $bla_{CTX\text{-}M\text{-}2}$ gene would provide a clue to elucidate the relatedness and origins of the plasmids.

The CTX-M-9 group of enzymes, including CTX-M-14, have so far been found worldwide in the species belonging to the family *Enterobacteriaceae* (7–9). However, almost all of the CTX-M-9 group of enzymes were found in *E. coli* in the present study, and some of them were suggested to be CTX-M-14. Precise analysis of the genetic environments mediating the $bla_{CTX\text{-}M\text{-}9}$ group of genes among these strains as well as their genome profiles would explain the presence of CTX-M-producing pandemic strains in Japan.

In conclusion, the aim of the present study was to make a rough estimate of the current status of CTX-M-type β-lactamases produced by nosocomial gram-negative bacilli isolated from Japanese medical facilities. The findings obtained imply that various plasmid-mediated genetic determinants for CTX-M-type β-lactamases have already been disseminated in Japanese clinical environments. Since CTX-M-2 was also identified in livestock (24), we must take special precautions against the further proliferation of gram-negative bacterial strains that harbor plasmids carrying genes for CTX-M-type β-lactamases, together with the other classes of plasmid-mediated β-lactamases, such as CMY-type cephamycinases and MBLs.

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Global Spread of Multiple Aminoglycoside Resistance Genes

Kunikazu Yamane,* Jun-ichi Wachino,* Yohei Doi,* Hiroshi Kurokawa,* and Yoshichika Arakawa*

Emergence of the newly identified 16S rRNA methylases RmtA, RmtB, and ArmA in pathogenic gram-negative bacilli has been a growing concern. ArmA, which had been identified exclusively in Europe, was also found in several gram-negative pathogenic bacilli isolated in Japan, suggesting global dissemination of hazardous multiple aminoglycoside resistance genes.

Multidrug-resistant gram-negative super microbes have been emerging worldwide. Since carbapenems and fluoroquinolones are the last resort against infections caused by gram-negative bacilli (1,2), the proliferation and dissemination of such clinical isolates that produce metallo-β-lactamases and acquire mutations in *gyrA* and *parC* genes have become a global threat (3,4). Aminoglycosides, including amikacin and tobramycin, are still potent agents for use against resistant bacilli. One of the most common resistance mechanisms against aminoglycosides is the production of aminoglycoside-modifying enzymes, such as aminoglycoside acetyltransferases, aminoglycoside phosphorylases, and aminoglycoside adenyltransferases (5), which are mainly mediated by transferable large plasmids.

Recently, a series of special methylases that protect microbial 16S rRNA, the main target of aminoglycosides, was identified in several nosocomial pathogens, including *Pseudomonas aeruginosa* (6), *Serratia marcescens* (7), and *Klebsiella pneumoniae* (8). The newly identified 16S rRNA methylases RmtA and RmtB were reported from Japan in 2003 and 2004, respectively (6,7). The gene for ArmA was initially sequenced in *Citrobacter freundii* isolated in Poland (GenBank accession no. AF550415) and later characterized in *K. pneumoniae* isolated in France in 2003 (8). In 2004, nosocomial spread of ArmA- or RmtB-producing *Escherichia coli* and *K. pneumoniae* was reported from Taiwan (9).

These enzymes are capable of conferring an extraordinary high level of resistance (MIC >512 mg/L) against most clinically important aminoglycosides as was observed among aminoglycoside-producing actinomycetes, suggesting their probable phylogenic relationship with the intrinsic 16S rRNA methylases of actinomycetes (Figure). RmtA shared 82% amino acid identity with RmtB, but the amino acid sequence similarities between 16S rRNA methylases isolated from pathogenic gram-negative microbes and those from aminoglycoside-producing actinomycetes were relatively low (≤33%). From analyses of the genetic environments of genes encoding 16S rRNA methylases, the *rmtA* gene is likely associated with the mercury-resistant transposon Tn*5041* (10); the *rmtB* gene was found in the flanking region of Tn*3*-like structure (7). The *armA* gene was found on a large plasmid which carries a type 1 integron (8) that mediates various gene cassettes responsible for multiple antimicrobial resistance. The structure of these genetic environments implied that the genes for these 16S rRNA methylases are mediated by mobile genetic elements carried by transferable large plasmids (7,8,10). In fact, the *rmtA* gene was transferred from *P. aeruginosa* strain AR-2 to an aminoglycoside-susceptible *P. aeruginosa* strain 105 by conjugation in vitro (6). The *rmtB* gene was also transferred from *S. marcescens* S-95 to *E. coli* by transformation (7). The *armA* gene was located on a composite transposon Tn*1548* (11).

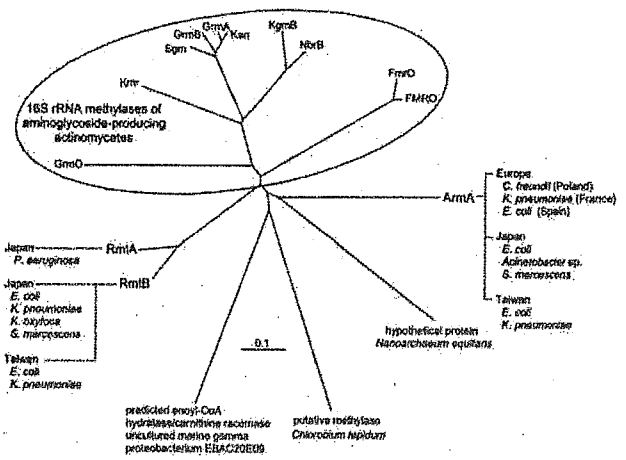

Figure. Phylogenic relationship among the 16S rRNA methylases. Each amino acid sequence was subjected to the analysis referred to the following sources: FmrO, accession no. JN0651; Kmr, accession no. AB164642; GrmA, accession no. M55520; GrmB, accession no. M55521; GrmO, accession no. AY524043; Kan, accession no. AJ414669; Sgm, accession no. A45282; KgmB, accession no. S60108; NbrB, accession no. AF038408; FMRO, Q08325; RmtA, (6); RmtB, (7); ArmA, (8); predicted enoyl-CoA hydratase/carnithine racemase of uncultured marine gamma proteobacterium EBAC20E09, accession no. AAS73112; putative methylase of *Chlorobium tepidum*, accession no. AAM72273; hypothetical protein of *Nanoarchaeum equitans*, accession no. AAR39385. The ClustalW program provided by the DNA Data Bank of Japan (http://www.ddbj.nig.ac.jp/search/clustalw-e.html) was used in this study.

*National Institute of Infectious Diseases, Tokyo, Japan

Thus, the growing concern was that these newly identified aminoglycoside-resistance genes could easily spread and be further disseminated among the glucose-nonfermentative gram-negative bacilli, including *P. aeruginosa* and *Acinetobacter* spp. and the genera belonging to the family *Enterobacteriaceae*.

The Study

We conducted a preliminary screening of the 16S rRNA methylase-producing bacilli on our gram-negative microbial stock of 2,877 strains isolated from Japanese hospitals within the past several years. Arbekacin, a semisynthetic aminoglycoside belonging to the kanamycin group, requires 2 modifications at the (6′) aminogroup and the (2″) hydroxyl group for inactivation, so this agent is not inactivated by known plasmid-mediated aminoglycoside-modifying enzymes. Therefore, a high-level arbekacin resistance (MIC >512 mg/L) was used as a marker for screening the 16S rRNA methylase-producing strains. All arbekacin-resistant strains were subjected to polymerase chain reaction (PCR) analysis to detect *rmtA*, *rmtB*, or *armA*, and all strains were PCR positive, except for a strain of *Acinetobacter* demonstrating a very high level of resistance to arbekacin (MIC 1,024 mg/L). This strain was later shown to produce both aminoglycoside 6′-acetyltransferase and 2″-adenyltransferase (12), so arbekacin was inactivated in this strain by both 6′-acetylation and 2″-adenylation. Each PCR primer set was used to detect *rmtA* and *rmtB* genes as in our previous reports (6,7). The PCR primers for amplification of *armA* were newly designed (forward: 5′-AGG TTG TTT CCA TTT CTG AG-3′, reverse: 5′-TCT CTT CCA TTC CCT TCT CC-3′), and the predicted size of the amplicon was 590 bp. These 3 sets of PCR primers were very reliable in detecting *rmtA*, *rmtB*, and *armA* genes, respectively. Each PCR amplicon was then subjected to sequencing analyses on both strands to confirm its nucleotide sequences for detecting mutations in the methylase genes.

As reported in our previous study, *rmtA* and *rmtB* genes had been found in *P. aeruginosa* isolates (6,10) and in 1 strain of *S. marcescens* (7), respectively. As shown in the Table, 5 *P. aeruginosa* strains isolated after our previous report (6) were *rmtA* positive. The *rmtB* gene was additionally identified in 4 *K. pneumoniae*, 2 *E. coli*, and 1 *K. oxytoca* strains in Japan. To our surprise, the *armA* gene, which had been found in various gram-negative microbial species belonging to the family *Enterobacteriaceae* exclusively in Europe as reported by Galimand et al. (13), was also identified in Japan in 1 strain each of *E. coli*, *S. marcescens*, and *Acinetobacter* sp. Notably, the *armA* and *rmtB* genes were also recently identified in *K. pneumoniae* and *E. coli* in Taiwan (9). Furthermore, the genetic environment of the *armA* gene found in *C. freundii* isolated in Poland was similar to that of *K. pneumoniae* isolated in France. The genetic environments of the *armA* gene found in the 3 Japanese microbial species, *E. coli*, *S. marcescens*, and *Acinetobacter* sp. (GenBank accession nos. AB116388 and AB117519), were also similar to those found in Europe (GenBank accession nos. AF550415 and AY220558). These findings suggest that the ArmA-producing gram-negative nosocomial microbes that harbor a very similar genetic environment carrying the *armA* gene have spread globally.

Conclusions

As described previously, arbekacin still shows a very broad antimicrobial spectrum from gram-positive to gram-negative nosocomial microbes and has been approved solely to treat methicillin-resistant *Staphylococcus aureus* (MRSA) infections in Japan since 1990 to ensure the prudent use of this agent. The emergence and presence of the 16S rRNA methylase-producing gram-negative bacilli,

Table. Methylase-producing strains of 16S rRNA identified after previous study (6)

Species and strain	Type	Year of isolation	Hospital	Prefecture
Pseudomonas aeruginosa P122	RmtA	2002	A	Aichi
P. aeruginosa P340	RmtA	2002	B	Gifu
P. aeruginosa 02-386	RmtA	2002	C	Saitama
P. aeruginosa 03-29	RmtA	2003	D	Aichi
P. aeruginosa 03-230	RmtA	2003	E	Shizuoka
Escherichia coli 01-139	RmtB	2001	H	Yamanashi
Klebsiella pneumoniae 01-140	RmtB	2001	H	Yamanashi
Klebsiella oxytoca 01-141	RmtB	2001	H	Yamanashi
K. pneumoniae 01-142	RmtB	2001	H	Yamanashi
E. coli C316	RmtB	2002	F	Hyogo
Serratia marcescens S95	RmtB	2002	G	Kohchi
K. pneumoniae 03-252	RmtB	2003	H	Yamanashi
K. pneumoniae 03-518	RmtB	2003	H	Yamanashi
E. coli C316-2	ArmA	2003	F	Hyogo
S. marcescens ARS8	ArmA	2003	I	Tochigi
Acinetobacter sp. ARS6	ArmA	2003	J	Kanagawa

however, has not been well recognized in Japan to date; arbekacin has not been listed among the antimicrobial agents for daily antimicrobial susceptibility testing of gram-negative microbes.

