

## Complete nucleotide sequencing and analysis of the 65-kb highly conjugative *Enterococcus faecium* plasmid pMG1: identification of the transfer-related region and the minimum region required for replication

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### Keywords

*Enterococcus faecium*; conjugative plasmid; *tra*  
genes; DNA replication.

### Introduction

Enterococci have several efficient systems of conjugative plasmid transfer that play a key role in genetic exchange, including the transfer of drug resistance (Clewell & Dunny, 2002). Enterococci have two types of highly efficient conjugation systems: the pheromone-responsive plasmids and the pheromone-independent plasmids. The pheromone-responsive plasmid is mainly found in *Enterococcus faecalis* (Clewell & Dunny, 2002) and is unique to this species. These plasmids transfer between *E. faecalis* strains at a high frequency of  $10^0$ – $10^{-2}$  per donor cell within a few hours of broth mating. A sex pheromone induces the synthesis of a surface aggregation substance that facilitates the formation of mating aggregates. The sex pheromones also induce a series of genes required for plasmid transfer. Plasmid-free recipients secrete multiple sex pheromones, each specific for a donor harboring a related pheromone-responsive plasmid.

### Abstract

pMG1 (65.1 kb) is a highly conjugative, pheromone-independent *Enterococcus faecium* plasmid that carries a Tn4001-like transposon encoding gentamicin resistance. The complete nucleotide sequence (65 029 bp) of the pMG1 plasmid was determined and 73 ORFs lying in the same transcription orientation were identified. Sixty-one of the 73 ORFs showed a high degree of similarity (90–100% identity at the amino acid level) to the ORFs of the pHT $\beta$  plasmids. Like the pHT $\beta$  plasmid, 22 of the pMG1 plasmid ORFs showed homology with ORFs present on the pXO2 plasmid (96.2 kb), which is the virulence plasmid essential for capsular formation by *Bacillus anthracis*. Analysis of *tra* mutants created by Tn917 insertion and Northern analysis of transcripts indicated that ORFs 15–49, lying in the 31.7 kb region between 13.6 and 45.3 kb on the plasmid map, were related to transfer. This region was designated as the Tra I region of pMG1. A 5.9-kb HindIII fragment that replicates autonomously in *Enterococcus faecalis* was cloned and analysis of this fragment by deletion and *in vitro* insertion mutations showed that ORF10 (*rep*) and the inverted repeat sequence in the noncoding region between ORF8 and ORF9 were necessary for pMG1 replication.

The pheromone-independent plasmids are found in *Enterococcus faecium* strains (Ike *et al.*, 1998; Tanimoto & Ike, 2002; Tomita *et al.*, 2002, 2003; Tomita & Ike, 2005). Previously, we described the first isolation of the pheromone-independent gentamicin-resistance conjugative plasmid pMG1 (Gm<sup>r</sup>; 65.1 kb) from an *E. faecium* clinical isolate in Japan (Ike *et al.*, 1998). pMG1-like plasmids were also found to be widely disseminated in vancomycin-resistant *E. faecium* clinical isolates obtained from a hospital in the United States (Tomita *et al.*, 2002). We have also reported the isolation of the pMG1-like vancomycin-resistance pHT plasmids from clinical isolates of *E. faecium* and *Enterococcus avium* strains in Japan (Tomita *et al.*, 2003). pMG1 transfers among *Enterococcus* strains during broth mating at a frequency of about  $10^{-4}$  per donor strain. pHT plasmids, including pHT $\alpha$  (65.9 kbp), pHT $\beta$  (63.7 kbp) and pHT $\gamma$  (66.5 kbp), are highly conjugative plasmids carrying Tn1546-like transposons that encode vancomycin resistance. DNA

sequencing and genetic analysis of pHT $\beta$ , which is the prototype pHT plasmid, have identified three (I–III) transfer-related regions and the *oriT* region (Tomita & Ike, 2005).

In this report, we describe the DNA sequence of the pMG1 plasmid and a comparative study of pMG1 and pHT $\beta$  DNA sequences. We have also shown that the *Tra* region genes form operon structures and are transcribed on long polycistronic RNAs. We have also identified the *rep* region of pMG1 by cloning the region.

## Materials and methods

### Bacterial strains and plasmids

The bacterial strains and plasmids used in this experiment are listed in Table 1. pMG1 is a gentamicin and kanamycin-resistance plasmid originally isolated from a clinical strain of *E. faecium* (Ike *et al.*, 1998). pBluescriptSK (Stratagene, CA) was used to clone pMG1 fragments and the spectinomycin-resistance vector pKO101 was constructed to isolate the autonomously replicating fragment of pMG1.

### Media and antibiotics

Todd–Hewitt broth (THB) (Difco, MI) was used for growth of *E. faecalis* and in conjugation experiments. Luria–Bertani

medium was used for growth of *Escherichia coli* (Miller, 1972). Antibiotics were used at the following concentrations: ampicillin, 100  $\mu\text{g mL}^{-1}$ ; chloramphenicol, 10  $\mu\text{g mL}^{-1}$ ; erythromycin, 10  $\mu\text{g mL}^{-1}$ ; gentamicin, 500  $\mu\text{g mL}^{-1}$ ; kanamycin, 20  $\mu\text{g mL}^{-1}$  for *E. coli* and 500  $\mu\text{g mL}^{-1}$  for *E. faecalis*; rifampin, 25  $\mu\text{g mL}^{-1}$ ; fusidic acid, 25  $\mu\text{g mL}^{-1}$ ; spectinomycin, 40  $\mu\text{g mL}^{-1}$  for *E. coli* and 100  $\mu\text{g mL}^{-1}$  for *E. faecalis*.

### Plasmid DNA preparation

The Qiaprep Spin Miniprep Kit (Qiagen Sciences, MD) was used for plasmid DNA preparation from *E. coli*. The alkaline lysis method was modified (Sambrook *et al.*, 1989) for DNA preparation from *E. faecalis* to include lysozyme treatment (10  $\mu\text{g mL}^{-1}$ , 20 min at 37 °C) before lysis with the NaOH/SDS solution. In addition, phenol/chloroform extraction was carried out before ethanol precipitation.

### DNA sequencing of pMG1

A series of pMG1 relational clones were constructed as described previously (Fujimoto *et al.*, 1995). pMG1 DNA was partially digested with HindIII and cloned into the HindIII site of pBluescriptSK. The resulting clones contained a variety of pMG1 fragments. The GPS–LS linker

Table 1. Bacterial strains and plasmids used in this study

Strains or plasmid	Relevant genotype or phenotype	Comment(s)	Reference or sources
<b>Bacterial strains</b>			
<i>E. faecalis</i>			
FA2-2	<i>rif fus</i>		Franke & Clewell (1981) Ike <i>et al.</i> (1983)
OG1X	<i>str</i>	Protease-negative derivative of OG1-10	
<i>E. coli</i>			
DH5 $\alpha$	<i>recA1 endA1 gyrA96 thi-1 relA1 hsdR17 supE44 DlacU169 f80 lacZDM15</i>		Invitrogen
<b>Plasmids</b>			
pMG1	Gm <sup>r</sup> , Km <sup>r</sup>	65.1-kb conjugative plasmid from <i>E. faecium</i> strain	Ike <i>et al.</i> (1998)
pTV1B	Em <sup>r</sup> , Cm <sup>r</sup>	Transposon delivery vector, temperature sensitive; pE194Ts(Cm <sup>r</sup> ::Tn917(Em <sup>r</sup> ))	Youngman (1987)
pBluescriptSK	<i>amp</i>	<i>E. coli</i> cloning vector	Stratagene
pMW119	<i>amp</i>	<i>E. coli</i> cloning vector	Nippon Gene
pDL278	<i>spc</i>	<i>E. coli</i> – <i>E. faecalis</i> shuttle vector	LeBlanc & Lee (1991)
pKO101	<i>amp spc</i>	<i>Spc</i> <sup>r</sup> gene of pDL278 was cloned in pMW119	This study
pMG230	Gm <sup>r</sup> , Km <sup>r</sup> , Em <sup>r</sup> , Tra	pMG1::Tn917, Tn917 was inserted at the position of 13 575*	This study
pMG231	Gm <sup>r</sup> , Km <sup>r</sup> , Em <sup>r</sup> , Tra	pMG1::Tn917, Tn917 was inserted at the position of 16 167*	This study
pMG232	Gm <sup>r</sup> , Km <sup>r</sup> , Em <sup>r</sup> , Tra	pMG1::Tn917, Tn917 was inserted at the position of 16 656*	This study
pMG233	Gm <sup>r</sup> , Km <sup>r</sup> , Em <sup>r</sup> , Tra	pMG1::Tn917, Tn917 was inserted at the position of 27 284*	This study
pMG234	Gm <sup>r</sup> , Km <sup>r</sup> , Em <sup>r</sup> , Tra	pMG1::Tn917, Tn917 was inserted at the position of 29 138*	This study
pMG235	Gm <sup>r</sup> , Km <sup>r</sup> , Em <sup>r</sup> , Tra	pMG1::Tn917, Tn917 was inserted at the position of 36 393*	This study
pMG236	Gm <sup>r</sup> , Km <sup>r</sup> , Em <sup>r</sup> , Tra	pMG1::Tn917, Tn917 was inserted at the position of 40 579*	This study
pMG237	Gm <sup>r</sup> , Km <sup>r</sup> , Em <sup>r</sup> , Tra	pMG1::Tn917, Tn917 was inserted at the position of 41 528*	This study
pRep	<i>amp spc</i>	5.9-kb HindIII fragment of pMG1 was cloned in pKO101, Rep <sup>+</sup> in <i>E. faecalis</i>	This study

\*The positions given are from the first base of the pMG1 sequence (AB206333).



scanning system, a transposon-based sequencing method, was used for DNA sequencing (New England Biolabs, Beverly, MA). Transprimer-5, a Tn7-based transposon ( $Km^r$ ) with specific sequencing primer sites at both ends, was transposed *in vitro* into the pMG1 fragment cloned in pBluescript ( $Ap^r$ ). *In vitro* transposition of Transprimer-5 and DNA sequencing were carried out according to the manufacturer's instructions. The pMG1 regions that were not isolated by relational cloning were amplified by long PCR (Expand Long Template PCR system, Roche Diagnostics GmbH, Mannheim, Germany). The PCR product was run on an agarose gel and purified from an agarose block with a Wizard SV Gel and PCR Clean-Up System (Promega, Madison, WI). The purified PCR product was used for sequencing by the primer walking method (Machida *et al.*, 2002). The DNA sequence was determined using the Dye Primer Cycle Sequencing FS Ready Reaction Kit (Perkin-Elmer, CA) with primers N and S (New England Biolabs), and an ABI Prism 377 and 310 Sequencer (Perkin-Elmer). ORF analysis and search for the  $\sigma^{70}$ -dependent promoter sequence were performed with GENETYX ver 5.0 (Genetyx, Tokyo, Japan), and a homology search using BLAST was performed through the NCBI website (<http://www.ncbi.nlm.nih.gov/Tools/index.html>).