The use of semisynthetic aminoglycosides, including arbekacin, in Japanese clinical settings for >10 years may have promoted the emergence and dissemination of the 16S rRNA methylase-producing gram-negative microbes in Japan. The large amount of various aminoglycosides used in livestock-farming environments could have also been a selective pressure for the emergence and spread of pathogenic microbes that harbor genetic determinants for the newly identified 16S rRNA methylases, as exemplified by recent isolation of ArmA-producing *E. coli* from swine in Spain (GenBank accession no. AY522431).

Since acquisition of multidrug resistance against clinically important antimicrobial agents such as carbapenems and fluoroquinolones has been developing rapidly worldwide, the acceleration of even greater aminoglycoside resistance among gram-negative bacilli promises to become an actual clinical concern in the near future, just as vancomycin-resistant enterococci (VRE) did in the 1990s (14). The emergence of gram-positive cocci including MRSA and VRE that acquire the 16S rRNA methylase could also be a grave clinical matter, although fortunately no such hazardous microbes have been identified. Thus, steps must be taken to block further proliferation of these multidrug-resistant gram-negative super microbes, including *P. aeruginosa*, *K. pneumoniae*, and *Acinetobacter* spp., as well as multidrug-resistant cocci such as MRSA and VRE, which have acquired an extraordinarily high level of resistance to various aminoglycosides through production of 16S rRNA methylases, especially in clinical environments.

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Dr. Yamane, a physician with background in intensive care and emergency medicine, is currently working at National Institute of Infectious Diseases, Japan. His current research interest is molecular mechanisms of antimicrobial resistance in nosocomial bacteria.

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Address for correspondence: Yoshichika Arakawa, Department of Bacterial Pathogenesis and Infection Control, National Institute of Infectious Diseases, 4-7-1 Gakuen, Musashi-Murayama, Tokyo 208-0011, Japan; fax: 81-42-461-7173; email: yarakawa@nih.go.jp

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Genetic Analysis of Transfer-Related Regions of the Vancomycin Resistance *Enterococcus* Conjugative Plasmid pHTβ: Identification of *oriT* and a Putative Relaxase Gene†

Haruyoshi Tomita[1]* and Yasuyoshi Ike[1,2]

Department of Bacteriology and Bacterial Infection Control, Gunma University Graduate School of Medicine, Maebashi, Gunma 371-8511, Japan,[1] and Laboratory of Bacterial Drug Resistance, Gunma University Graduate School of Medicine, Maebashi, Gunma 371-8511, Japan[2]

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The pHT plasmids pHTα (65.9 kbp), pHTβ (63.7 kbp), and pHTγ (66.5 kbp) are highly conjugative pheromone-independent pMG1-like plasmids that carry Tn*1546*-like transposons encoding vancomycin resistance. pHTβ is the prototype plasmid, and the pHTα and pHTγ plasmids are derivatives of the insertion into pHTβ of an IS*232*-like (2.2 kbp) element and a group II intron (2.8 kbp), respectively. The complete nucleotide sequence of the pHTβ plasmid was determined and, with the exception of the Tn*1546*-like insertion (10,851 bp), was found to be 52,890 bp. Sixty-one open reading frames (ORFs) having the same transcript orientation were identified. A homology search revealed that 22 of the pHTβ (pHT) plasmid ORFs showed similarities to the ORFs identified on the pXO2 plasmid (96.2 kbp), which is the virulence plasmid essential for capsule formation by *Bacillus anthracis*; however, the functions of most of the ORFs remain unknown. Most other ORFs did not show any significant homology to reported genes for which functions have been analyzed. To investigate the highly efficient transfer mechanism of the pHT plasmid, mutations with 174 unique insertions of transposon Tn*917-lac* insertion mutants of pHTβ were obtained. Of the 174 derivatives, 92 showed decrease or loss in transfer frequency, and 74 showed normal transfer frequency and LacZ expression. Eight derivatives showed normal transfer and no LacZ expression. Inserts within the 174 derivatives were mapped to 124 different sites on pHTβ. The Tn*917-lac* insertions which resulted in altered transfer frequency mapped to three separate regions designated I, II, and III, which were separated by segments in which insertions of Tn*917-lac* did not affect transfer. There was no region homologous to the previously reported *oriT* sequences in the pHT plasmid. The *oriT* was cloned by selection for the ability to mobilize the vector plasmid pAM401. The *oriT* region resided in a noncoding region (192 bp) between ORF31 and ORF32 and contained three direct repeat sequences and two inverted repeat sequences. ORF34, encoding a 506-amino-acid protein which was located downstream of the *oriT* region, contains the three conserved motifs (I to III) of the DNA relaxase/nickase of mobile plasmids. The transfer abilities of the Tn*917-lac*-insertion mutants of ORF34 or a mutant of ORF34 with an in-frame motif III deletion were completely abolished. The sequence of the *oriT* region and the deduced relaxase/nickase protein of ORF34 showed no significant similarity to the *oriT* and relaxase/nickase of other conjugative plasmids, respectively. The putative relaxase/nickase protein of ORF34 could be classified as a new member of the MOB_{MG} family.

The isolation of vancomycin-resistant enterococci (VRE) was first reported in 1988 in the United Kingdom (43) and France (25), and shortly thereafter, VRE were detected in hospitals in the United States (34). Since then, VRE have emerged with unanticipated rapidity and are now encountered in most hospitals, especially in the United States (28).

Increased drug resistance is linked to direct selective pressure by the use of antibiotics and often the presence of a genetic transfer system for spread of resistance (5). Most VRE clinical isolates are *Enterococcus faecium* strains (28). Little is known about systems of efficient plasmid transfer in *E. faecium*. Previously, we described the isolation of the pheromone-independent gentamicin resistance conjugative plasmid pMG1 (65.1 kbp) from an *E. faecium* clinical strain in Japan, which was the first report describing efficient plasmid transfer in *E. faecium* (21). pMG1 transfers among enterococcus strains during broth mating at a frequency of about 10^{-4} per donor strain. Southern hybridization analysis revealed no similarity to other gram-positive conjugative plasmids, and pMG1 was categorized as a new type of conjugative plasmid. Our epidemiological study revealed that pMG1-like plasmids are widely disseminated in vancomycin-resistant *E. faecium* clinical isolates from a hospital in the United States, suggesting that pMG1-like plasmids may contribute to the efficient dissemination of vancomycin resistance in enterococcus strains (41).

Recently, we reported the isolation of pMG1-like vancomycin resistance pHT plasmids from clinical *Enterococcus faecium* and *Enterococcus avium* strains in Japan (42). pHT plasmids, including pHTα (65.9 kbp), pHTβ (63.7 kbp), and pHTγ (66.5 kbp), are highly conjugative plasmids carrying Tn*1546*-like transposons (3) that encode vancomycin resistance (VanA). The pHT plasmids are related to the gentamicin resistance conjugative plasmid pMG1 with respect to DNA hy-

* Corresponding author. Mailing address: Bacteriology and Bacterial Infection Control, Gunma University Graduate School of Medicine, Maebashi, Gunma 371-8511, Japan. Phone: (81) 27 220 7992. Fax: (81) 27 220 7996. E-mail: tomitaha@med.gunma-u.ac.jp.

† Supplemental material for this article may be found at http://jb.asm.org/.

TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant features	Reference
Strains		
E. faecalis		
FA2-2	*rif fus*	12
JH2SS	*spc str*	37
UV202	*fir fus*, recombination-deficient mutant of JH2-2	46
OG1X	*str*	20
OG1RF	*rif fus*	8
OG1SS	*spc str*	12
E. faecium		
BM4105RF	*rif fus*	21
BM4105SS	*spc str*	21
E. coli DH5α	*endA1 recA1 gyrA96 thi-1 hsdR17 supE44 relA1* Δ(*argE-lacZYA*)*U169*	Bethesda Research Laboratories
Plasmids		
pHTα	pHTβ carrying IS*232*-like element	42
pHTβ	pMG1-like vancomycin resistance (Tn*1546*) conjugative plasmid	42
pHTγ	pHTβ carring group II intron	42
pAM401	*E. coli-E. faecalis* shuttle; *cat tet*	45
pTV32Ts	Transposon delivery vector, temperature sensitive; pE194Ts(Cm^r)::Tn*917-lac*(Em^r)	31
pMW119	*E. coli* cloning vector; Amp^r	Nippon Gene
pBluescript SKII(+)	*E. coli* cloning vector; Amp^r	Stratagene
pACYC184	*E. coli* cloning vector; Tc^r Cm^r	New England Biolabs
pHTβ/ORF34del	pHTβ derivative mutant carrying a deletion of motif III of ORF34 (Tral)	This study

bridization, and they are thought to contain the same efficient conjugation system. The transfer gene *traA*, which is involved in the formation of mating aggregates, is conserved in all pMG1-like plasmids (36, 42).

In this report, sequence comparisons and genetic analysis of pHTβ obtained by generating Tn*917-lac* insertion mutants led to identification of a novel type of *oriT* region and a putative relaxase/nickase gene designated *traI*.

MATERIALS AND METHODS

Bacterial strains, plasmids, oligonucleotides, media, and reagents. The bacterial strains, plasmids, and oligonucleotides used in this study are listed in Table 1 (and see Table S6 in the supplemental material). *E. faecalis* strains were grown in Todd-Hewitt broth (THB) (Difco Laboratories) at 37°C. The following antibiotic concentrations were used for selection of *E. faecalis*: erythromycin, 12.5 μg ml^{-1}; streptomycin, 250 μg ml^{-1}; kanamycin, 250 μg ml^{-1}; spectinomycin, 250 μg ml^{-1}; chloramphenical, 20 μg ml^{-1}; rifampin, 25 μg ml^{-1}; and fusidic acid, 25 μg ml^{-1}. Antibiotic concentrations for selection of *Escherichia coli* were as follows: ampicillin, 100 μg ml^{-1}; kanamycin, 40 μg ml^{-1}; chloramphenicol, 50 μg ml^{-1}; and spectinomycin, 50 μg ml^{-1}. All antibiotics were obtained from Sigma Chemical Co. X-Gal (5-bromo-4-chloro-3 indolyl-β-D-galactopyranoside) was used at 40 μg ml^{-1}.

Plasmid/DNA methodology. Recombinant DNA methodology, analyses of plasmid DNA with restriction enzymes, and agarose gel electrophoresis were carried out by standard methods (35). Introduction of plasmid DNA into bacterial cells was by electrotransformation, as described previously (13). Plasmid DNA was purified from *E. faecalis* as previously described (44). Restriction enzymes were purchased from New England Biolabs and Roche Co. The PCR was performed with a Perkin-Elmer Cetus apparatus. Specific primers were purchased from Invitrogen, and *Taq* DNA polymerase was obtained from Takara.

Construction of the clone sets of the pHTβ plasmid. For determination of the physical map of the pHTβ and for DNA sequence analysis, the clone sets of the pHTβ were constructed as previously described (14, 39, 40). The partially HindIII-digested fragments of pHTβ plasmid were cloned into vector plasmid pMW119. Plasmid pACYC184 was also used for cloning of the partially BclI-digested pHTβ plasmid DNA.