#### Transposon mutagenesis of pMG1 with Tn917 and isolation of *tra* mutants

Tn917 was transposed into pMG1 from the temperature-sensitive plasmid pTV1Ts (Youngman, 1987; Weaver & Clewell, 1990). OG1X (pMG1, pTV1Ts) was grown overnight at 30 °C in the presence of chloramphenicol ( $10 \mu\text{g mL}^{-1}$ ) and erythromycin ( $0.5 \mu\text{g mL}^{-1}$ ). After dilution (1/1000) in fresh THB, cells were grown overnight in the absence of antibiotics at 42 °C (the restriction temperature for pTV1Ts replication). The culture was diluted and plated on THB agar plates containing kanamycin plus erythromycin and incubated at 42 °C overnight. Temperature-resistant colonies were picked up and grown at 42 °C to isolate single colonies. Temperature-resistant colonies were assumed to contain a Tn917 insert and were examined for the ability to transfer pMG1 in broth. Broth mating was performed as described previously (Ike *et al.*, 1998). Overnight cultures of the donor (Tn917 insertion mutant) and the recipient (FA2-2) were prepared, and 0.05 mL of the donor and 0.5 mL of the recipient were added to 4.5 mL of fresh THB. The mixture was then incubated at 37 °C with gentle shaking for 4 h. One hundred microliters of the mating mixture was plated onto a selective plate containing kanamycin, fusidic acid and rifampin. Transconjugants were counted after 24 h of incubation at 37 °C. The mutant strains unable to transfer pMG1 were studied further.

#### Mapping of Tn917 insertion

The insertion sites of Tn917 in the transfer-deficient mutants of pMG1 were mapped by the Easy Gene Walking method (Harrison *et al.*, 1997). After three consecutive rounds of PCR with a random primer and three nested primers for the left end of Tn917, the region flanking the left end of Tn917 was amplified. The Tn917 insertion site was determined by sequencing the PCR product purified from an agarose block after agarose gel electrophoresis. The three nested primers (Tn917L, Tn917L1 and Tn917L2) were designed based on sequences close to the left inverted repeat of Tn917. The sequences of the three nested primers and random primer are as follows: Tn917L (5'-GAGAGATGTC ACCGTCAAGT-3'), Tn917L1 (5'-TTAAAACGGTTGAAAA CTG-3'), Tn917L2 (5'-TGTACCACTAATAACTCACAATAG-3') and random primer (5'-NNNNNNNNNGATC-3'). Total DNA from the mutant strains was prepared with ISO-PLANT2 (Nippon Gene, Toyama, Japan) for use as templates.

#### Construction of pKO101

The spectinomycin-resistance *E. coli* vector pKO101 was constructed from the *E. coli* cloning vector pMW119 (Nippon Gene) for the isolation of an autonomously replicating fragment from pMG1 in *E. faecalis*. The spectinomycin-resistance gene, which is expressed in both *E. coli* and *E. faecalis*, was cloned into pMW119 XmaI site from pDL278, an *E. coli*-*E. faecalis* shuttle vector, by PCR using the specific primers *spcF* (5'-TCCCCCGGGGCGATTTTCGGTTCGTG AATACATG-3') and *spcB* (5'-TCCCCCGGGCCAAATTAG AATGAATATTTCCC-3'), which were designed based on the database sequence (accession no. M69221) and have an XmaI recognition sequence at the 5' end. The resulting plasmid was designated pKO101.

#### Transformation and DNA manipulation

DNA manipulation was performed using standard protocols (Ausubel *et al.*, 1987; Sambrook *et al.*, 1989). Transformation of *E. coli* and *E. faecalis* was performed by electroporation (Fujimoto *et al.*, 1991).

#### Nucleotide sequence accession number

The nucleotide sequence data reported here have been deposited in the DDBJ, EMBL and the GenBank nucleotide sequence databases under the accession number AB206333.

#### Results and discussion

##### DNA sequences and genetic organization of the pMG1 plasmid

The pMG1 plasmid is 65 029 bp in total size. The HindIII site upstream of the *traA* gene (ORF2) was designated as bp 1

(Tanimoto & Ike, 2002). The G+C content of pMG1 is 31.59%, lower than *E. faecalis* (33.5–38%) and *E. faecium* (38.3–39%) chromosomes (Schleifer, 1986). ORF analysis performed with GENETYX ver 5.0 (Genetyx) indicated that pMG1 contains 73 ORFs with putative ribosome-binding sites. All the ORFs were found on the same DNA strand and are predicted as being transcribed in the same direction. A homology search for 73 of the pMG1 ORFs was performed by BLAST against the protein database through the NCBI website (<http://www.ncbi.nlm.nih.gov/Tools/index.html>), and the results are shown in Table 2.

Sixty-one of the 73 ORFs showed a high degree of similarity at the amino acid level to ORFs from the pHT $\beta$  plasmids (Tomita & Ike, 2005). The degree of identity between the deduced amino acid residues of the majority of

the 61 homologous ORFs and the pHT $\beta$  residues ranged from 90% to 100%. Like the pHT $\beta$  plasmid (Tomita & Ike, 2005), 22 ORFs (gray-colored ORFs shown in Fig. 1) located between ORF10 and -54 showed similarity at the amino acid level to the ORFs encoded on the *Bacillus anthracis* virulence plasmid pXO2 (96 231 bp, accession number NC002146; 94 829 bp, accession number NC003981). The pXO2 plasmid carries capsule genes and is necessary to produce the disease anthrax (Makino *et al.*, 1989). The degree of amino acid identity between the deduced amino acid residues of the homologous ORFs of pMG1 and pXO2 ranged from about 10% to 50% (Table 2).

Although the functions of most of the pMG1 homologues are not known, the putative functions of a number of proteins can be assigned based on their similarity to other

**Table 2.** Comparison of ORFs identified in the pMG1 plasmid with those of pHT $\beta$  and other elements

ORF	pMG1		pHT $\beta$			Other elements			
	5'	3'	Amino acid size	Matching ORF (pHT $\beta$ )	Amino acid size	Identity <sup>†</sup> (%)	Identification (organism)/protein family	Identity <sup>†</sup> (%)	E-value
1	268	831	187	56	187	100	Hypothetical protein		
2	842	1702	286	57	286	98	<i>traA</i> (pMG1, <i>E. faecium</i> )	100	
3	2096	2593	165				Dehydrofolate reductase ( <i>S. haemolyticus</i> , <i>L. monocytogenes</i> )	73	5e-64
4	3830	3955	41				Hypothetical protein		
5	3996	4286	96				Transposase, IS1485 ( <i>E. faecium</i> )	96	2e-37
6	4514	5158	214				Transposase, IS1485 ( <i>E. faecium</i> )	100	
7	5522	5608	28				Hypothetical protein		
8	5799	6356	185				Hypothetical protein		
9	6858	7112	84	58	84	100	Hypothetical protein		
10	7509	9110	533	59	533	99	Rep protein, pRE25 ( <i>E. faecalis</i> ), pXO2-38 ( <i>B. anthracis</i> )	32	2e-71
11	9345	9644	99	60	99	98	PrgN ( <i>E. faecalis</i> , pCF10), replication and negative control of conjugation	68	2e-33
12	9779	10156	125	61	125	100	Hypothetical protein		
13	10205	12568	787	1	787	99	Tral topoisomerase ( <i>L. lactis</i> ), pXO2-7776 ( <i>B. anthracis</i> )	34	2e-98
14	12886	13485	199	2	199	99	AbiQ ( <i>L. lactis</i> )	19*	0.029
15	13606	13821	71	3	71	100	Hypothetical protein		
16	14203	14409	68	4	68	98	Hypothetical protein		
17	14489	15343	284	5	284	99	Putative ATPase, pXO2-39 ( <i>B. anthracis</i> )	29	8e-28
18	15356	15793	145	6	145	100	Yyfu ( <i>B. subtilis</i> ), two-component sensor protein	19*	3.6
19	15811	16110	99	7	99	100	Hypothetical protein		
20	16113	16556	147	8	147	98	Hypothetical protein		
21	16701	17024	107	9	107	98	Hypothetical protein		
22	17442	21071	1209	10	1209	99	Surface protein, pXO2-28 ( <i>B. anthracis</i> )	33*	5e-36
23	21131	21493	120	11	120	92	Hypothetical protein		
24	21507	21659	50	12	50	94	Hypothetical protein		
25	21766	22149	127	13	127	98	pXO2-26 ( <i>B. anthracis</i> )	26	3e-9
26	22161	23576	471	14	471	99	VirB11 family sec protein, pXO2-25 ( <i>B. anthracis</i> )	40	1e-99
27	23614	24408	264	15	264	99	pXO2-23/24 ( <i>B. anthracis</i> )	50	6e-77
28	24408	25205	265	16	265	99	pXO2-22 ( <i>B. anthracis</i> )	43	8e-51
29	25223	25585	120	17	120	95	pXO2-21 ( <i>B. anthracis</i> )	51	6e-30
30	25599	25745	48	18	48	100	Hypothetical protein		

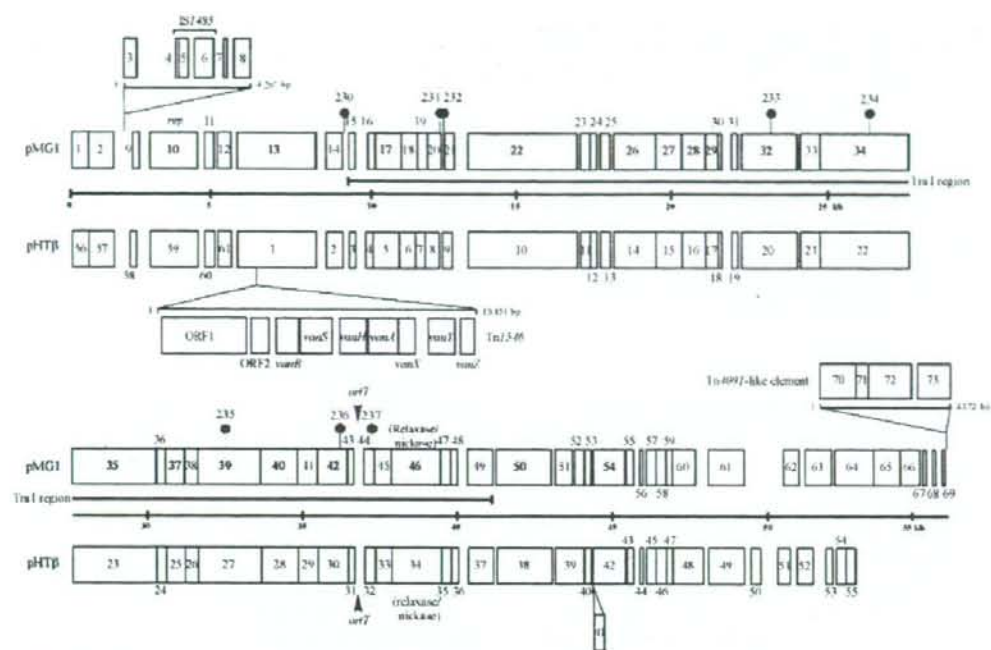


Table 2. Continued.

ORF	pMG1		pHT $\beta$			Identity <sup>†</sup> (%)	Identification (organism)/protein family	Identity <sup>†</sup> (%)	E-value
	5'	3'	Amino acid size	Matching ORF (pHT $\beta$ )	Amino acid size				
31	25997	26272	91	19	91	100	pXO2-18 ( <i>B. anthracis</i> )	47	5e-15
32	26381	28207	608	20	608	97	pXO2-17 ( <i>B. anthracis</i> )	10*	5e-8
33	28220	28936	238	21	238	97	Hypothetical protein		
34	28949	31807	952	22	952	99	traG/D family, pXO2-15/16 ( <i>B. anthracis</i> )	54*/52*	1e-50/1e-170
35	31858	34494	878	23	913	95	pXO2-14 ( <i>B. anthracis</i> )	25*	1e-30
36	34542	34853	103	24	103	98	pXO2-11 ( <i>B. anthracis</i> )	35	2e-12
37	34850	35467	205	25	205	100	pXO2-10 ( <i>B. anthracis</i> )	35	9e-24
38	35483	35950	155	26	155	99	Hypothetical protein		
39	35966	37921	651	27	651	100	pXO2-9 ( <i>B. anthracis</i> )/TraE ( <i>L. lactis</i> )	48/22	1e-168/7e-12
40	37943	39079	378	28	378	100	pXO2-8 ( <i>B. anthracis</i> )/ORF14(Tn916: <i>E. faecalis</i> ), NLP/P60 family cell wall-associated hydrolase	43/32	7e-78/2e-50
41	39094	39744	216	29	216	98	Hypothetical protein		
42	39758	40654	298	30	298	99	pXO2-5 ( <i>B. anthracis</i> )	25	4e-19
43	40690	40920	76	31	76	100	Hypothetical protein		
44	41271	41567	98	32	98	98	pXO2-4 ( <i>B. anthracis</i> )	22	0.013
45	41564	42031	155	33	155	96	Hypothetical protein		
46	42113	43633	506	34	506	99	pXO2-84 ( <i>B. anthracis</i> ), relaxase/nickase family	21*	3e-35
47	43655	43984	109	35	109	100	Hypothetical protein		
48	43986	44249	87	36	87	98	Hypothetical protein		
49	44424	45290	288	37	288	97	Hypothetical protein		
50	45445	47271	608	38	608	99	pXO2-81 ( <i>B. anthracis</i> )/LtrC(pMRC01: <i>L. lactis</i> )	44*/27*	5e-55/1e-19
51	47336	48013	225	39	338	99	Hypothetical protein		
52	48027	48353	108	39	338	98	Hypothetical protein		
53	48362	48622	86	40	85	96	Hypothetical protein		
54	48659	49663	334	42	334	99	pXO2-78 ( <i>B. anthracis</i> )/LtrC-like protein ( <i>S. epidermidis</i> )	40*/32*	2e-49/4e-28
55	49754	50044	96	43	96	97	Hypothetical protein		
56	50206	50397	63	44	63	100	Hypothetical protein		
57	50505	50723	72	45	72	97	Hypothetical protein		
58	50760	51077	105	46	105	95	Hypothetical protein		
59	51088	51285	65	47	65	100	Hypothetical protein		
60	51282	52100	272	48	322	93	Putative ATPase ( <i>Clostridium acetobutylicum</i> )	36*	2e-17
61	52480	53667	395	49	395	87	Hypothetical protein		
62	55007	55549	180	51	135	75	Transcriptional regulator, Cro/C1 family (bacteriophage bIL311, <i>L. lactis</i> prophage ps3)	45*	2e-11
63	55744	56640	298				Transcriptional regulator, Cro/C1 family (bacteriophage bIL311, <i>L. lactis</i> prophage ps3)	41*	3e-11
64	56689	58071	460	51	135	72	Transcriptional regulator, Cro/C1 family (bacteriophage bIL311, <i>L. lactis</i> prophage ps3)	40*	9e-8
65	58085	58867	260	51	135	95	Transcriptional regulator, Cro/C1 family (bacteriophage bIL311, <i>L. lactis</i> prophage ps3)	29*	8e-9
66	58907	59518	203	52	203	100	Sin recombinase (pSK1: <i>S. aureus</i> )	47	2e-43
67	59678	59794	38				Hypothetical protein		
68	59962	60111	49	54	102	100	Hypothetical protein		
69	60282	60452	56	55	105	100	Hypothetical protein		
70	60549	61721	390				Transposase, IS256	100	
71	61724	62170	148				Hypothetical protein (Tn4001: <i>S. aureus</i> )	100	
72	62171	63610	479				Bifunctional AAC/APH (Tn4001: <i>S. aureus</i> )	100	
73	63748	64920	390				Transposase, IS256	100	

The columns list the ORF number, 5' and 3' ends of the ORF, and percentage identity of the pMG1 ORF compared with the matching ORF of pHT $\beta$ , the matching gene in the BLAST search and the name of a similar gene in another organism.

<sup>†</sup>Amino acid sequence identity spans the entire gene. The number with asterisk indicates amino acid sequence identity in conserved domain of the gene.



**Fig. 1.** Genetic map and ORFs deduced from the complete plasmid sequence of pMG1 plasmid and the pHT $\beta$  plasmid. Boxes indicate the ORFs identified on pMG1 and pHT $\beta$ . All ORFs are transcribed from left to right, except ORF1 of Tn1546 on pHT $\beta$ . The locations of the Tn917 insertions in pMG1 (vertical bar with black circle) are shown on the map. The number on the insert indicates the pMG plasmid construct (pMG1::Tn917), which is also shown in Table 1 and Table 3. Gray-colored ORFs had significant degrees of similarity to the ORFs encoded on the *Bacillus anthracis* virulence plasmid pXO2. The horizontal bar designated as Tra region I indicates the transfer-related region. The nucleotide sequence data reported here have been deposited in the DDBJ, EMBL and GenBank nucleotide sequence databases under the accession number AB206333.

well-characterized proteins (Table 2). ORF10 was homologous (32% identity at the amino acid level) to ORF1 of pRE25, a putative Rep protein from an *E. faecalis* multi-resistance plasmid (Schwarz *et al.*, 2001). In addition, ORF10 was homologous at the amino acid level with the Rep proteins of other plasmids, such as pIP501 (31%; *Streptococcus agalactiae*), pAM $\beta$ 1 (31%; *E. faecalis*) and pSM19035 (32%; *Streptococcus pyogenes*). These plasmids are representative of the inc18 plasmid family broadly distributed among the low G+C gram-positive bacteria (Weaver *et al.*, 2002).

The five ORFs 11, -13, -26, -34 and -39 showed similarities to the transfer-related genes of other plasmids: plasmid pCF10 (Hedberg *et al.*, 1996), plasmid pMRC01 (Dougherty *et al.*, 1998) and the Ti plasmid (Goodner *et al.*, 2001; Nelson *et al.*, 2004; Rasko *et al.*, 2004). ORF46 of pMG1 exhibited 99% (503/506) amino acid identity to ORF34 of pHT $\beta$ , which encodes the DNA relaxase/nickase gene essential for conjugation. The DNA sequence of the 192-bp noncoding region between ORF43 and -44 of pMG1 was identical to the 192-bp noncoding region be-

tween ORF31 and -32 of pHT $\beta$ , which has *oriT* activity (Tomita & Ike, 2005).

The DNA sequence of the ORF5-ORF6 region (3921-5286 bp of the pMG1 map) was identical to IS1485, which was originally identified in *E. faecium*, and ORF5 and ORF6 corresponded to *orfA* and *orfB* of IS1485, respectively (Cheng *et al.*, 1999). IS1485 was flanked by a 4-bp direct repeat (TATC) at both ends. There are four ORFs (ORF70, -71, -72 and -73) in the region between 60 448 and 64 970 bp of the plasmid map. ORF70 and ORF73 were identical to the transposase gene of IS256. The regions between 60 448 and 61 771 bp, and 63 647 and 64 970 bp of the plasmid map were identical to IS256 and these two IS256 sequences were located in the same orientation. ORF72 was located between these two IS256 sequences and was identical to the aminoglycoside-resistance gene found in Tn4001 encoding the bifunctional aminoglycoside-modifying enzyme, AAC (*aac6'*)/APH (*aph2''*) conferring kanamycin and gentamicin resistance; however, the corresponding gene in Tn4001 is flanked by two IS256 sequences located in different orientations (Rouch *et al.*, 1987). The DNA



**Table 3.** Transcripts of ORFs produced in pMG1::Tn917 mutant plasmids

Plasmid	ORF15	ORF17	ORF20	ORF21	ORF22	ORF26	ORF32	ORF34	ORF39	ORF40	ORF42	ORF44	ORF49	ORF50
pMG230	Tn917*	+	+	+	+	+	+	+	+	+	+	+	NT	+
pMG231	+	+	Tn917	+	+	+	+	+	+	+	+	+	NT	NT
pMG232	+	+	+	Tn917	-	-	-	-	-	-	-	+	+	+
pMG233	NT	NT	NT	+	NT	+	Tn917	-	-	-	-	+	NT	NT
pMG234	NT	NT	NT	NT	NT	NT	+	Tn917	-	-	-	+	NT	NT
pMG235	+	NT	NT	NT	NT	NT	NT	+	Tn917	-	-	+	NT	NT
pMG236	NT	NT	NT	+	+	+	+	+	+	+	Tn917	+	NT	NT
pMG237	NT	NT	NT	+	+	NT	NT	+	NT	NT	+	Tn917	-	+

\*Tn917 insertions were mapped in the ORFs listed above, except that the Tn917 insert in pMG230 was mapped to a position downstream of the putative promoter sequence for ORF15; +, transcript was detected; -, transcript was not detected; NT, not tested.

sequence of the region (60 448–64 970 bp) was identical to that of Tn4001, with the exception of the orientation of IS256R and a 43-bp deletion upstream from the left end of IS256R. This Tn4001-like transposon was flanked by an 8-bp direct repeat (TAGAATTA) at both ends. The direct repeats at both ends of the Tn4001-like transposon and IS1485 would be generated by target duplication of the insertion sites upon their insertion events. The presence of such duplications will give rise to the plausibility of evolutionarily recent insertion events of those transposable elements without any further rearrangements of the plasmids, which could result from recombination between the directly repeated IS modules.