DNA sequence analysis. Sequence analysis was performed using the Dye primer and Dye terminator cycle sequencing kit (Applied Biosystems) and a 377 DNA Sequencer and 310 Gene Analyzer (ABI PRISM). To determine the DNA sequence of the pHTβ plasmid, a GPS in vitro transposition system kit (New England BioLabs) and shotgun cloning method were used (35). To determine the DNA sequences in the gap regions, PCR amplification was performed (LA-Taq; Takara, Japan) to obtain PCR products covering the gaps. The PCR products were sequenced directly using custom primers. Open reading frames (ORFs) were identified and initially analyzed using Genetyx version 5.1 computer software and the BLAST (1) database to search for putative genes.

Conjugation experiments. Filter matings and solid surface matings were performed as previously described (7, 20). Broth matings (in THB) were for 4 h. Transfer frequencies are expressed as the number of transconjugants per donor cell (at the end of mating).

Identification and genetic analyses of the *oriT* region of the pHT plasmid. Various segments of pHTβ containing sequences that were related to the consensus *oriT* sequence of IncP or IncQ plasmids were amplified by PCR (2, 22). Segments containing inverted repeat (IR) or direct repeat (DR) sequences were also amplified. The amplified DNAs were cloned into the pAM401 vector plasmid. Two clones, pAM401::119/113 and pAM401::119/96, were constructed as chimeric plasmids between pAM401 and the clones pMW119::113 and pMW119::96, respectively (see Fig. 1 and see Table S6 in the supplemental material). Each of the pAM401 derivatives carrying pHTβ segments to be tested for *oriT* activity was introduced by electrotransformation into *Enterococcus faecalis* UV202, which is defective in homologous recombination (46). Then, the conjugative plasmid pHTβ was introduced into each of the UV202 transformants carrying the pAM401 derivative (Cm^r) by conjugation. Both broth matings and filter matings were performed using the transconjugants carrying the two plasmids as donor strains and JH2SS as the recipient strain.

Transposon mutagenesis and isolation of pHTβ derivatives with Tn*917-lac* insertions. The transposon delivery vector pTV32Ts (31) was used for mutagenesis (19, 44). Strain OG1X/pTV32Ts was originally constructed by protoplast formation (45), and plasmid pHTβ was introduced into this strain by conjugation. The isolation of pHTβ::Tn*917-lac* insertion mutants was performed as previously described (44). The locations of transposon inserts in the pHTβ plasmid were determined by PCR amplification and DNA sequencing, using primers that amplify the segment containing the junction of the inserts.

Construction of the ORF34 in-frame mutant. The overlapping PCR technique was used to construct the ORF34 deletion mutant. The internal region of

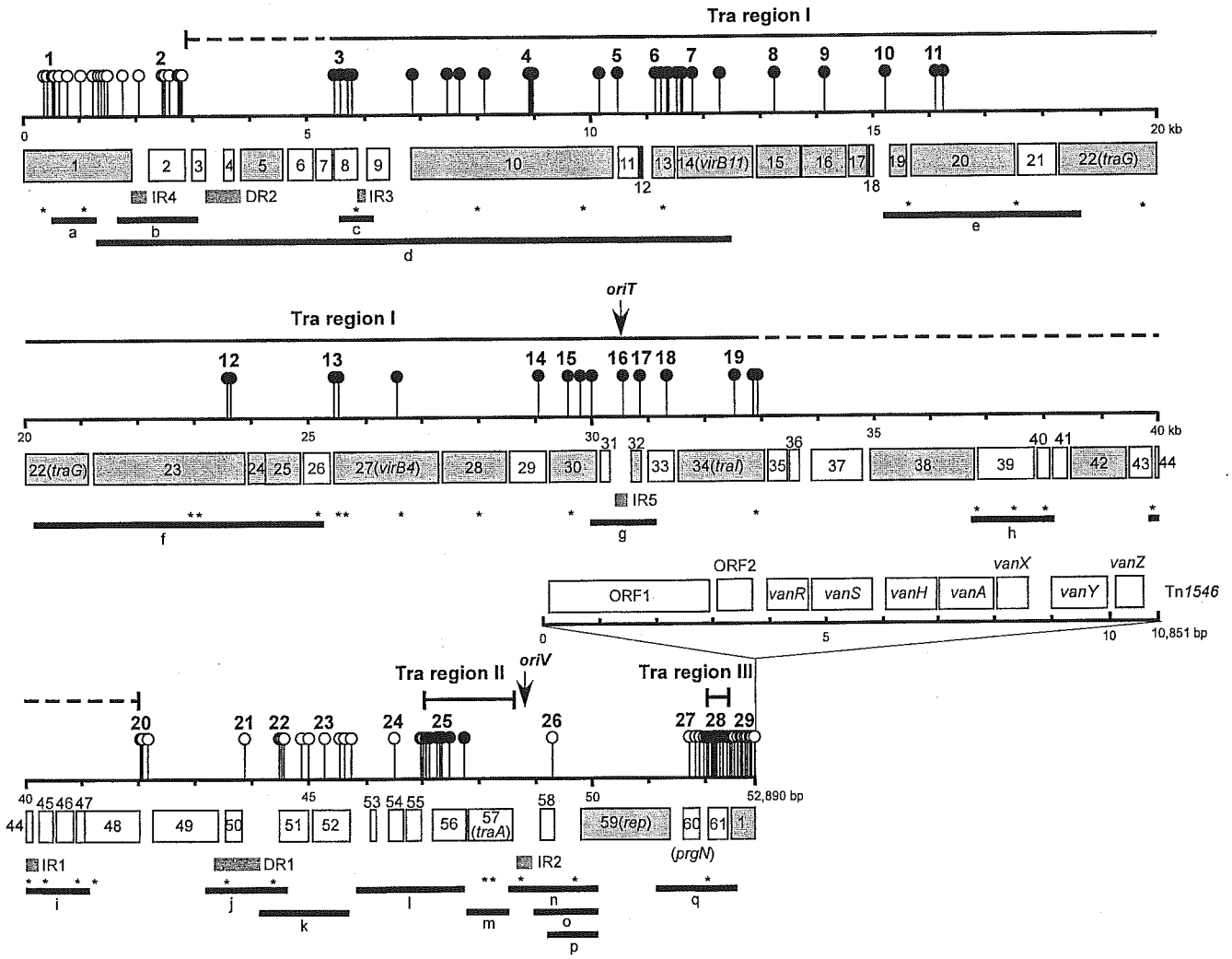

FIG. 1. Genetic map and ORFs deduced from the complete plasmid sequence of pHTβ plasmid. Boxes indicate ORFs identified on the pHT plasmid. The locations of the Tn*917-lac* insertions of pHTβ (vertical bar with circular head) are shown on the map. Each head color shows the transfer frequency of the derivatives (white; transfer frequency the same as parent plasmid, black; transfer frequency less than one-fifth of the frequency of the parent plasmid or no transfer by broth mating). The number on the insert (1 through 29) indicates the representative plasmids of the derivatives, which are also shown in Fig. 2 and Table 3. Horizontal bars designated as Tra regions I, II, and III on the map indicate the transfer-related regions. DR and IR indicate the direct repeat sequences and inverted repeat sequences. The asterisks under the map indicate the sequence resembling the consensus *oriT* sites of IncP, IncQ, or *oriT* of the F plasmid. Black horizontal bars designated a through q under the map show the cloned DNA segments used for the identification of the *oriT* region of the pHT plasmid. The clones are as follows: a, pAM401::ORF1; b, pAM401::IR4; c, pAM401::IR3; d, pAM401::119/113; e, pAM401::ORF19/21; f, pAM401::119/96; g, pAM401::IR5-1; h, pAM401::ORF39/40; i, pAM401::IR1; j, pAM401::DR1; k, pAM401::ORF51/52; l, pAM401::ORF53/56; m, pAM401::traA; n, pAM401::IR2; o, pAM401::ORF58; p, pAM401::rep5′; and q, pAM401::ORF60/61.

ORF34, which contained the 30-bp deletion corresponding to 10 amino acid residues (HRNTEHIHIH) of motif III, was amplified using the specific primer set (see Fig. 5 and see Table S6 in the supplemental material). The amplified DNA was cloned into the pBluescript vector plasmid, resulting in pBS::ORF34del. The 1.1-kbp DNA fragment amplified by PCR, which carries a spectinomycin resistance gene, *aad(9)*, was cloned into pBS::ORF34del, to give pBS::ORF34del-Spc (24). pBS::ORF34del-Spc was introduced into *E. faecalis* FA2-2/pHTβ by electrotransformation, and recombinants occurring via double homologous recombination were selected as previously described (38). A representative recombinant carrying the 30-bp deletion of motif III of ORF34, the deduced relaxase gene, was designated as pHTβ/ORF34del.

Nucleotide sequence accession number. The complete nucleotide sequence of the pHTβ plasmid has been deposited in DDBJ/EMBL/GenBank under accession no. AB183714.

RESULTS AND DISCUSSION

DNA sequence and gene organization of the pHT plasmid. The total size of the pHTβ plasmid, excluding the Tn*1546*-like insertion, was 52,890 bp. The nucleotide sequence of the Tn*1546*-like element inserted in the pHTβ plasmid was almost identical to that of the prototype Tn*1546* (10,851 bp). There was no amino acid substitution of the VanS protein found in Japanese VRE isolates that show low-level teicoplanin resistance (17). The G-C content of the core region of the pHT plasmid excluding the Tn*1546*-like transposon was 31.35, and that of the Tn*1546*-like region was 38.66. The Tn*1546*-like

TABLE 2. Open reading frames identified in pHT plasmids[a]