Sequence comparison of the pMG1 ORFs suggested that ORFs 11–39 were involved in conjugation. However, many pMG1 ORFs did not show any significant level of similarity to any reported proteins; therefore, the plasmid was categorized as a new type of conjugative plasmid based on the data obtained in our previous genetic analysis. Twenty-two ORFs located in the region between 7.5 and 49.7 kb of the pMG1map showed significant homology with ORFs encoded on the pXO2 plasmid, suggesting that both plasmids have the same ancestor plasmid.

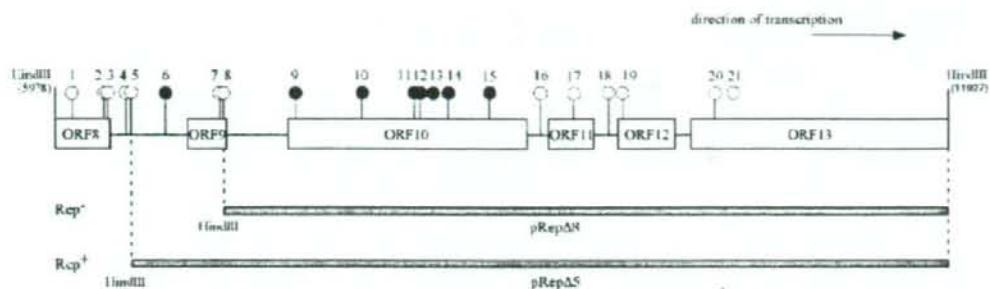
### Transposon insertion mutants and analysis of the transcript

Transposon-insertional mutagenesis of the pMG1 plasmid using Tn917 was performed and 12 mutants that were unable to transfer at a frequency  $> 10^{-7}$  per donor cell in broth mating were isolated. The insertion points in the eight mutants were mapped by DNA sequencing and the locations are shown in Fig. 1. There were potential  $\sigma^{70}$ -dependent promoters (-10 and -35) upstream of ORF15, ORF16, ORF21, ORF22 and ORF44 in the 27.9-kb region that lies between the Tn917 insertion at 13 575 bp (pMG230) and 41 528 bp (pMG237) (Table 1 and Fig. 1). Transcripts were examined in ORFs downstream of insertion of each mutant to examine the possibility of polar effects produced by the insertions. A transcript from each insertion mutant was

examined by Northern analysis with a probe specific for each ORF, as shown in Table 3; these were randomly selected from the ORFs in the region between ORF15 and ORF50. In the case of pMG230 and pMG231, which have Tn917 insertions downstream of the putative promoter for ORF15, and in the C-terminal region of ORF20, respectively, transcripts were detected in ORFs located downstream of each insertion. In the case of pMG232, 233, 234, 235 and 237, polar effects were produced in the genes downstream of the transposon insertions in the region lying between ORF21 and ORF43 or in the region lying between ORF44 and ORF49 (Table 3). These results also suggested that one transcription might initiate from a promoter upstream of ORF21 and terminate downstream of ORF43, and another transcript might initiate from a promoter upstream of ORF44 and terminate downstream of ORF49. These regions corresponded to the Tra I region of the three separate Tra regions (i.e. I, II, and III) of the pHT $\beta$  plasmid (Tomita & Ike, 2005). The minimum Tra I region of the pHT $\beta$  plasmid consisted of ORFs 8–34 of the pHT $\beta$  plasmid, which corresponded to ORFs 20–46 of the pMG1 plasmid. The 31.7-kb region lying between 13.6 and 45.3 kbp of the plasmid map contained ORF15 and ORF49, and was designated as the Tra I region of pMG1.

### Isolation of autonomously replicating HindIII fragment of pMG1 in *E. faecalis*

pKO101 was constructed as described in Materials and methods for the isolation of autonomously replicating fragments (i.e. mini-replicon) from pMG1. pKO101 is an *E. coli* vector with a spectinomycin-resistance gene expressed in both *E. coli* and *E. faecalis*. pMG1 DNA digested with HindIII and HindIII-digested pKO101 were ligated by T4 DNA ligase. The ligation product was used to transform *E. faecalis* OG1X by electroporation, and the transformants were selected for spectinomycin resistance. Transformants were expected to contain the pKO101 plasmid with the pMG1 mini-replicon, which contains the replication origin and *rep* gene(s) of pMG1, because pKO101 cannot replicate



**Fig. 2.** Autonomously replicating 6-kb HindIII fragment of pMG1 in *Enterococcus faecalis*. Open boxes indicate ORFs. ORF8 and ORF12 are truncated; ORF8 does not have the N-terminal region and ORF12 does not have the C-terminal region. The locations of Transprimer-5 insertions in the pRep plasmid (vertical bar with a circular head) are shown on the map. The numbers on the inserts indicate the mutant plasmids (pRep::Transprimer-5). A white head indicates the ability of the mutant plasmid to replicate in *E. faecalis*, and a black head indicates that the mutant plasmid is unable to replicate in *E. faecalis*. The thick gray lines represent the segments contained in the deletion-mutant plasmids pRep $\Delta$ 5 and pRep $\Delta$ 8. The HindIII site at the left end of each gray line is located in Transprimer-5. The Rep<sup>+</sup> or Rep<sup>-</sup> designation beside the gray line indicates the replication phenotype of the corresponding plasmid in *E. faecalis*.

in *E. faecalis* without the pMG1 mini-replicon. Twelve transformants were examined for plasmid content by HindIII digestion and agarose gel electrophoresis. All of them were found to have a 6-kb HindIII fragment. One of the plasmids was named pRep.

#### Sequencing of the 6-kb HindIII fragment

The fragment was 5950-bp long and contained four complete ORFs (ORF9, ORF10, ORF11 and ORF12), and truncated versions of ORF8 and ORF13 (Fig. 2). The N-terminal part was defective in ORF8 and the C-terminal part was defective in ORF13. As shown in Table 2, the predicted amino acid sequences of ORF10 and ORF13 had significant homology with the *rep* gene of pRE25, a multi-resistance plasmid in *E. faecalis*, and the *traI* gene of pMRC01 plasmid from *Lactococcus lactis*, respectively (Dougherty *et al.*, 1998; Schwarz *et al.*, 2001). ORF11 had significant homology with PrgN of the pheromone-responsive *E. faecalis* plasmid pCF10. The PrgN protein is involved in the negative regulation of expression of the mating aggregation substance (Hedberg *et al.*, 1996). ORF8, ORF9 and ORF12 did not show any significant homology with other genes in the database.

To determine the gene(s) involved in pMG1 replication, insertion mutants of pRep were constructed *in vitro* with Transprimer-5 of the GPS-LS Linker Scanning System (New England Biolabs). Twenty-one insertional mutants of the pRep plasmid were isolated, and the locations of the insertions were mapped within the 6-kb HindIII fragment by DNA sequencing. Plasmid DNAs of the insertion mutants were prepared from *E. coli* strains and were used to transform *E. faecalis* OG1X by electroporation. Transformants were selected for spectinomycin resistance. Transformants

were obtained from 13 of the 21 mutant plasmids. No transformants were obtained from eight mutant plasmids. As shown in Fig. 2, seven of the eight insertions were mapped in ORF10, and the other insertion in mutant plasmid #6 was mapped to 6663 bp of the pMG1 map within the noncoding region between ORF8 and ORF9. As these insertions abolished the ability to replicate, it was not possible to transform OG1X to spectinomycin resistance with these plasmids. These results implied that ORF10 and the DNA sequence in the noncoding region between ORF8 and ORF9 were required for the replication of pMG1. In our previous study (Tomita & Ike, 2005), DNA sequence analysis suggested that ORF59 of pHT $\beta$  was a *rep* gene, and corresponded to ORF10 of pMG1 based on its amino acid identity (Table 2). There were two inverted repeat sequences in the noncoding region between the insertion site of mutant plasmid #5 and ORF9. The insertion of mutant plasmid #6 was located in one of the two inverted repeat sequences (Fig. 3). These data implied that the inverted repeat sequences might be necessary as a *cis*-acting site for plasmid replication. The insertional mutant plasmids had three HindIII sites: one located in Transprimer-5 and one at each end of the 5950-bp HindIII fragment. Two deletion-mutant plasmids, pRep $\Delta$ 5 and pRep $\Delta$ 8, were constructed from mutant plasmids #5 and #8, respectively, to confirm the requirement for the region located between ORF8 and ORF9 (Fig. 2) by partial digestion by HindIII, followed by self-ligation. These were then used to transform *E. coli* DH5 $\alpha$ . Plasmid DNAs were prepared from the transformants, and their structures were examined by agarose gel electrophoresis. A deletion mutant lacking ORF8 and ORF9 was obtained from plasmid #8 and was designated as pRep $\Delta$ 8. A deletion mutant lacking ORF8 was obtained



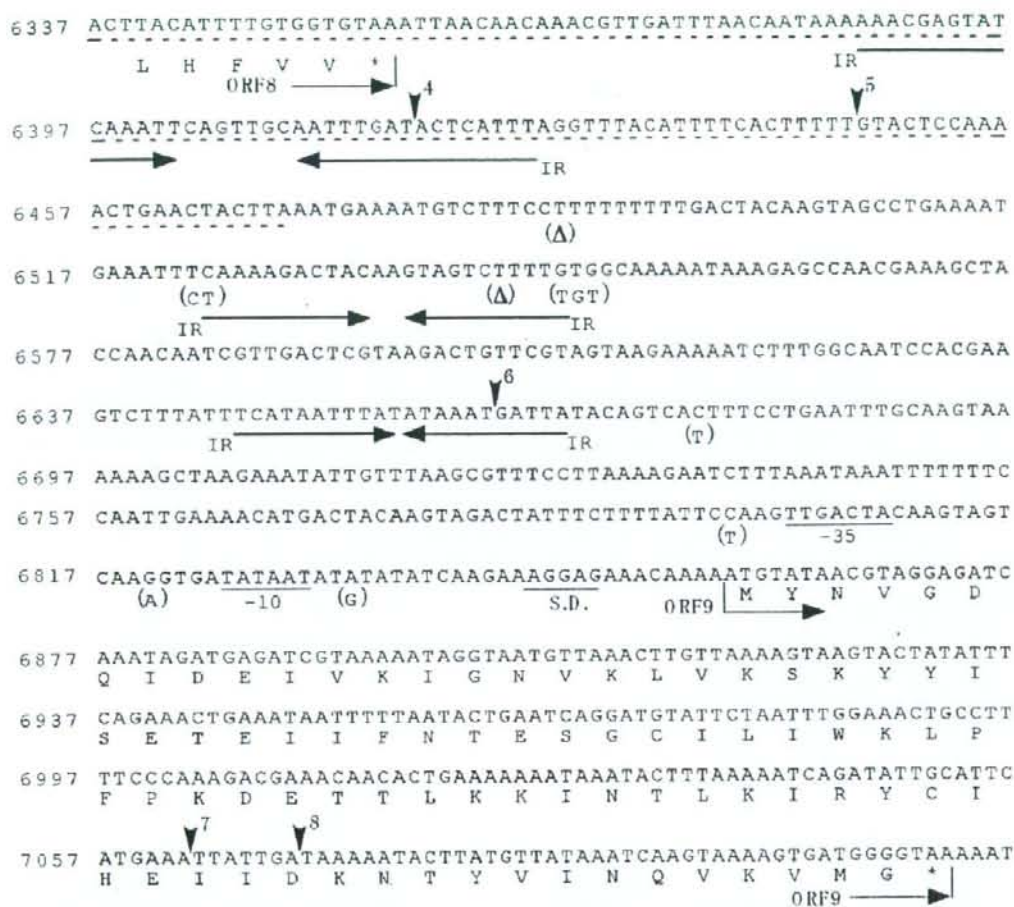


Fig. 3. Nucleotide sequences of the region between ORF8 and ORF9 of the pMG1 plasmid. The 780-bp DNA sequence of the region between ORF8 and ORF9 is shown. The horizontal arrows labeled with IR indicate the inverted repeats. The numbered arrowheads facing downwards indicate the locations of the Transprimer-5 insertion shown in Fig. 2. The nucleotide substitutions in pHT $\beta$  are shown in parentheses below the sequences.  $\Delta$ , indicates deletion. pMG1 and pHT $\beta$  do not share any homology in the region underlined with a dotted line. The putative promoter region (-35 and -10) and ribosome-binding site [Shine-Dalgarno sequence (S.D.)] are shown.

from plasmid #5, and was designated as pRep $\Delta$ 5. The two deletion-mutant plasmids were used to transform *E. faecalis* OG1X to examine their ability to replicate. Spectinomycin-resistant transformants were obtained with pRep $\Delta$ 5, but not with pRep $\Delta$ 8. This result indicated that the DNA sequence in the noncoding region between ORF9 and the insertion point of #5 was required for the replication of pMG1 (Fig. 2).

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## Isolation of VanB-Type *Enterococcus faecalis* Strains from Nosocomial Infections: First Report of the Isolation and Identification of the Pheromone-Responsive Plasmids pMG2200, Encoding VanB-Type Vancomycin Resistance and a Bac41-Type Bacteriocin, and pMG2201, Encoding Erythromycin Resistance and Cytolysin (Hly/Bac)<sup>∇</sup>

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Eighteen identical VanB-type *Enterococcus faecalis* isolates that were obtained from different hospitalized patients were examined for their drug resistance and plasmid DNAs. Of the 18 strains, 12 strains exhibited resistance to erythromycin (Em), gentamicin (Gm), kanamycin (Km), tetracycline (Tc), and vancomycin (Van) and produced cytolysin (Hly/Bac) and a bacteriocin (Bac) active against *E. faecalis* strains. Another six of the strains exhibited resistance to Gm, Km, Tc, and Van and produced a bacteriocin. Em and Van resistance was transferred individually to *E. faecalis* FA2-2 strains at a frequency of about  $10^{-4}$  per donor cell by broth mating. The Em-resistant transconjugants and the Van-resistant transconjugants harbored a 65.7-kbp plasmid and a 106-kbp plasmid, respectively. The 106-kbp and 65.7-kbp plasmids isolated from the representative *E. faecalis* NKH15 strains were designated pMG2200 and pMG2201, respectively. pMG2200 conferred vancomycin resistance and bacteriocin activity on the host strain and responded to the synthetic pheromone cCF10 for pCF10, while pMG2201 conferred erythromycin resistance and cytolysin activity on its host strain and responded to the synthetic pheromone cAD1 for pAD1. The complete DNA sequence of pMG2200 (106,527 bp) showed that the plasmid carried a Tn1549-like element encoding vanB2-type resistance and the Bac41-like bacteriocin genes of pheromone-responsive plasmid pY114. The plasmid contained the regulatory region found in pheromone-responsive plasmids and encoded the genes *prgX* and *prgQ*, which are the key negative regulatory elements for plasmid pCF10. pMG2200 also encoded TraE1, a key positive regulator of plasmid pAD1, indicating that pMG2200 is a naturally occurring chimeric plasmid that has a resulting *prgX-prgQ-traE1* genetic organization in the regulatory region of the pheromone response. The functional *oriT* region and the putative relaxase gene of pMG2200 were identified and found to differ from those of pCF10 and pAD1. The putative relaxase of pMG2200 was classified as a member of the MOB<sub>MG</sub> family, which is found in pheromone-independent plasmid pHTβ of the pMG1-like plasmids. This is the first report of the isolation and characterization of a pheromone-responsive highly conjugative plasmid encoding vanB resistance.

Multiple-drug-resistant enterococci and vancomycin-resistant enterococci (VRE), in particular, are opportunistic pathogens and major causes of nosocomial infections in immunocompromised patients (4, 7, 44). The isolation of VRE (VanA type) was first reported in 1988 in the United Kingdom (67) and France (41), and shortly thereafter it was reported in the United States (55). Since then, VRE have been identified in many countries. VRE have caused an increasing number of treatment-related problems, especially in the United States (4, 43), where they are estimated to account for about 15% of nosocomial enterococcal isolates (15). In Asia, VRE have been isolated from hospitalized patients or food animals in China, Japan, South Korea, Taiwan, and Thailand (49, 74). In particular, they have frequently been isolated in South Korea (72)

and Taiwan (40). Since the first report of the isolation of VanA-type VRE from a patient in Japan, VRE have been isolated from both sporadic individual cases and outbreaks of nosocomial infections in several hospitals (29, 49). However, an outbreak of VRE nosocomial infection is still a very rare event in university teaching hospitals in Japan.

VRE isolates of the VanA and VanB types are the most commonly identified VRE isolates to be acquired. Their genomes are composed of operon gene clusters, and isolates of the VanA and VanB types have the same basic mechanism of resistance (12). The VanA-type determinant is encoded on the Tn1546 transposon or a Tn1546-like transposon (2), which frequently resides on a conjugative plasmid in VanA-type *Enterococcus faecium* (41). The *vanB* gene has been divided into three subtypes, *vanB1*, *vanB2*, and *vanB3*, on the basis of differences in the sequence of the *vanB* ligase (13, 22, 50). The *vanB2* determinant is encoded on conjugative transposon Tn1549 (34 kb) (30) and the closely related transposon Tn5382 (27 kb) (3), which have similarities with the Tn916 family of conjugative transposons (10, 26). The transposable elements

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TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant features	Reference or source
<b>Strains</b>		
<i>E. faecalis</i>		
FA2-2	<i>rif fus</i>	11
JH2SS	<i>spc str</i>	59
UV202	<i>rif fus</i> , recombination-deficient mutant of JH2-2	71
OG1S(OG1-10)	<i>str</i> , derivative of OG1	18
OG1X	<i>str</i> , protease-negative derivative of OG1-10	37
NKH15	Representative of vancomycin-resistant ( <i>vanB2</i> -type) first hospital outbreak strain, pMG2200 (Van <sup>r</sup> , Bac), pMG2201 (Em <sup>r</sup> , Cyt)	This study; 47
<i>E. faecium</i>		
BM4105RF	<i>rif fus</i> , derivative of plasmid-free <i>E. faecium</i> BM4105	65
BM4105SS	<i>spc str</i> , derivative of plasmid-free <i>E. faecium</i> BM4105	65
<i>E. coli</i> DH5 $\alpha$	<i>endA1 recA1 gyrA96 thi-1 hsdR17 supE44 relA1 <math>\Delta</math>(argE-lacZYA)U169</i>	Bethesda Research Laboratories
<b>Plasmids</b>		
pMG2200	<i>vanB2</i> , Bac44, 106.5-kb pheromone (cCF10)-responsive conjugative plasmid from NKH15	This study
pMG2201	Em <sup>r</sup> , cytolsin (Hly/Bac), 60-kb pheromone (cAD1)-responsive conjugative plasmid from NKH15	This study
pMG2210	pAM401 containing the 322-bp <i>oriT</i> region of pMG2200	This study
pAM401	<i>E. coli-E. faecalis</i> shuttle, <i>cat tet</i>	70
pUC18	<i>E. coli</i> cloning vector, Amp <sup>r</sup>	Nippon gene
pMG326	pMW119 containing a 16.7-kbp EcoRI-SalI fragment of pPD1; pheromone regulatory region	28, 58
pY114	Bac41, a 61-kb pheromone-responsive conjugative plasmid from Y1714	64

can be located on a conjugative or a nonconjugative plasmid or on the chromosome (3, 30, 53, 54). To our knowledge, there has been no report of a *vanB* determinant located on the pheromone-responsive highly conjugative plasmid.