ORF	5′ end	3′ end	Amino acid size	Identification [organism]	Protein family/domain	% Identity
1	52467	1946	787	TrsI (TraI) [*L. lactis*]/pXO2-77/76 [*B. anthracis*]	DNA topoisomerase I/III	34/29
2	2264	2863	199	AbiQ [*L. lactis*]	Phage abortive infection mechanism	19
3	2984	3199	71	Hypothetical protein		
4	3515	3721	68	Hypothetical protein		
5	3801	4655	284	ParA [*E. faecalis*; pTEF3]/pXO2-39 [*B. anthracis*]	Replication-related protein	29/27
6	4668	5105	145	YvfU [*B. sutilis*]	Two-component sensor histidine kinase	19
7	5123	5422	99	Hypothetical protein		
8	5425	5868	147	Hypothetical protein		
9	6014	6337	107	Hypothetical protein		
10	6755	10384	1,209	pXO2-28 [*B. anthracis*]	Putative cell surfrace protein	10
11	10444	10806	120	Hypothetical protein		
12	10820	10972	50	Hypothetical protein		
13	11079	11462	127	pXO2-26 [*B. anthracis*]		26
14	11474	12889	471	pXO2-25 [*B. anthracis*]	VirB11 family, secretory protein	40
15	12927	13721	264	pXO2-24/23 [*B. anthracis*]		29/12
16	13721	14518	265	pXO2-22 [*B. anthracis*]		43
17	14536	14898	120	pXO2-21 [*B. anthracis*]		51
18	14912	15058	48	Hypothetical protein		
19	15310	15585	91	pXO2-18 [*B. anthracis*]		47
20	15694	17520	608	pXO2-17 [*B. anthracis*]	Chromosome segregation ATPases	10
21	17533	18249	238	Hypothetical protein		
22	18262	21120	952	VirD4 [*E. faecium*]/pXO2-16/15 [*B. anthracis*]	VirD4/TraG/TraD family (coupling protein)	33/33/10
23	21138	23912	924	AidA [*E. faecium*]/pXO2-14 [*B. anthracis*]	Amino acid transporters	71/25
24	23960	24271	103	pXO2-11 [*B. anthracis*]		35
25	24268	24885	205	pXO2-10 [*B. anthracis*]		35
26	24902	25369	155	Hypothetical protein		
27	25385	27340	651	TrsE (TraE) [*L. lactis*; pMRC01] pXO2-09 [*B. anthracis*]	VirB4 family (transfer complex protein)	22/48
28	27362	28498	378	ORF14 [*E. faecalis*; Tn*916*]/pXO2-08 [*B. anthracis*]	NLP/P60 family, cell wall-associated hydrolase	32/43
29	28512	29162	216	Hypothetical protein		
30	29176	30072	298	pXO2-05 [*B. anthracis*]		25
31	30108	30338	76	Hypothetical protein		
32	30689	30985	98	pXO2-04 [*B. anthracis*]	ABC transport system, permease component	22
33	30982	31449	155	Hypothetical protein		
34	31532	33052	506	pXO2-84 [*B. anthracis*]	Three motifs conserved in DNA relaxase/nickase	21
35	33074	33403	109	Hypothetical protein		
36	33405	33668	87	Hypothetical protein		
37	33890	34756	288	HlyD [*E.. coli*]	Hemolysin secretion protein	11
38	34911	36737	608	LtrC [*L. lactis*; pMRC01]/pXO2-81 [*B. anthracis*]	Catalytic active site (HEXXH)	15/23
39	36799	37815	338	Hypothetical protein	ABC transpoter	
40	37824	38081	85	Hypothetical protein		
41	38122	38412	96	Hemolysin-related protein [*Thermotoga maritima*]		21
42	38437	39441	334	LtrC-like protein [*S. epidermidis*]/pXO2-78 [*B. anthracis*]		26/34
43	39532	39822	96	Hypothetical protein		
44	39984	40175	63	Hypothetical protein		
45	40284	40502	72	Hypothetical protein		
46	40539	40856	105	Hypothetical protein		
47	40867	41064	65	Hypothetical protein		
48	41061	42029	322	Hypothetical protein	Predicted ATPase	
49	42255	43442	395	Hypothetical protein		
50	43553	43846	97	Hypothetical protein		
51	44498	44905	135	Prophage ps3 protein 15 [*L. lactis*]	Cro/CI family, transcriptional regulator	21
52	45057	45668	203	PinR [*E.. coli*; lambdoid prophage Rac]	Site-specific recombinases, DNA invertase	33
53	46046	46219	57	Hypothetical protein		
54	46387	46695	102	Hypothetical protein		
55	46707	47024	105	Hypothetical protein		
56	47191	47754	187	71ORF1 [*E. faecium*; pMG1]		100
57	47765	48625	286	*traA* [*E. faecium*; pMG1]	Related to formation of mating aggregate	98
58	49122	49376	84	Hypothetical protein		
59	49773	51374	533	RepS [*E. faecalis*; V583]/pXO2-38 [*B. anthracis*]	Replication protein	34/27
60	51608	51907	99	PrgN [*E. faecalis*; pCF10]	Replication and negative control of conjugation	38
61	52041	52418	125	Hypothetical protein		

[a] The colums list open reading frame number, 5′ end of the ORF, 3′ end of the ORF, deduced amino acid size, identification of similar proteins and organisms, functional protein family or conserved domain, and percentage identity of the pHT plasmid ORF to the matching protein(s).

transposon is inserted at the same location in the three pHT plasmids α, β, and γ. The hexa-oligonucleotide sequence 5′-G ATTAT-3′ was duplicated at the junctions. These results suggested that the pHT plasmid resulted from the transposition of the Tn*1546*-like element into an original plasmid without a drug resistance determinant. Sixty-one open reading frames were identified in the 52,884-bp core region of the pHT plasmid, excluding the Tn*1546*-like element and the 6-bp repeat (i.e., 5′-GATTAT-3′), as shown in Fig. 1 and as listed in Table 2. All the ORFs were transcribed in the same direction on the plasmid, with the exception of ORF1 of Tn*1546*-like transposon (Fig. 1). The nucleotide next to the right end of the Tn*1546*-like transposon was designated as the first base pair of the plasmid. Transcription of the genes encoded on the pHTβ plasmid was in a counterclockwise direction from the physical map described in our previous report (42).

A homology search of 61 ORFs encoded on the pHT plasmid was performed by BLAST against the protein databases, and the results are shown in Table 2. Only six ORFs (ORF1, -14, -22, -27, -57, and -60) showed significant similarity to transfer-related genes of other plasmids (Fig. 1) (16). Twenty-two ORFs that are located between ORF1 and -42 and ORF59 (gray-colored ORFs shown in Fig. 1) had significant degrees of similarity to the ORFs encoded on the *Bacillus anthracis* virulence plasmid pXO2 (96,231 bp; accession no. NC_002146; 94,829 bp, accession no. NC_003981) (15, 32). The pXO2 plasmid carries capsule genes and is necessary to cause the disease anthrax (27). The degree of identity between the deduced amino acid residues of the homologous ORFs ranged from about 10 to 50% (Table 2).

Although most of the functions of the homologues are not known, the putative functions of several proteins were assigned based on their similarity to other well-characterized proteins (Table 2). ORF1 encoded 787 amino acid residues, but the Tn*1546*-like insert at the 142nd amino acid residue (Trp) separated ORF1 into an N-terminal 142-amino-acid residue portion and a C-terminal 645-amino-acid residue portion. The intact protein was related (about 40% identity) to the *trsI* gene carried on the *Lactococcus lactis* bacteriocin plasmid pMRC01 (60.2 kbp) (6). TrsI belongs to the DNA topoisomerase family, which is frequently found in the transfer-related region of conjugative plasmids (26). Three pHT plasmids, α, β, and γ, that carried Tn*1546*-like insertions within ORF1 could transfer at high frequencies. This suggested that the topoisomerase homologue (ORF1) was not essential for plasmid transfer.

The sequence comparisons of the ORFs on the pHT plasmid suggested that a relatively large portion of the plasmid from ORF57 through ORF61 and from ORF1 through ORF28 (a region spanning about 33 kb) could be associated with the transfer region of the pHT plasmid (Fig. 1). However, many pHT ORFs did not have significant homology with any reported proteins, and the plasmid was therefore categorized as a new type of conjugative plasmid, as shown by our previous genetic analysis (21). There are reports describing highly efficient self-transferable large plasmids in *Bacillus* species and the mobilization of the anthrax toxin plasmid pXO1 (181.7 kbp) and pXO2 (4, 29, 33). The pMG1-like plasmids, including the pHT plasmids, could be closely related to these efficient conjugative plasmids.

Analysis of Tn*917-lac* transposon insertion mutants of pHTβ plasmid. For genetic analysis of the transfer system of pHT plasmids, we isolated mutants which altered the transferability of the pHTβ plasmid. Transposon-insertional mutagenesis of pHTβ plasmid using Tn*917-lac* (Em^r) was performed, and 1,000 independent insertion derivatives were obtained. Each derivative was examined for transferability in broth mating and LacZ expression in OG1X harboring the plasmid on THB plates containing X-Gal reagent. A total of 352 derivatives which showed altered transferability or expressed LacZ activity were chosen for further analysis. Agarose gel electrophoresis analysis of NdeI-digested plasmid DNA showed that 289 of 352 derivatives had a single insertion of Tn*917-lac* with no deletion or recombination. Of the 289 derivatives carrying a single insertion, the location of each insertion in 174 representative derivatives was determined by DNA sequencing and mapped on pHTβ, excluding the Tn*1546*-like region (Fig. 1). Of 174 derivatives, 92 showed altered transfer frequency and 74 showed normal transfer and positive LacZ expression. Eight derivatives showed normal transfer and no LacZ expression after repeated examinations. There were hot spots for Tn*917-lac* insertion on pHTβ plasmid, and the inserts were mapped to 124 different sites within the pHTβ plasmid. The locations of the Tn*917-lac* insertions into the ORFs within the 124 derivatives are shown in Fig. 1. The transfer frequency of each of the 124 representative derivatives was examined in broth mating. The mating experiment was repeated using different hosts: *E. faecalis* FA2-2 and JH2SS, *E. faecalis* OG1RF and OG1SS, or *E. faecium* BM4105RF and BM4105SS, respectively. Ninety-two derivatives showed an altered transfer frequency, which was either a reduced transfer frequency or an inability to transfer at a frequency of greater than 10^{-7} per donor cell. Some representative plasmids and the results of the mating experiments are shown in Table 3.

Inserts that decreased in the transfer frequency of pHTβ were mapped within ORFs in three separate regions designated I, II, and III (Fig. 1). Region I could span a relatively large portion of the plasmid totaling 39.3 kb lying between 2.8 kbp and 42.1 kbp and contained 46 ORFs from ORF3 to ORF48. The precise borders of region I were not defined, since the insertions within the ORFs between ORF3 and ORF7 or between ORF35 and ORF48 could not be obtained (Fig. 1). Region II spanned a small portion of 1.7 kb between 47.0 kb and 48.7 kb and contained ORF56 and ORF57, and region III consisted of ORF61 located between 52.0 kb and 52.4 kb. Of the 22 ORFs on pHTβ which showed homology with the ORFs on the pXO2 plasmid of *B. anthracis*, 20 were in region I. Inserts in 11 of these 20 ORFs and insertion within the noncoding region upstream of ORF19 were obtained, and all of these inserts resulted in decreased transfer frequency.

Inserts in ORF56 of region II resulted in the inability to transfer in broth mating. ORF57 downstream of region II was almost identical to *traA* of pMG1, which is involved in the formation or stability of mating aggregate and is expressed in the early stage of mating (21, 36, 42). Although an insert in pHTβ ORF57 has not been isolated, insertion into *traA* of pMG1 resulted in the inability to transfer in broth mating (36). Insertion into ORF61 of region III resulted in a reduced transfer frequency. The predicted amino acid sequence of ORF60, which lies upstream of ORF61, was homologous with PrgN of

TABLE 3. Transfer of pHTβ::Tn*917-lac* derivatives

No. in Fig. 1 and 2	Representative plasmid	Location of Tn*917-lac* insertion	Position of insertion (bp)	Frequency of transfer (broth mating)[a]	Relative transfer frequency (broth mating)
Wild type	pHTβ	Wild type	Wild type	1.1×10^{-4}	100
1	pHTβ::Tn*917-lac*/315	ORF1 (C terminal)	492	1.9×10^{-4}	172
2	pHTβ::Tn*917-lac*/261	ORF2	2445	2.8×10^{-4}	254
3	pHTβ::Tn*917-lac*/268	ORF8	5589	$<1.1 \times 10^{-7}$	<0.1
4	pHTβ::Tn*917-lac*/136	ORF10	8937	4.2×10^{-7}	0.4
5	pHTβ::Tn*917-lac*/55	ORF10/ORF11	10425	$<2.0 \times 10^{-7}$	<0.2
6	pHTβ::Tn*917-lac*/154	ORF13	11171	$<2.1 \times 10^{-7}$	<0.2
7	pHTβ::Tn*917-lac*/60	ORF14	11798	$<1.8 \times 10^{-7}$	<0.2
8	pHTβ::Tn*917-lac*/148	ORF15	13221	$<1.5 \times 10^{-7}$	<0.2
9	pHTβ::Tn*917-lac*/328	ORF16	14123	$<1.5 \times 10^{-7}$	<0.2
10	pHTβ::Tn*917-lac*/331	ORF18/ORF19	15259	$<1.7 \times 10^{-7}$	<0.2
11	pHTβ::Tn*917-lac*/57	ORF20	16119	$<1.5 \times 10^{-7}$	<0.2
12	pHTβ::Tn*917-lac*/274	ORF23	23643	7.3×10^{-7}	0.7
13	pHTβ::Tn*917-lac*/230	ORF27	25416	$<2.0 \times 10^{-7}$	<0.2
14	pHTβ::Tn*917-lac*/321	ORF29	29049	$<2.2 \times 10^{-7}$	<0.2
15	pHTβ::Tn*917-lac*/142	ORF30	29612	$<1.7 \times 10^{-7}$	<0.2
16	pHTβ::Tn*917-lac*/323	ORF31/ORF32 (*oriT*)	30573	$<1.5 \times 10^{-7}$	<0.2
17	pHTβ::Tn*917-lac*/275	ORF32	30821	$<1.3 \times 10^{-7}$	<0.2
18	pHTβ::Tn*917-lac*/278	ORF33	31343	$<1.9 \times 10^{-7}$	<0.2
19	pHTβ::Tn*917-lac*/95	ORF34	32626	$<1.5 \times 10^{-7}$	<0.2
20	pHTβ::Tn*917-lac*/250	ORF48/49	42073	8.5×10^{-5}	77
21	pHTβ::Tn*917-lac*/203	ORF50/51	43864	9.1×10^{-5}	83
22	pHTβ::Tn*917-lac*/22	ORF51	44414	1.4×10^{-4}	127
23	pHTβ::Tn*917-lac*/309	ORF52	45297	9.5×10^{-5}	86
24	pHTβ::Tn*917-lac*/304	ORF54	46466	4.8×10^{-5}	44
25	pHTβ::Tn*917-lac*/82	ORF56	47472	$<1.4 \times 10^{-7}$	<0.2
26	pHTβ::Tn*917-lac*/10	ORF58	49350	8.0×10^{-5}	73
27	pHTβ::Tn*917-lac*/19	ORF60	51712	1.3×10^{-4}	118
28	pHTβ::Tn*917-lac*/210	ORF61	52052	1.7×10^{-5}	15
29	pHTβ::Tn*917-lac*/17	ORF1 (N terminal)	52618	1.0×10^{-4}	91

[a] The mating time was 4 h. The mating experiments were performed between *E. faecalis* OG1RF and OG1SS when the transconjugants were obtained by solid surface mating.

the pheromone responsive plasmid pCF10, which is the protein involved in the negative regulation of expression of the mating aggregation substance, and insertion into ORF60 did not affect the transfer frequency (18).

We cannot exclude any potential polar effects on an adjacent gene or genes by transposon insertions in region I and region II. Research is now under way to determine the function of each ORF. Analysis of transferability and mapping of the insertion mutants implied that many ORFs in region I occupied a relatively large portion of pHTβ plasmid and could be related to transfer of the plasmid. The transfer-related ORFs in region I showed significant homology to ORFs on the pXO2 plasmid of *B. anthracis*. Regions I, II, and III were separated by Tn*1546* or several ORFs where inserts did not affect the transfer frequency. The ORFs in region II and region III might be necessary for *trans*-regulating expression of the ORF(s) of region I.

Identification of the fragment containing the *oriT* region of pHTβ. The transfer origin (*oriT*) is thought to be characteristic of the conjugative plasmid and essential for the transfer of the transferable or mobile element (11, 47). The *oriT* functions in *cis* to generate the single-stranded plasmid intermediate, after DNA relaxase cleaves a specific phosphodiester bond of the *nic* site. The known *oriT*s are classified into several groups based on sequence similarities (47). To identify the *oriT* region, which involves identification of the relaxase recognition sequence (i.e., direct repeat sequences) and *oriT* (site), the degree of homology between the DNA sequences determined for the pHT plasmid and the reported consensus sequences of the *oriT* region was analyzed. No sequence that was identical or similar to known *oriT* regions of gram-positive conjugative plasmids was found in the pHTβ sequences. It is characteristic of the *oriT* region that direct repeat sequences flank the *oriT* site and that the *oriT* sites are present within inverted repeat sequences. Thus, segments containing direct repeats (DR) and inverted repeats (IR) were selected as candidates for the *oriT* region. These candidates for the *oriT* region in the plasmid are indicated by DR1 and DR2 and from IR1 through IR5 in Fig. 1. Sequences which had similarities (more than 80% identity) to sequences near the *oriT* site (*nic* site) of the IncP, IncQ, or F plasmids (see Fig. 4) were also screened as candidates for the *oriT* site.

DNA segments containing potential candidates for the *oriT* region, which were indicated by black horizontal bars marked from a to q in Fig. 1, were cloned into pAM401. Each plasmid clone (Cm^r) was tested for its ability to be mobilized by the pHTβ plasmid (Vm^r). Two of the clones, pAM401::IR5-1 and pAM401::IR2, were mobilized by the pHTβ plasmid (Table 4). The issue of the mobilization of pAM401::IR2 clone will be discussed later as a possible *oriV* candidate. The segment containing the IR5 region between ORF31 and ORF32 (g in Fig. 1), conferred the ability to transfer the pAM401::IR5-1 chimeric plasmid at a high frequency comparable to that of the pHT plasmid (Table 4). Of the transconjugants selected on the

TABLE 4. Mobilization frequencies of pAM401 derivatives (Cm^r) carrying the various segments of pHTβ by coresident pHTβ plasmid (Vm^r)

Segment in Fig. 1	Plasmids	5′/3′ end of segment on map (bp)	Length (bp)	Region	Transfer frequency (no. of conjugants/donor; broth mating)	
					Vm^r	Cm^r
a	pHTβ/pAM401::ORF1	525/1300	776	ORF1 internal region	6.8×10^{-5}	$<10^{-7}$
b	pHTβ/pAM401::IR4	1657/3060	1,404	IR4 and ORF2	7.7×10^{-6}	$<10^{-7}$
c	pHTβ/pAM401::IR3	5618/6116	499	IR3	5.1×10^{-5}	$<10^{-7}$
d	pHTβ/pAM401::119/113	1300/12476	11,177	IR4 to ORF13	8.0×10^{-5}	$<10^{-7}$
e	pHTβ/pAM401::ORF19/21	15213/18648	3,436	ORF19, −20, and −21	4.6×10^{-5}	$<10^{-7}$
f	pHTβ/pAM401::119/96	20168/25254	5,087	ORF23, −24, and −25	5.3×10^{-5}	$<10^{-7}$
g	pHTβ/pAM401::IR5-1	29918/31161	1,244	IR5, ORF31 and −32	6.0×10^{-5}	4.1×10^{-5}
h	pHTβ/pAM401::ORF39/40	36696/38127	1,432	ORF39 and ORF40	5.7×10^{-5}	$<10^{-7}$
i	pHTβ/pAM401::IR1	39726/41121	1,396	IR1, ORF44 to −47	6.0×10^{-5}	$<10^{-7}$
j	pHTβ/pAM401::DR1	43218/44620	1,403	DR1 and ORF50	6.5×10^{-5}	$<10^{-7}$
k	pHTβ/pAM401::ORF51/52	44171/45708	1,538	ORF51 and ORF52	4.3×10^{-5}	$<10^{-7}$
l	pHTβ/pAM401::ORF53/56	45810/47759	1,950	ORF53 to ORF56	8.2×10^{-5}	$<10^{-7}$
m	pHTβ/pAM401::traA	47762/48505	744	*traA* internal region	5.2×10^{-5}	$<10^{-7}$
n	pHTβ/pAM401::IR2	48482/50108	1,627	IR2 and ORF58	9.1×10^{-6}	3.4×10^{-6}
o	pHTβ/pAM401::ORF58	48943/50108	1,166	ORF58	6.5×10^{-5}	$<10^{-7}$
p	pHTβ/pAM401::rep5′	49257/50108	852	Upstream region of *rep*	5.0×10^{-5}	$<10^{-7}$
q	pHTβ/pAM401::ORF60/61	51124/52626	1,503	ORF60 and ORF61	7.5×10^{-5}	$<10^{-7}$

agar plates containing vancomycin, about 40% of the transconjugants were resistant to vancomycin, and 60% were resistant to both vancomycin and chloramphenicol. The vancomycin-resistant transconjugants contained only the pHTβ plasmid, and the vancomycin- and chloramphenicol-resistant transconjugants contained both the pHTβ plasmid and the pAM401::IR5-1 chimeric plasmid. Of the transconjugants selected on agar plates containing chloramphenicol, about 10% were resistant to chloramphenicol and 90% were resistant to chloramphenicol and vancomycin. The chloramphenicol-resistant transconjugants contained only the pAM401::IR5-1 chimeric plasmid; the chloramphenicol- and vancomycin-resistant transconjugants contained both pAM401::IR5-1 and the pHTβ plasmid. These results indicated that the chimeric plasmid pAM401::IR5-1 was mobilized in *trans* by the coresident pHTβ plasmid. None of the other fragments of pHTβ except fragment n containing IR2 and ORF58 could mobilize transfer, and transfer of pAM401::IR5-1 required the presence of pHTβ.

Cloning and genetic analysis of *oriT* region of pHTβ plasmid. IR5 contained direct repeats of 13 bp and two inverted repeats (Fig. 2 and 3). The direct repeats were composed of two copies of a 13-bp sequence and 7 bp that were part of the 13-bp sequence in the same orientation (i.e., ACTATGACC AAAA [DR-a], ACTATGACCAAAA [DR-b], and TAT GACC [DR-c]). The inverted repeats were composed of short and long inverted repeats, AGTTGGC/GCCAACT (IR5S) and TAGCcACCTTCCT/AGGAAGGTGCTA (IR5L), respectively. Detailed analysis of this IR5 region was performed. Deletion mutants of the IR5-1 clone were constructed (Fig. 2). Deletion mutant IR5-9, which possessed a 192-bp fragment lying between 30,409 bp and 30,600 bp of the pHTβ map, was mobilized in *trans* by the coresident pHTβ plasmid and was the smallest fragment retaining the ability to transfer with a frequency equivalent to that of the pHTβ plasmid. This region contained one (DR-b) of the two copies of the 13-bp direct repeat sequences and the 7 bp (DR-c) of the 13-bp sequence, as well as two inverted repeat sequences (i.e., IR5S and IR5L).

Deletion mutants containing only two inverted repeats, such as pAM401::IR5-3 and pAM401::IR5-10, were still mobilized at low frequencies by filter mating (Table 5). The pAM401::IR5-8 deletion mutant, which had a deletion of two inverted repeat sequences, was completely incapable of transfer, and the transfer frequency was less than 10^{-8} in filter mating. Two Tn*917-lac* mutants (numbered as 16 in Fig. 1 and 2) near the *oriT* region of pHTβ were obtained, and the insertion was mapped to 10 bp downstream of the long inverted repeats (IR5L) (Fig. 3). The mutants, pHTβ::Tn*917-lac*/323 and -327, could not transfer at all, even on a solid surface. These results indicated that the IR5 region could be the *oriT* region and that two inverted repeat sequences were essential for plasmid transfer. The sequence of the putative *oriT* region (IR5) showed no significant similarity to the reported *oriT* sequences.

Five families of *oriT* core sequences have been defined through comparison of a wide range of transfer origins (Fig. 4) (9, 11, 47). There are conserved sequences within the core sequences of an *oriT* family. A closer inspection revealed a consensus sequence that is common even among the apparently phylogenetically remote *oriT* families (47). The most conserved sequence found among the families contains a centrally located TG or CG site, which is the *nic* site for the relaxase, and an A residue 4 bp away, which represents the highest level of conservation among the nucleotides in the *nic* sites of *oriT*s and *dso*s. IR5L of pHT contained a centrally located TG and an A residue 4 bp away (Fig. 4). It was possible that the *nic* site might be located within this region in the inverted repeat region in the pHTβ plasmid (Fig. 4).