The first outbreak of a VRE nosocomial infection in Japan was caused by a VanB-type *Enterococcus faecalis* strain in a hospital setting in July 1999. Twenty VanB-type *E. faecalis* isolates were obtained from three clinical specimens, nine rectal swab specimens from asymptomatic carriers, and eight swab specimens from the hospital environment and were examined for drug resistance by pulsed-field gel electrophoresis (PFGE) (47). Southern blot analysis of the PFGE gel with a *vanB* probe implied that the VanB-type determinants resided on a 110-kbp plasmid in 19 strains obtained from among the 20 isolates (47). As described in this report, we examined the plasmids carried by the VanB-type VRE and identified two pheromone-responsive plasmids: one plasmid encoding vancomycin resistance and a bacteriocin and the other plasmid encoding erythromycin resistance and cytolsin.

#### MATERIALS AND METHODS

**Bacterial strains, plasmids, and media.** The bacterial strains and plasmids used in this study are listed in Table 1 and Table 2. Of the 18 isolates studied, the results of PFGE and Southern hybridization analysis with the *vanB* probe for 11 isolates (i.e., isolates NKH1 to NKH7 and NKH15 to NKH18) have been described in a previous study (47). The *E. faecalis* strains were grown in brain heart infusion broth and agar (Difco Laboratories) or Todd-Hewitt broth (Difco Laboratories) at 37°C. *Escherichia coli* strains were grown in Luria-Bertani medium (GIBCO BRL, Life Technologies). The following antibiotics were used at the indicated concentrations for the selection of *E. faecalis*: erythromycin, 12.5  $\mu\text{g ml}^{-1}$ ; streptomycin, 250  $\mu\text{g ml}^{-1}$ ; spectinomycin, 250  $\mu\text{g ml}^{-1}$ ; chloramphenicol, 20  $\mu\text{g ml}^{-1}$ ; rifampin (rifampicin), 25  $\mu\text{g ml}^{-1}$ ; and fusidic acid, 25  $\mu\text{g ml}^{-1}$ . The following antibiotics were used at the indicated concentrations for the selection of *E. coli*: ampicillin, 100  $\mu\text{g ml}^{-1}$ , and chloramphenicol, 50  $\mu\text{g ml}^{-1}$ . All

antibiotics were obtained from Sigma Chemical Co. 5-Bromo-4-chloro-3-indolyl- $\beta$ -D-galactopyranoside was used at 40  $\mu\text{g ml}^{-1}$ .

**Antimicrobial susceptibility testing.** The MICs of the antibiotics were determined by the agar dilution method. An overnight pure culture of each strain grown in Mueller-Hinton broth (Nissui, Tokyo, Japan) was diluted 100-fold with fresh broth. An inoculum of approximately  $5 \times 10^5$  cells was plated on a series of Mueller-Hinton broth (Eiken, Tokyo, Japan) cultures containing a range of concentrations of the test drug. The plates were incubated at 37°C, and the susceptibility results were finalized at 24 h of incubation. Susceptibility testing and interpretation of the results were in compliance with standards recommended by Clinical and Laboratory Standards Institutes (formerly NCCLS). *E. faecium* ATCC 9790 was used as a control strain.

**Soft agar assay for bacteriocin production and immunity.** The bacteriocin production assay was performed as described previously (36, 62). The test for immunity to the bacteriocin was performed essentially as described previously (36).

**Plasmid and DNA methodology.** Recombinant DNA techniques, analyses of plasmid DNA with restriction enzymes, and agarose gel electrophoresis were carried out by standard methods (56). The introduction of plasmid DNA into bacterial cells was carried out by electrotransformation, as described previously (27). Plasmid DNA was purified from *E. faecalis* as described previously (68). Restriction enzymes were purchased from New England Biolabs and Roche Co. PCR was performed with a Perkin-Elmer Cetus apparatus. *Taq* DNA polymerase was obtained from Takara.

**DNA sequence analysis.** Sequence analysis was performed with a Dye primer and a Dye Terminator cycle sequencing kit (Applied Biosystems) and with a 377 DNA sequencer and 310 gene analyzer (ABI Prism). To determine the DNA sequence of plasmid pMG2200, a shotgun cloning method was used (56). To determine the DNA sequences in the gap regions, PCR amplification was performed to obtain PCR products covering the gaps. The PCR products were sequenced directly by using custom primers. Open reading frames (ORFs) were identified and initially analyzed with Genetyx (version 5.1) computer software and the BLAST database to search for putative genes (1).

**Conjugation experiments.** Filter mating was performed as described previously (16, 37). Broth mating was carried out for 4 h. Transfer frequencies were expressed as the number of transconjugants per donor cell (at the end of mating).

**Pheromone response (clumping) assay.** Pheromone response assays were performed as described previously (18). The synthetic enterococci pheromones



TABLE 2. VanB-type vancomycin-resistant first outbreak *E. faecalis* strains isolated from a Japanese hospital

Strain <sup>a</sup>	Date of isolation (yr/mo/day)	Source	Diagnosis or underlying disease	VCM	TEIC	ABPC	CPFX	EM	GM	KM	SM	CP	TC	Transfer frequency <sup>c</sup> (per donor cell)				Bacteriocin production <sup>d</sup>	Cytolysin (Hly/Bac)	pMG2200-like	pMG2201-like
														Vancomycin resistance	Broth mating (4 h)	Filter mating (16 h)	Erythromycin resistance				
NKH1	1999/7/9	Sputum	Urinary tract infection	128	0.25	4	64	0.25	>1,024	>1,024	32	8	64	10 <sup>-7</sup>	10 <sup>-3</sup>	<10 <sup>-7</sup>	<10 <sup>-7</sup>	P	N	P	N
NKH2	1999/7/19	Urine	Cerebral infarction	256	0.25	4	64	>1,024	>1,024	>1,024	32	8	64	10 <sup>-5</sup>	10 <sup>-4</sup>	10 <sup>-4</sup>	10 <sup>-2</sup>	P	P	P	P
NKH3	1999/7/31	Rectal swab	Diarrhea	64	0.25	4	64	>1,024	>1,024	>1,024	32	8	32	10 <sup>-5</sup>	10 <sup>-4</sup>	10 <sup>-4</sup>	10 <sup>-2</sup>	P	P	P	P
NKH4	1999/7/27	Urine	Cerebral infarction	64	0.25	4	64	>1,024	>1,024	>1,024	32	8	32	10 <sup>-5</sup>	10 <sup>-2</sup>	10 <sup>-2</sup>	10 <sup>-2</sup>	P	P	P	P
NKH5	1999/8/1	Rectal swab	Gall bladder cancer	64	0.25	4	64	0.125	>1,024	>1,024	32	4	64	10 <sup>-5</sup>	10 <sup>-4</sup>	<10 <sup>-7</sup>	<10 <sup>-7</sup>	P	N	P	N
NKH6	1999/8/1	Rectal swab	Esophagus cancer	64	0.25	4	64	>1,024	>1,024	>1,024	32	8	64	10 <sup>-4</sup>	10 <sup>-2</sup>	10 <sup>-2</sup>	10 <sup>-2</sup>	P	P	P	P
NKH7	1999/8/1	Rectal swab	Parkinson's disease	64	0.25	8	64	0.125	>1,024	>1,024	32	4	64	10 <sup>-5</sup>	10 <sup>-3</sup>	<10 <sup>-7</sup>	<10 <sup>-7</sup>	P	N	P	N
NKH8	1999	Rectal swab	Pneumonia	256	0.25	8	64	>1,024	>1,024	>1,024	32	8	64	10 <sup>-3</sup>	10 <sup>-3</sup>	10 <sup>-3</sup>	10 <sup>-2</sup>	P	P	P	P
NKH9	1999	Rectal swab	Melroy-Weiss syndrome	256	0.25	8	64	>1,024	1,024	>1,024	32	8	64	10 <sup>-7</sup>	10 <sup>-3</sup>	10 <sup>-3</sup>	10 <sup>-2</sup>	P	P	P	P
NKH10	1999	Rectal swab	Bedsores	256	0.25	4	64	>1,024	>1,024	>1,024	32	8	64	10 <sup>-6</sup>	10 <sup>-2</sup>	10 <sup>-3</sup>	10 <sup>-2</sup>	P	P	P	P
NKH11	1999	Rectal swab	Ileocecal abscess	64	0.25	4	64	0.125	>1,024	>1,024	32	8	64	10 <sup>-5</sup>	10 <sup>-2</sup>	<10 <sup>-7</sup>	<10 <sup>-7</sup>	P	N	P	N
NKH12	1999	Rectal swab	Lung cancer	256	0.25	4	64	>1,024	>1,024	>1,024	32	8	64	10 <sup>-4</sup>	10 <sup>-2</sup>	10 <sup>-3</sup>	10 <sup>-2</sup>	P	P	P	P
NKH13	1999	Rectal swab	Cerebral infarction	256	0.125	8	64	>1,024	>1,024	>1,024	32	8	64	10 <sup>-5</sup>	10 <sup>-2</sup>	10 <sup>-3</sup>	10 <sup>-2</sup>	P	P	P	P
NKH14	1999	Sputum	Pneumonia	256	0.25	8	64	>1,024	>1,024	>1,024	32	8	64	10 <sup>-6</sup>	10 <sup>-2</sup>	10 <sup>-3</sup>	10 <sup>-2</sup>	P	P	P	P
NKH15	1999/8/1	Rectal swab	Hepatic cancer	64	0.25	4	64	>1,024	>1,024	>1,024	32	8	32	10 <sup>-4</sup>	10 <sup>-2</sup>	10 <sup>-2</sup>	10 <sup>-2</sup>	P	P	P	P
NKH16	1999/8/1	Rectal swab	Cholelithiasis	64	0.25	4	64	0.125	>1,024	>1,024	32	4	32	10 <sup>-5</sup>	10 <sup>-2</sup>	<10 <sup>-7</sup>	<10 <sup>-7</sup>	P	N	P	N
NKH17	1999/8/1	Rectal swab	Cholecystitis	64	0.125	8	64	0.125	>1,024	>1,024	32	4	32	10 <sup>-3</sup>	10 <sup>-2</sup>	<10 <sup>-7</sup>	<10 <sup>-7</sup>	P	N	P	N
NKH18	1999/8/1	Rectal swab	Dementia	64	<0.125	8	64	>1,024	>1,024	>1,024	32	8	32	10 <sup>-3</sup>	10 <sup>-2</sup>	10 <sup>-4</sup>	10 <sup>-2</sup>	P	P	P	P

<sup>a</sup> The Tn 1546-like element was located on plasmids in all strains.

<sup>b</sup> Abbreviations: VCM, vancomycin; TEIC, teicoplanin; ABPC, ampicillin; CPFX, ciprofloxacin; EM, erythromycin; GM, gentamicin; KM, kanamycin; SM, streptomycin; CP, chloramphenicol; TC, tetracycline.

<sup>c</sup> The wild-type strains and *E. faecalis* FA2-2 were used as the donor and recipient, respectively (11).

<sup>d</sup> The indicator strains used for bacteriocin activity were *Staphylococcus aureus* FDA209P, *E. faecalis* FA2-2 and DG15, *E. faecium* BM4105RF, and *Enterococcus hirae* ATCC 9790 (61). P, positive; N, negative. \* The pMG2200-like plasmid is 106.5 kb, vanB2, Bac41, and cF10 responsive. The pMG2201-like plasmid is 65.7 kb, EMV, cytolysin (Hly/Bac) positive, and cadI responsive. P, positive; N, negative.

TABLE 3. Sequences of oligonucleotides used in the study

Oligonucleotide name	Sequence (5'-3')	Plasmid generated with the primer or use
V43622F	CCG <u>GGA TCC</u> AGA ACA ATC AAC AAC TAA TTA GGC	pMG2210
V43943R	GCG <u>GGA TCC</u> TAT TTA TTC TCT AGT ATT CGC	pMG2210
cylL <sub>1</sub>	GAT GGA GGG TAA GAA TTA TGG	57
cylL <sub>2</sub>	GCT TCA CCT CAC TAA GTT TTA TAG	57
cylL <sub>3</sub>	GAA GCA CAG TGC TAA ATA AGG	57
cylL <sub>2</sub>	GTA TAA GAG GGC TAG TTT CAC	57
cylB-TE15	ATT CCT ACC TAT GIT CTG TTA	20
cylB-TE16	AAT AAA CTC TTC TTT TCC AAC	20
cylA-TE17	TGG ATG ATA GTG ATA GGA AGT	20
cylA-TE18	TCT ACA GTA AAT CTT TCG TCA	20

\* Underlining indicates the BamHI restriction endonuclease recognition sequence GGATCC.

cAD1, cCF10, cPD1, cOB1, and cAM373 were prepared by Sawaday Technology Co., Ltd. (Tokyo, Japan).

**Identification and genetic analyses of the *oriT* region of the pMG2200 plasmid.** The amplified DNAs were cloned into the pAM401 vector plasmid. The oligonucleotides used as PCR primers were V43622F and V43943R, respectively (Table 3). Each of the pAM401 derivatives carrying pMG2200 segments to be tested for *oriT* activity was introduced by electrotransformation into *E. faecalis* UV202, which is defective in homologous recombination (63, 71). Conjugative plasmid pMG2200 was then introduced into each of the transformants carrying the pAM401 derivative (Cm<sup>r</sup>) by conjugation. Both broth matings and filter matings were performed with the transconjugants carrying the two plasmids as donor strains and JH2SS as the recipient strain.

**Southern hybridization analysis.** Southern hybridization was performed with the digoxigenin-based nonradioisotope system of Boehringer GmbH (Mannheim, Germany), and all procedures were based on the manufacturer's manual and standard protocols (56). Plasmid pMG326 was used as the probe, as it contains the regulatory region of pheromone plasmid pPD1 (58).

**Detection of the cytotoxin (Hly/Bac) genes in the VRE isolates.** To detect the cytotoxin (Hly/Bac) gene encoded on the pAD1-like plasmid (31, 32, 34, 35, 36), PCR amplification with primer sets specific for the *cyl* genes *cylL<sub>1</sub>*, *cylL<sub>2</sub>*, *cylL<sub>3</sub>*, *cylA*, and *cylB* was performed as described in the literature (20, 57).

**PFGE.** PFGE was carried out in a 1% agarose gel with 0.5% Tris-borate-EDTA buffer; and the following settings were applied: 1 to 23 s, 6 V/cm, and 22 h (with the CHEF Mapper system [Bio-Rad]) (49).

**Nucleotide sequence accession number.** The nucleotide sequence data reported in this article are available from the DDBJ, EMBL, and GenBank nucleotide sequence databases under accession number AB374546.

## RESULTS AND DISCUSSION

**Antimicrobial susceptibilities of the VRE isolates.** The MICs of the various antimicrobial agents used to test the 18 VRE isolates are shown in Table 2. All of the VRE isolates showed high levels of resistance to vancomycin (MICs, 32 to 256 µg/ml) and susceptibility to teicoplanin (MIC, 0.125 to 0.25 µg/ml). There were high levels of resistance to the aminoglycosides gentamicin and kanamycin and to tetracycline. Of the 18 VRE isolates, 12 isolates had high levels of resistance to erythromycin, and the remaining 6 isolates were susceptible to erythromycin.

PFGE analysis of SmaI-digested total DNA from the 18 VRE isolates showed that there were two PFGE patterns which differed with regard to the positions of two bands in the lower portion of the gels (data not shown); these observations are indicative of differences in plasmid contents (Fig. 1). These data indicate that the strains were identical but that the identical host strains contained different plasmids.

**Bacteriocin production.** All of the 18 isolates showed bacteriocin activity against the *E. faecalis* strain among the indicator strains examined (Table 2). Of the 18 isolates, the 12 isolates that were resistant to erythromycin also showed cytotoxic activity.

**Conjugative experiments with drug resistance.** The conjugative transfer of each of the vancomycin, erythromycin, gentamicin, and tetracycline resistance determinants from each of the VRE isolates to *E. faecalis* FA2-2 or *E. faecium* BM4105RF was examined by broth mating for 4 h or filter mating for 18 h at 37°C. The vancomycin resistance of the 18 isolates and the erythromycin resistance of the 12 isolates transferred to *E. faecalis* FA2-2 at frequencies of about 10<sup>-3</sup> to 10<sup>-5</sup> per donor cell in the broth mating experiments. Resistance to the other drugs was not transferred to *E. faecalis* FA2-2 at a detectable frequency (less than 10<sup>-8</sup> per donor cell), even by filter mating, suggesting that resistance to the other drugs might be encoded on a nonconjugative plasmid(s) or the chromosome. The transconjugants of each strain were examined for their drug resistance and bacteriocin production. The vancomycin- and erythromycin-resistant transconjugants showed both bacteriocin and cytotoxic activities, the vancomycin-resistant transconjugants showed only bacteriocin activity, and the erythromycin-resistant transconjugant showed only cytotoxic activity.

The EcoRI restriction profiles of the plasmids found in the vancomycin-resistant transconjugants and the erythromycin-



FIG. 1. Agarose gel electrophoresis of restriction endonuclease-digested DNA of pMG2200 and pMG2201 and Southern hybridization with genes specific from the pheromone-responsive plasmid. Agarose gel electrophoresis of EcoRI-digested plasmid DNA (A) and Southern hybridization with pMG326 (58) (B). Lanes: 1, HindIII-digested bacteriophage lambda DNA; 2, wild-type strain NKH15; 3, pMG2200; 4, pMG2201; 5, pAD1.



TABLE 4. ORFs identified in pMG2200

ORF	Direction <sup>a</sup>	5' residue no.	3' residue no.	Gene (bp)/ protein (aa)	Homology	% Identity	% Similarity	Organism or plasmid	Function
1	CW	9	2012	2,004/667	PCP50	23	44	<i>Clostridium perfringens</i> pCP13	Transmembrane protein, ATPase
2	CW	2019	2399	381/126	PCP49	25	43	<i>Clostridium perfringens</i> pCP13	
3	CW	2636	4351	1,716/571	PCP48/46 (combined)	30	49	<i>Clostridium perfringens</i> pCP13	TraC-like transmembrane bound ATPase
4	CW	4404	5084	1,077/359	PCP46	30	53	<i>Clostridium perfringens</i> pCP13	
5	CW	5294	6445	1,149/383	LtrC-like	28	45	<i>Bacillus thuringiensis</i>	
6	CW	6442	7524	1,083/360	ORF14	48	66	<i>Enterococcus faecalis</i> Tn916	
7	CW	7579	7821	243/80	Hypothetical protein	42	58	<i>Lactobacillus plantarum</i>	
8	CW	7822	8148	327/108	PemK-like protein	47	68	<i>Enterococcus faecium</i>	Toxin of the ChpA-ChpR toxin-antitoxin system, endoribonuclease
9	CW	8240	8579	341/113					Interrupted by Tn1549-like insertion at 114th amino acid of the original ORF
10	CW	8831	10030	1,200/399	ORF13	100	100	<i>Enterococcus faecalis</i> VS83 Tn1549	
11	CW	10052	10264	213/70	ORF14	100	100	<i>Enterococcus faecalis</i> VS83 Tn1549	
12	CW	10339	10815	477/158	ORF15	100	100	<i>Enterococcus faecalis</i> VS83 Tn1549	
13	CW	10812	12620	1,806/564	ORF16, TrsK-like protein	99	99	<i>Enterococcus faecalis</i> VS83 Tn1549	TraG/TraD/VirD4 family involved in type IV secretion
14	CW	13004	13867	864/287	ORF17	100	100	<i>Enterococcus faecalis</i> VS83 Tn1549	
15	CW	13898	14440	543/180	ORF18, Muni-like protein	100	100	<i>Enterococcus faecalis</i> VS83 Tn1549	Methyltransferase
16	CW	14454	14876	423/140	ORF19	100	100	<i>Enterococcus faecalis</i> VS83 Tn1549	
17	CW	14806	17205	2,400/799	ORF20, TrsE-like protein	100	100	<i>Enterococcus faecalis</i> VS83 Tn1549	VirB4
18	CW	17237	19228	1,992/663	ORF21	100	100	<i>Enterococcus faecalis</i> VS83 Tn1549	
19	CW	19251	19502	252/83	ORF22	100	100	<i>Enterococcus faecalis</i> VS83 Tn1549	
20	CW	19492	20721	1,230/409	ORF23	100	100	<i>Enterococcus faecalis</i> VS83 Tn1549	
21	CW	20718	22799	2,082/693	ORF24	100	100	<i>Enterococcus faecalis</i> VS83 Tn1549	DNA topoisomerase III-like protein
22	CW	22948	26868	3,921/1,306	ORF25, LtrC-like protein	100	100	<i>Enterococcus faecalis</i> VS83 Tn1549	
23	CW	26869	27813	945/314	ORF26	100	100	<i>Enterococcus faecalis</i> VS83 Tn1549	
24	CCW	28766	28320	447/148	ORF27	100	100	<i>Enterococcus faecalis</i> VS83 Tn1549	
25	CCW	30435	29107	1,329/442	ORF28, relaxase	100	100	<i>Enterococcus faecalis</i> VS83 Tn1549	
26	CCW	30725	30396	330/109	ORF29	100	100	<i>Enterococcus faecalis</i> VS83 Tn1549	
27	CCW	31354	30983	372/123	ORF30	100	100	<i>Enterococcus faecalis</i> VS83 Tn1549	
28	CW	32627	33286	660/219	VanR <sub>B</sub>	100	100	<i>Enterococcus faecalis</i> VS83 Tn1549	Two-component regulatory system, regulator protein
29	CW	33289	34632	1,344/447	VanS <sub>B</sub>	99	99	<i>Enterococcus faecalis</i> VS83 Tn1549	Two-component regulatory system, sensor protein
30	CW	34803	35609	807/268	VanY <sub>B</sub>	100	100	<i>Enterococcus faecalis</i> VS83 Tn1549	DD-Carboxypeptidase
31	CW	35627	36454	828/275	VanW	100	100	<i>Enterococcus faecalis</i> VS83 Tn1549	
32	CW	36451	37422	972/323	VanH <sub>B</sub>	94	97	<i>Enterococcus faecalis</i> VS83 Tn1549	D-2-Hydroxyacid dehydrogenase
33	CW	37415	38443	1,029/342	VanB2	99	99	<i>Enterococcus faecalis</i> VS83 Tn1549	D-Ala-D-Lac ligase
34	CW	38449	39057	609/202	VanX <sub>B</sub>	95	97	<i>Enterococcus faecalis</i> VS83 Tn1549	DD-Dipeptidase
35	CW	39645	40076	432/143	ORF7	99	100	<i>Enterococcus faecalis</i> VS83 Tn1549	
36	CW	40083	40316	234/77	ORF8	85	88	<i>Enterococcus faecalis</i> VS83 Tn1549	