A clone, pAM401::IR2, carrying the IR2 segment (segment n in Fig. 1) was also mobilized by the pHTβ plasmid at low frequencies, of around 10^{-6} per donor cell, which was about 10% of the transfer frequency of a plasmid containing the *oriT* region, by broth mating (Table 4). The donor strain UV202 was shown to carry the pHTβ plasmid and the cloned pAM401::IR2 plasmid by agarose gel electrophoresis of plasmid DNAs from the donor strains (data not shown). The

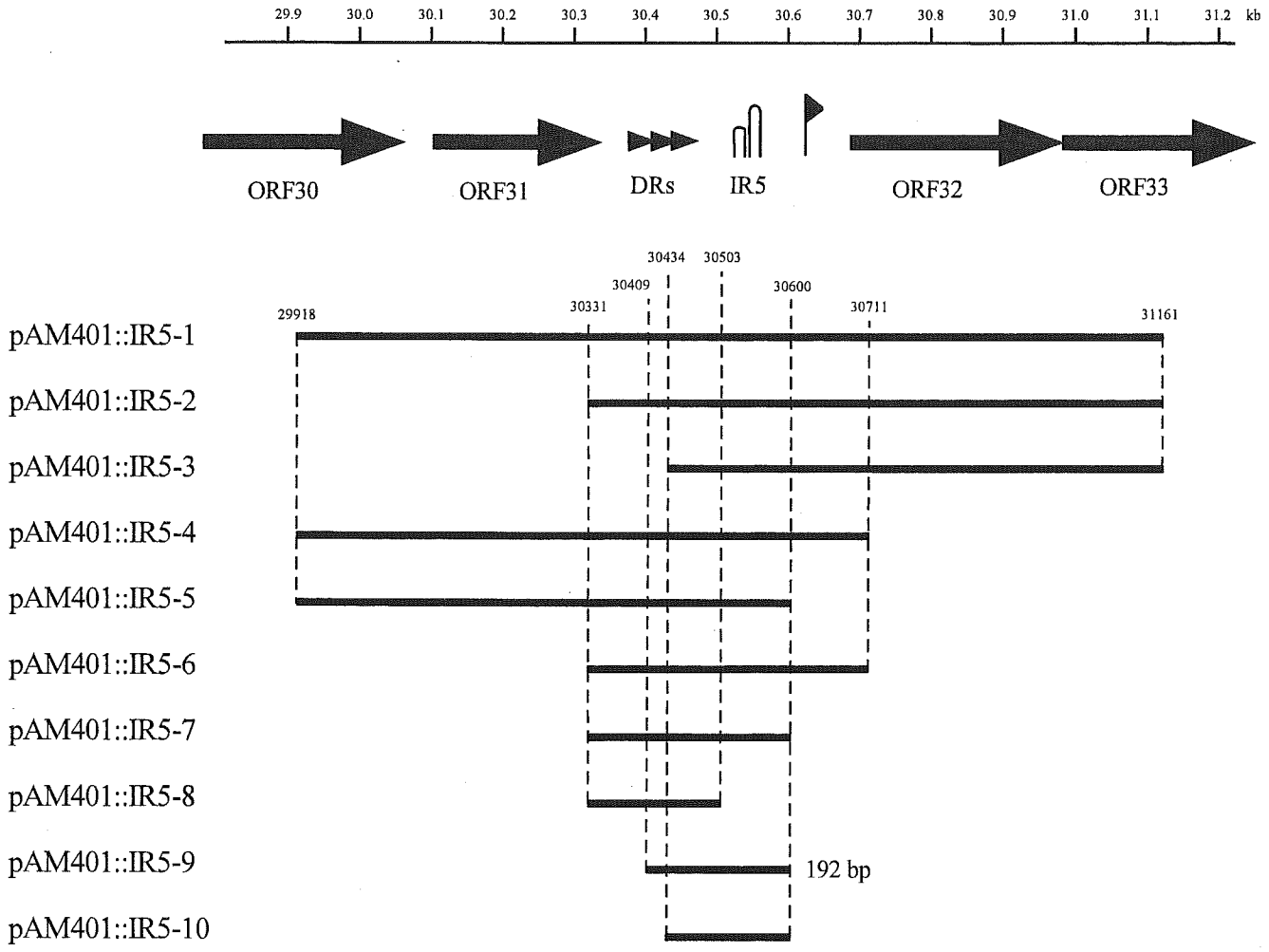

FIG. 2. Genetic analysis and determination of *oriT* region of pHT plasmids. The horizontal bars indicate the cloned PCR fragments of the pAM401 derivatives, and the bar numbers show the end positions of the segments. The transfer frequency of each of the pAM401 derivatives is shown in Table 5.

transconjugants of JH2SS were resistant to both chloramphenicol and vancomycin and harbored one chimeric plasmid formed between pHTβ and the pAM401 derivative (data not shown), which could result from cointegration between pHTβ and pAM401::IR2. These data indicated that the mode of mobilization of pAM401::IR2 was different from that of pAM401::IR5-1. Analysis of the chimeric plasmid formed between a fragment of the F plasmid and pSC101 shows that chimeric plasmids that lack *oriT* but contain *oriV1* of the F plasmid were mobilized in *cis* via cointegration with the coresident F plasmid at *oriV1* in a RecA-independent recombination (23). The *oriV* region is essential for plasmid replication and is the start site for replication. The IR2 region of the pHTβ plasmid is located just upstream of ORF59, which is highly related to the *rep* genes of rolling circle replication (RCR)-type gram-positive plasmids. It was probable that IR2 was not a second *oriT* region but was the *oriV* region of the pHTβ plasmid (2, 10). Site-specific recombination could occur between the predicted *oriV*s of the pAM401::IR2 and pHTβ plasmids.

The putative DNA relaxase/nickase gene, ORF34. In addition to the *oriT* sequence, the relaxase/nickase is an important feature of conjugative plasmids (11, 47) needed for the initiation of DNA transfer. None of the ORFs encoded on the pHT plasmid showed significant similarity to reported relaxase/nickase genes. Similarities between amino acid residues in relaxases encoded by different conjugative systems have been reported (30), and three common motifs are seen (11, 47). These suggest a shared DNA relaxation mechanism. Motif I contains the catalytic Tyr residue involved in DNA cleavage-joining activity. Motif II was reported to be involved in DNA-protein contacts through the 3′ end of the nick region, and a Ser residue is usually present. Motif III contains three conserved His residues and is known as the His_3 motif. It has been suggested that the His residues aid the nucleophilic activity of the Tyr residue in motif I coordinate the required Mg^{2+} ions and direct activation of the active Tyr. These three motifs are thought to form part of the catalytic center of the relaxase. We examined the pHT plasmid for the presence of a relaxase by

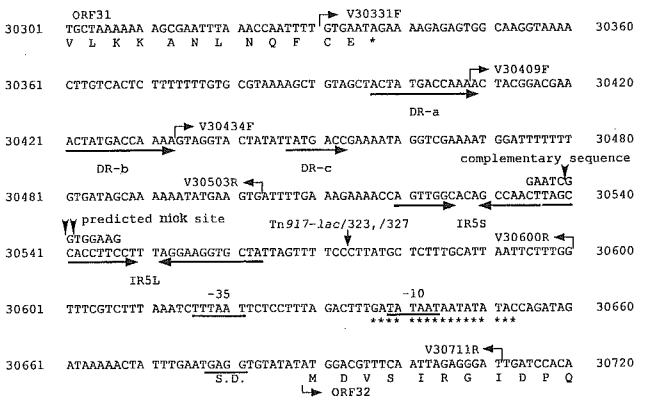

FIG. 3. Nucleotide sequences of the *oriT* region of the pHT plasmid. The 420-bp noncoding DNA sequence region between ORF31 and ORF32 is shown. The horizontal arrows under the sequences indicate the direct repeats (DR-a, DR-b, and DR-c) and inverted repeats (IR5S and IR5L) in the *oriT* region. The names and locations of oligonucleotide primers used for the analysis of the *oriT* region are shown on the sequence with the angled arrows. Two downward arrowheads on the complementary sequence show the possible nick sites in the *oriT* region based on the sequence comparisons with other defined nick sites (see the text and Fig. 5). The vertical arrows that are numbered as 323 and 327 indicate the locations of the two Tn*917-lac* insertion mutants of pHTβ that abolished transfer ability. The putative promoter region (−35 and −10) and ribosome binding site (Shine-Dalgarno sequence [S.D.]) for ORF32 are shown upstream of the start codon. The asterisk marks indicate the dyad symmetric sequences overlapping the promoter region.

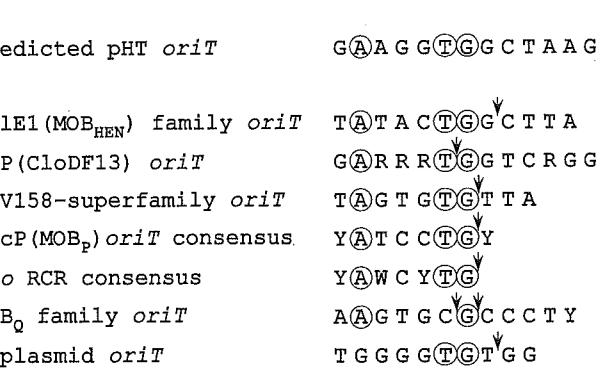

FIG. 4. Comparison of the *oriT* consensus nucleotide sequences (*nic* region) of the representative mobile plasmids and the possible nick site of the pHTβ plasmid. The *oriT* region containing the possible nick site of the pHT plasmid found in the inverted repeat sequences is shown. The consensus *oriT* region found in the pheromone-responsive conjugative plasmids (PRP) and the previously reported consensus *oriT* sites found in mobile plasmids are shown. The consensus *oriT* sequences of IncP (MOB_P), the pMV158 superfamily, the MOB_Q family (R1162, etc.), and the F plasmid are indicated. The consensus double-stranded replication origin (*dso*) of RCR plasmids is also shown. The circular marks indicate the conserved nucleotides, centrally located TG site, and an A located four residues away. The black arrowheads indicate the nick sites determined in the *oriT* region.

searching for the conserved motifs (residues) of the relaxase. ORF34 encoding 506 amino acid residues was found to contain the three conserved motifs I, II, and III, which contained Tyr, Ser, and three His residues, respectively, and could be the relaxase for the pHT plasmid (Fig. 5).

Three Tn*917-lac* insertion mutants of the pHTβ plasmid, pHTβ::Tn*917-lac*/95, -197, and -223, were obtained in the ORF34 gene, and each of the insertions was mapped. The transposons were inserted into residues Leu^{366}, Gln^{440}, and Val^{469} of the ORF34 protein, respectively. All the insertion mutants had completely lost the ability to transfer. Each phenotype of the mutant might result from the polar effects of insertion. To confirm whether ORF34 is essential for plasmid transfer, an in-frame deletion mutant of 10 amino acids (i.e.,

TABLE 5. Mobilization of pAM401 derivative plasmids by pHTβ[a]

Plasmid	Transfer frequency (no. of conjugants/donor)			
	Broth		Filter	
	Vm^r	Cm^r	Vm^r	Cm^r
pAM401	1.2×10^{-4}	$<10^{-7}$	4.7×10^{-2}	$<10^{-8}$
pAM401::IR5-1	6.0×10^{-5}	4.1×10^{-5}	5.3×10^{-2}	1.1×10^{-1}
pAM401::IR5-2	8.0×10^{-4}	3.8×10^{-5}	3.2×10^{-2}	1.0×10^{-1}
pAM401::IR5-3	1.1×10^{-4}	$<10^{-7}$	2.5×10^{-2}	3.2×10^{-4}
pAM401::IR5-4	1.1×10^{-4}	5.1×10^{-5}	4.0×10^{-2}	1.3×10^{-1}
pAM401::IR5-5	1.4×10^{-4}	6.9×10^{-5}	2.5×10^{-2}	1.2×10^{-1}
pAM401::IR5-6	9.0×10^{-5}	3.0×10^{-5}	4.4×10^{-2}	1.1×10^{-1}
pAM401::IR5-7	1.6×10^{-4}	6.7×10^{-5}	3.1×10^{-2}	1.2×10^{-1}
pAM401::IR5-8	7.0×10^{-5}	$<10^{-7}$	4.8×10^{-2}	$<10^{-8}$
pAM401::IR5-9	1.4×10^{-4}	4.2×10^{-5}	3.0×10^{-2}	1.2×10^{-1}
pAM401::IR5-10	1.8×10^{-4}	$<10^{-7}$	2.7×10^{-2}	2.5×10^{-4}

[a] The mating experiment was performed using *E. faecalis* UV202 as a donor carrying two plasmids and *E. faecalis* JH2SS as a recipient strain. The donor strains harbored both pHTβ (Vm^r) as a mobilizer plasmid and each of the pAM401 derivatives (Cm^r) containing various segments of the pHT plasmid as a tester plasmid. Each mating experiment was carried out in triplicate.

FIG. 5. Comparison of the N-terminal region of the deduced ORF34 protein (TraI) of pHTβ with the hypothetical proteins found in sequence databases. The bold characters indicate the conserved amino acid residues in each protein. The asterisks on the sequences show the key residues, Tyr, Ser, and His_3 (3His) in motifs I, II, and III, respectively. There are two motif I candidates (Ia and Ib) in most of the proteins. The gray box in motif III indicates the deleted 10-amino-acid residues of ORF34 resulting in pHTβ/ORF34del. The lowest sequence shows the putative consensus His_3 motif (motif III) of these proteins (designated as MOB_{MG} family), $H(x_2)T(x_3)HxH(x_4)E(x_4)R$. The accession numbers for the proteins are as follows: NP_569166 for *Listeria innocua* pLI100 (81,905 bp), NP_734852 for *Streptococcus agalactiae* MEN316, NP_765038 for *Staphylococcus epidermidis* ATCC 12228, NP_053238 for *B. anthracis* pXO2-84, and NP_150032 for *Clostridium perfringens* strain 13 plasmid pCP13 (54,310 bp), respectively.

HRNTEHIHIH) from amino acid residues 200 to 209, which is located within motif III of ORF34, was constructed in the pHTβ plasmid as described in Materials and Methods (Fig. 5). The pHTβ plasmid derivative mutant with the defective ORF34 could not transfer in broth or on a filter (data not shown). These results indicated that ORF34 was the essential transfer-related gene.

A FASTA/BLAST homology search showed that sequences homologous to the motifs of the putative relaxase ORF34 were found in ORFs of other bacterial species for which function has not been determined (Fig. 5). Most of the ORFs homologous to ORF34 were found to contain two putative motifs I, designated as motifs Ia and Ib, in the N-terminal region, and a Tyr residue was conserved in each of the motifs (Fig. 5). Although until now there has been no genetic information or characterization of the genes homologous to ORF34, the new pHT family relaxase represented by the putative relaxase ORF34 could be widespread throughout a variety of bacteria. The conserved His_3 sequence in motif III of the pHT family was designated as MOB_{MG}: i.e., $H(x_2)T(x_3)HxH(x_4)E(x_4)R$.

Concluding remarks. Sequence data for the pHT plasmid revealed that the pMG1-like plasmids had little similarity to well-characterized plasmids. The ORFs encoded on pXO2, a pathogenic capsule plasmid found in *B. anthracis*, shared sequence homologies with the pHT plasmids. Little is known about the transfer of pXO2 and about the function of each ORF, although there are reports about the mobilization of the plasmid by other highly conjugative plasmids found in *Bacillus* species (4, 33). Based on the genetic analysis by transposon insertional mutagenesis, a region containing ORFs from ORF3 to ORF48 that was designated as region I could be related to the transfer of the pHT plasmid: the other two regions, region II containing ORF56 and -57 and region III consisting of ORF61, were also necessary for efficient transfer.

Both *oriT* and nickase/relaxase are thought to be essential and important characteristic features of the conjugative plasmid (11, 47). The *oriT* region of the pHT plasmid was genetically determined, and the *traI* gene encoding the putative DNA relaxase/nickase resided in the transfer-related region I. The biochemical activity of the product has not yet been elucidated.

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A vancomycin-dependent VanA-type *Enterococcus faecalis* strain isolated in Japan from chicken imported from China

K. Tanimoto[1], T. Nomura[2], H. Hamatani[2], Y.-H. Xiao[3] and Y. Ike[1,2]

[1]Laboratory of Bacterial Drug Resistance, [2]Department of Bacteriology and Bacterial Infection Control, Gunma University Graduate School of Medicine, Maebashi, Gunma, Japan, and [3]Institute of Clinical Pharmacology, Peking University, Beijing, China

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ABSTRACT

K. TANIMOTO, T. NOMURA, H. HAMATANI, Y.-H. XIAO AND Y. IKE. 2005.

Aims: The characterization of KC122.1, which is a vancomycin-dependent VRE (Vancomycin-resistant enterococci) (*Enterococcus faecalis*) and the first case in Japan of a VRE isolate obtained from chicken meat imported from China.

Methods and Results: PCR amplification of *vanA*, *vanS* and *ddl* gene and direct sequencing of the PCR products were performed. KC122.1 was a VanA-type VRE showing high-level vancomycin resistance and low-level teicoplanin resistance, and its *vanS* gene had three point mutations. The *ddl* gene of KC122.1 was sequenced and two changes were found at the ninth codon (GCC–GAC) and the stop codon (TAA–CAA). The latter change was also found in the laboratory strain *E. faecalis* FA2-2.

Conclusions: Three point mutations in vanS resulted in high-level vancomycin resistance and low-level teicoplanin resistance. The change at the ninth codon resulted in the inactivation of the *ddl* gene and vancomycin-dependent growth. An eight amino acid extension at the C-terminal did not impair the function of the D-Ala : D-Ala ligase.

Significance and Impact of the Study: This is the first example of the isolation of VRE from chicken meat imported from China and the first vancomycin-dependent VRE from a nonhuman source.

Keywords: chicken meat, D-Ala : D-Ala ligase, VanA-type, vancomycin-dependent, Vancomycin-resistant enterococci.

INTRODUCTION

Vancomycin (glycopeptide)-resistant enterococci (VRE or GRE) can cause serious problems for hospitalized patients because of the limited options for the treatment of VRE infection. The number of infections caused by VRE is increasing (Murray 1990; Korten and Murray 1993; National Nosocomial Infections Surveillance System Report 1999). Among the acquired glycopeptide resistances, VanA and VanB are the most common resistance phenotypes. In both the cases, resistance resulted from the acquisition of genes encoding seven proteins (Arthur *et al.* 1996; Kak and Chow 2002). The VanS ($VanS_B$) and VanR ($VanR_B$) proteins, which are the sensor and the regulator, respectively, constitute a two-component regulatory system responsible for the recognition of glycopeptide in the culture medium and transcriptional activation of the resistance genes. Glycopeptide resistance can be induced by the presence of antibiotics such as vancomycin (Arthur *et al.* 1992). The VanA-type strains have high-level resistance to vancomycin and teicoplanin because both are inducers. However, the VanB-type strains remain

Correspondence to: K. Tanimoto, Laboratory of Bacterial Drug Resistance, Gunma University Graduate School of Medicine, Showa-machi 3-39-22, Maebashi, Gunma 371-8511, Japan (e-mail: tanimoto@med.gunma-u.ac.jp).

susceptible to teicoplanin because teicoplanin cannot be an inducer (Arthur *et al.* 1996). In a previous report, one VanA-type VRE had high-level resistance to vancomycin, and low-level resistance to teicoplanin. This VRE had three amino acid substitutions in the N-terminal region of the VanS protein and the glycopeptide resistance of this strain was not induced by teicoplanin, which implied that the mutated VanS protein could not recognize the presence of teicoplanin, resulting in the failure to activate the resistance genes (Hashimoto *et al.* 2000). Enterococci that require vancomycin in the culture medium for growth have been isolated (Green *et al.* 1995; Baptista *et al.* 1997). All have been isolated from patients, a rabbit model of experimental endocarditis or *in vitro*. Although many of these vancomycin-dependent strains are VanB-type *Enterococcus faecium* (Dever *et al.* 1995; Green *et al.* 1995), vancomycin-dependent *Enterococcus faecalis* (Fraimow *et al.* 1994; Farrag *et al.* 1996) and *Enterococcus avium* (Sifaoui and Gutmann 1997) with the *vanA* gene have also been described. Vancomycin-dependence results from a mutation that inactivates the D-Ala : D-Ala ligase gene (*ddl*) in the chromosome, so that the mutant strain no longer produces D-Ala : D-Ala-ending peptidoglycan precursors. Thus, cell wall synthesis in the mutant strain is dependent on the production of alternative peptidoglycan precursors. The D-Ala : D-Lac ligase activity of *vanA* and *vanB* can replace *ddl* activity by production of D-Ala : D-Lac-ending peptidoglycan precursors instead of the native D-Ala : D-Ala-ending precursors. As both resistances are inducible with vancomycin, the production of alternate precursors requires the presence of vancomycin and the mutant strain becomes vancomycin-dependent for growth (Kak and Chow 2002). In this work, we describe vancomycin-dependent *E. faecalis* isolated in Japan from chicken meat imported from China. This is the first vancomycin-dependent strain isolated from a nonhuman sample. Its *ddl* gene had one amino acid substitution (Gly9–Asp), and it had become dependent on the presence of vancomycin.

MATERIALS AND METHODS

Bacterial strains, media and antibiotics

Vancomycin-resistant *E. faecalis* KC122.1 and KC122.3 were isolated in Japan from imported chicken meat from China in 2001. Both strains were isolated from the same sample. *Enterococcus faecalis* FA2-2 (Clewell *et al.* 1982), which is rifampicin and fusidic acid resistant, was used for the sequencing of the *ddl* gene, and in mating experiments as a recipient. Todd–Hewitt broth (THB) (Difco, Detroit, MI, USA) was used for the growth of enterococci. Agar plates were made by the addition of agar (1·5%) to the broth medium. Mueller–Hinton (MH) broth and MH agar were used for the sensitivity disc agar-N (Nissui, Tokyo, Japan) assay to test the minimal inhibitory concentrations (MICs) of antibiotics.

MIC determination

The MICs were determined by the agar dilution method according to the criteria of the National Committee for Clinical Laboratory Standards (2000) using MH agar. Overnight cultures of the strains grown in MH broth were diluted 100 times with fresh broth. One loopful (5 μl: *c.* 5×10^3–10^4 cells) of each dilution was transferred to agar plates containing the relevant antibiotics.

Mating procedures

Filter matings were performed with a donor/recipient ratio of 1 : 4. Overnight cultures were prepared and 0·05 ml of the donor and 0·2 ml of the recipient were added to 4·5 ml of fresh THB broth, and the cells were then trapped on a membrane filter (Millipore, Bedford, MA, USA). The cells on the filters were incubated at 37°C for 4 h and were then suspended in 1 ml of THB broth. Appropriate dilutions of the mixture were plated onto the solid medium containing vancomycin (6 μg ml^{-1}), rifampicin (25 μg ml^{-1}) and fusidic acid (25 μg ml^{-1}). Broth matings were performed as previously described with a donor/recipient ratio of 1 : 10 (Dunny *et al.* 1979; Ike and Clewell 1984). Overnight cultures were prepared and 0·05 ml of donor and 0·5 ml of recipient were added to 4·5 ml of fresh THB broth. The mixtures were incubated at 37°C for 4 h. Appropriate dilutions of the mixture were then plated onto a solid medium containing vancomycin, rifampicin and fusidic acid. Colonies were counted after 24 h of incubation at 37°C for both filter matings and broth matings.

Sequencing of the *ddl* gene and *vanS* gene

The DNA fragments to be sequenced were amplified by PCR using a Mastercycler gradient (Eppendorf, Westbury, NY, USA) with *Taq* polymerase (Promega, Madison, WI, USA) as recommended by the manufacturer. The fragments were then separated by agarose gel electrophoresis and purified from an agarose gel block with Wizard SV Gel and the PCR Clean-Up System (Promega). Template DNAs for PCR were prepared with ISOPLANT II (Nippon Gene, Toyama, Japan). The sequencing reaction was carried out with a Dye Terminator Cycle Sequencing FS ready Reaction Kit (Perkin-Elmer, Wellesley, MA, USA). The nucleotide sequence was determined using an ABI PRISM 310 genetic analyzer (Perkin-Elmer). The template DNAs for *ddl* and *vanS* were amplified by PCR with the primers ddl/F4 and ddl/B1, and MS-up and MS-2 respectively

Table 1 Primers used for PCR amplification and sequencing

Target gene	Primer name	Oligonucleotide sequence (5′ → 3′)	Position(5′ → 3′)	Product size (bp)	Accession no.
ddl	ddl/F	tgagaggacaagcatt	173654 → 173669*		AE016949
	ddl/F1	cttcactaaatttgcttc	173557 → 173574*		
	ddl/F2	ttatgaagaagaagcgattgt	173926 → 173946*		
	ddl/F3	gaatgaattgaacaccatgcctgg	174586 → 174609*		
	ddl/F4	acagaccaagatagaacaacg	173466 → 173486*		
	ddl/B	ttatttaaaacgattca	174715 → 174699*		
	ddl/B1	gttttgctcctctgacac	174814 → 174797*		
	ddl/B2	aatttcacgtgcttcgatccc	174337 → 174317*		
	ddl/B3	gggccttttacccattgaccg	173817 → 173797*		
vanS	MS-up	ggggaatgtggttagctccg	4491 → 4510*		M97297
	MS-7	gaaagcatgacgcgacatag	4938 → 4919*		
	MS-1	ccgtgcatatccggcatttg	4571 → 4590*		
	MS-2	ctaccgtgtaagaacgagcc	5904 → 5885*		
vanA	A1	gggaaaacgacaattgc	176 → 191†	732	M97297
	A2	gtacaatgcggccgtta	907 → 891†		

All primers were designed in this study.

*The positions given are from the first base of the reported sequences in database listed in accession no.

†The positions given are from the first base of the coding sequences of the genes.

(Table 1). Additional primers were used to sequence the internal regions of the amplified genes (Table 1). A homology search with BLAST was performed through the website of NCBI (http://www.ncbi.nlm.nih.gov/Tools/index.html).

Pulsed field gel electrophoresis

Lysis of the cells in an agarose plug was performed according to the standard protocol (Sambrook *et al.* 1989) except that cells were treated with lysozyme at a concentration of 20 mg ml^{-1} for 2 h. For restriction endonuclease digestion of total DNA, the agarose plugs were placed in 300 μl of reaction mixture containing 50 U of *Sma*I, and were then incubated at 25°C overnight. After digestion, the plugs were washed with TE for 1 h at room temperature. The plugs were placed in wells of a 1·0% agarose gel made with 0·5× TBE (10× TBE is 0·89 mol l^{-1} Tris, 0·89 mol l^{-1} boric acid and 0·025 mol l^{-1} EDTA), and the wells were sealed with the same agarose. The gels were electrophoresed with a clamped homogeneous electric field (6 V cm^{-1}, 15°C for 24 h, Switch times ramped from 1 to 25 s, CHEF-DR II; Bio-Rad Laboratories, Richmond, CA, USA). After electrophoresis, the gels were stained with ethidium bromide and photographed with a UV light source.

Nucleotide sequence accession number

The nucleotide sequence data reported here have been deposited in the DDBJ, EMBL and GenBank nucleotide sequence databases under accession numbers AB186053 (*ddl* of FA2-2) and AB186052 (*ddl* of KC122.1).

RESULTS

Characterization of the strains

Vancomycin-resistant *E. faecalis* KC122.1 and KC122.3 were isolated in Japan from chicken imported from China in 2001. Both strains were isolated from the same sample. KC122.1 grew only in the presence of glycopeptide in the culture media, which is characteristic of the vancomycin-dependent enterococci, while KC122.3 grew both in the absence and in the presence of glycopeptide. The two strains were compared by examining the restriction fragment pattern of the chromosome produced by pulsed-field gel electrophoresis. They had identical restriction fragment patterns, implying that KC122.1 had been derived from KC122.3 or *vice versa*. KC122.1 and KC122.3 showed a high level of resistance to vancomycin with a MIC of 256 μg ml^{-1}, and a relatively low-level of resistance to teicoplanin with MICs of 8 and 4 μg ml^{-1} respectively. PCR amplification with specific primers for the D-Ala : D-Lac ligase gene was performed to determine the type of vancomycin resistance, and a PCR product was obtained using the *vanA*-specific primers listed in Table 1. Nucleotide sequence analysis of the *vanS* gene from each of the two strains was performed by sequencing the PCR products with the *vanS*-specific primers listed in Table 1. Each of the two strains had three amino acid substitutions in the N-terminal

region of the deduced VanS sequence. Leu50 was converted to Val, Glu54 was converted to Gln and Gln69 was converted to His compared with the *vanS* gene sequence of Tn*1546*. Based on these results, it is concluded that KC122.1 is a vancomycin-dependent *E. faecalis* with a VanA-type determinant.

Transferability of vancomycin resistance

Mating experiments were performed to examine the transferability of the vancomycin resistance of KC122.1 and KC122.3. Both strains were used as the donor and FA2-2 was used as the recipient. Broth matings (4 h) and filter matings (4 h) were performed, and vancomycin resistance was transferred from both donor strains to FA2-2 only in filter mating at a frequency of *c.* 10^{-5} per donor cell.

Sequencing of the *ddl* gene

The *ddl* genes of KC122.1, KC122.3 and the laboratory strain *E. faecalis* FA2-2 were sequenced. As shown in Fig. 1, KC122.1 had one point mutation at the ninth codon (GGC was converted to GAC), which resulted in an amino acid substitution (Gly was converted to Asp), and one point mutation at the stop codon [TAA was converted to GAA (Glu)] when compared with the reported *ddl* gene sequence of *E. faecalis* V583 (accession no. U00457). The *ddl* genes of K122.3 and FA2-2 were identical and both had a point mutation at the stop codon [TAA was converted to GAA (Glu)] compared with the reported *ddl* gene sequence of *E. faecalis* V583. The nucleotide sequence and the deduced amino acid sequence of the *ddl* gene showed that the mutation at the stop codon resulted in an eight amino acid extension of the *ddl* amino acid residues from the original stop codon (Fig. 1). FA2-2 and KC122.3 do not require vancomycin for growth, indicating that the mutation at the stop codon does not affect the growth of these strains. These results implied that the amino acid substitution (Gly9–Asp) produced by the point mutation in the *ddl* gene resulted in the inactivation of D-Ala : D-Ala ligase and the generation of a requirement for *vanA* ligase activity for growth.

DISCUSSION

The KC122.1 was a VanA-type VRE showing high-level resistance to vancomycin and low-level resistance to

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                    ▼
FA2-2       1:LKIILLYGGRSEEHDVSVLSAYSVLNAIYYKYYQVQLVFISKDGQWVKGPLLSERPQNKE 60
KC122·1     1:LKIILLYGDRSEEHDVSVLSAYSVLNAIYYKYYQVQLVFISKDGQWVKGPLLSERPQNKE 60
V583        1:LKIILLYGGRSEEHDVSVLSAYSVLNAIYYKYYQVQLVFISKDGQWVKGPLLSERPQNKE 60

FA2-2      61:VLHLTWAQTPEETGEFSGKRISPSEIYEEEAIVFPVLHGPNGEDGTIQGFMETINMPYVG 120
KC122·1    61:VLHLTWAQTPEETGEFSGKRISPSEIYEEEAIVFPVLHGPNGEDGTIQGFMETINMPYVG 120
V583       61:VLHLTWAQTPEETGEFSGKRISPSEIYEEEAIVFPVLHGPNGEDGTIQGFMETINMPYVG 120

FA2-2     121:AGVLASVNAMDKIMTKYLLQTVGIPQVPFVPVLRSDWKGNPKEVFEKCEGSLIYPVFVKP 180
KC122·1   121:AGVLASVNAMDKIMTKYLLQTVGIPQVPFVPVLRSDWKGNPKEVFEKCEGSLIYPVFVKP 180
V583      121:AGVLASVNAMDKIMTKYLLQTVGIPQVPFVPVLRSDWKGNPKEVFEKCEGSLIYPVFVKP 180

FA2-2     181:ANMGSSVGISKVENREELQEALEEAFRYDARAIVEQGIEAREIEVAILGNEDVRTTLPGE 240
KC122·1   181:ANMGSSVGISKVENREELQEALEEAFRYDARAIVEQGIEAREIEVAILGNEDVRTTLPGE 240
V583      181:ANMGSSVGISKVENREELQEALEEAFRYDARAIVEQGIEAREIEVAILGNEDVRTTLPGE 240

FA2-2     241:VVKDVAFYDYDAKYINNTIEMQIPAHVPEEVAHQAQEYAKKAYIMLDGSGLSRCDFFLTS 300
KC122·1   241:VVKDVAFYDYDAKYINNTIEMQIPAHVPEEVAHQAQEYAKKAYIMLDGSGLSRCDFFLTS 300
V583      241:VVKDVAFYDYDAKYINNTIEMQIPAHVPEEVAHQAQEYAKKAYIMLDGSGLSRCDFFLTS 300

FA2-2     301:KNELFLNELNTMPGFTDFSMYPLLWENMGLKYSDLIEELIQLALNRFKERQEFYNN     356
KC122·1   301:KNELFLNELNTMPGFTDFSMYPLLWENMGLKYSDLIEELIQLALNRFKERQEFYNN     356
V583      301:KNELFLNELNTMPGFTDFSMYPLLWENMGLKYSDLIEELIQLALNRFK--------     348
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Fig. 1 Deduced amino acid sequence of the D-Ala : D-Ala ligase of FA2-2, KC122.1 and *Enterococcus faecalis* V583. The amino acid substitution at the ninth codon (Gly–Asp) in KC122.1 is shown in bold and is indicated by an arrowhead. The eight amino acid extension caused by the mutation at the stop codon is shown in bold. The conserved amino acids predicted to play a key role in enzymatic activity on the basis of the X-ray structure of the *Escherichia coli* enzyme are indicated by dots (Gholizadeh *et al.* 2001). The numbers on the left side refer to the first amino acid in the corresponding sequence. The numbers on the right side refer to the last amino acid in the corresponding line

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