Continued on following page

TABLE 4—Continued

ORF	Direction <sup>a</sup>	5' residue no.	3' residue no.	Gene (bp)/ protein (aa)	Homology	% Identity	% Similarity	Organism or plasmid	Function
37	CW	40740	40940	201/66	Xis-TnJ549	98	100	<i>Enterococcus faecalis</i> V583 TnJ549	Excisionase
38	CW	41024	42217	1,194/397	Int-TnJ549	100	100	<i>Enterococcus faecalis</i> V583 TnJ549	Integrase
39	CW	42392	42492	101/32					
40	CW	42522	42695	174/57					
41	CW	42702	43643	942/313	PCP43	22	44	<i>Clostridium perfringens</i> pCP13	
42	CW	44015	44296	282/93	PCP41	30	50	<i>Clostridium perfringens</i> pCP13	Putative transcriptional regulator
43	CW	44289	44681	393/130	Hypothetical protein	26	47	<i>Agrobacterium tumefaciens</i>	Transcriptional regulator, AraC family
44	CW	44674	46734	2,061/686	PCP39	32	52	<i>Clostridium perfringens</i> pCP13	Putative relaxase (nickase)
45	CW	46811	48469	1,659/552					
46	CCW	49999	48656	1,341/447	PcJ, ORf63	86, 76	93, 86	<i>Enterococcus faecalis</i> pCF10, pAD1	
47	CCW	50772	49999	792/258	PefK, ORF62	78, 77	88, 87	<i>Enterococcus faecalis</i> pCF10, pAD1	Phage related
48	CCW	51467	50916	552/182	PcIL	100	100	<i>Enterococcus faecalis</i> pCF10	
49	CCW	51664	51491	174/57	PcIM	98	100	<i>Enterococcus faecalis</i> pCF10	
50	CCW	51852	51667	186/61	PcIN	100	100	<i>Enterococcus faecalis</i> pCF10	
51	CW	52148	52348	201/66	PcIP	100	100	<i>Enterococcus faecalis</i> pCF10	
52	CCW	52680	52450	231/76	PcIQ	100	100	<i>Enterococcus faecalis</i> pCF10	
53	CW	52837	53229	393/130	PcIR	98	100	<i>Enterococcus faecalis</i> pCF10	
54	CW	53295	53747	453/150	ORF1, PcIS	98, 98	98, 98	<i>Enterococcus faecalis</i> pY114, pCF10	Phage-related single-strand binding protein
55	CW	53761	53913	153/50	ORF2	88	92	<i>Enterococcus faecalis</i> pY114	
56	CW	53925	54515	591/196	ORF3, PefT	84, 81	91, 88	<i>Enterococcus faecalis</i> pY114, pCF10	Thermonuclease precursor
57	CW	54521	54841	330/109	ORF4, PefU	97, 95	100, 97	<i>Enterococcus faecalis</i> pY114, pCF10	
58	CW	54949	55194	246/81					
59	CW	55248	55430	183/60	ORF5	100	100	<i>Enterococcus faecalis</i> pY114	
60	CW	55625	56341	717/238	ORF6	100	100	<i>Enterococcus faecalis</i> pY114	
61	CW	56479	58266	1,788/595	BacL <sub>1</sub>	99	99	<i>Enterococcus faecalis</i> pY114	Prebacteriocin for Bac41
62	CW	58452	59087	636/211	BacL <sub>2</sub>	100	100	<i>Enterococcus faecalis</i> pY114	Prebacteriocin secretion
63	CW	59110	59541	432/133	ORF9	100	100	<i>Enterococcus faecalis</i> pY114	
64	CW	59544	60071	528/175	ORF10	97	99	<i>Enterococcus faecalis</i> pY114	
65	CW	60114	62294	2,181/726	BacA	100	100	<i>Enterococcus faecalis</i> pY114	Activator for Bac41
66	CW	62402	62944	543/180	BacI	99	100	<i>Enterococcus faecalis</i> pY114	Provides immunity against Bac41
67	CW	63011	63586	576/191	ORF13	98		<i>Enterococcus faecalis</i> pY114	
68	CW	63729	64061	333/110	ORF14	98	100	<i>Enterococcus faecalis</i> pY114	
69	CCW	65564	64245	1,317/439	Tnp	75	85	<i>Lactococcus lactis</i> Tn5721	Transposase
70	CW	65797	65937	141/46					
71	CW	66024	66854	831/276	ORF130	45	68	<i>Lactobacillus sakei</i>	
72	CW	66917	67126	210/69					
73	CW	68042	68464	423/140	Ej97D	58	80	<i>Enterococcus faecalis</i>	Enterocin EJ97 plasmid, CsbD stress protein
74	CW	68628	69194	567/188	EF0053	65	82	<i>Enterococcus faecalis</i>	
75	CW	69211	69408	198/65	EF0054	61	77	<i>Enterococcus faecalis</i>	
76	CW	69435	69971	537/178	Gis24	88	95	<i>Enterococcus faecalis</i> V583	Stress response
77	CCW	71158	70100	1,059/352	ExigDRAFT 2618	45	69	<i>Exiguobacterium sibiricum</i>	EzrA, septation ring formation regulator
78	CCW	71425	71183	243/80	EF2708	72	87	<i>Enterococcus faecalis</i> V583	Putative membrane protein
79	CW	71630	71872	243/80					
80	CW	71958	72170	213/70	Tnp	53	71	<i>Lactococcus lactis</i>	Transposase

Continued on following page



TABLE 4—Continued

ORF	Direction <sup>a</sup>	5' residue no.	3' residue no.	Gene (bp)/ protein (aa)	Homology	% Identity	% Similarity	Organism or plasmid	Function
81	CW	72346	72456	111/36					
82	CW	72694	73314	621/206	PcfY, Orf86	94, 92	97, 96	<i>Enterococcus faecalis</i> pCF10, pAD1	DNA invertase
83	CW	73331	73618	288/95	PcfZ, Orf87	90, 89	97, 96	<i>Enterococcus faecalis</i> pCF10, pAD1	
84	CW	73612	73716	105/35	UvrC-N (truncated)	97, 98	98, 100	<i>Enterococcus faecalis</i> pCF10, pAD1	
85	CCW	76852	73880	2,973/990	EP0008	99	100	<i>Enterococcus faecalis</i> pAM373	Transposase, Tn3 family
86	CW	76983	77588	606/201	EP0007	63	78	<i>Enterococcus faecalis</i> pAM373	Recombinase
87	CW	77651	77775	125/40	UvrC-C (truncated)	97, 98	98, 100	<i>Enterococcus faecalis</i> pCF10, pAD1	
88	CW	77836	78045	210/69	UvrB	92, 92	97, 97	<i>Enterococcus faecalis</i> pCF10, pAD1	
89	CW	78057	78359	303/100	UvaB	94, 94	100, 94	<i>Enterococcus faecalis</i> pCF10, pAD1	
90	CW	78786	80114	1,329/442	UvrA	99, 97	99, 98	<i>Enterococcus faecalis</i> pCF10, pAD1	UV resistance
91	CW	80111	80461	351/116	UvaE, OrfB	95, 94	98, 95	<i>Enterococcus faecalis</i> pCF10, pAD1	
92	CW	80418	80630	213/70	UvaF, OrfC	97, 92	98, 97	<i>Enterococcus faecalis</i> pCF10, pAD1	
93	CW	81062	81358	294/98	PrgN, OrfE	98, 91	100, 98	<i>Enterococcus faecalis</i> pCF10, pAD1	Replication control
94	CW	81612	82394	783/260	PatA	99	99	<i>Enterococcus faecalis</i> pTEF2	Plasmid partitioning
95	CW	82387	82743	354/118	EF B0065	100	100	<i>Enterococcus faecalis</i> pTEF2	
96	CW	83003	84010	1,008/335	RepA, PrgW	97, 64	98, 77	<i>Enterococcus faecalis</i> pTEF2, pCF10	Plasmid replication
97	CW	84169	85806	1,638/545	PrgZ	99	99	<i>Enterococcus faecalis</i> pCF10	Pheromone uptake
98	CW	85817	86971	1,155/384	PrgY	100	100	<i>Enterococcus faecalis</i> pCF10	pheromone shutdown
99	CCW	87957	87004	954/319	PrgX	100	100	<i>Enterococcus faecalis</i> pCF10	cCF10 pheromone receptor, negative regulator (DNA binding protein)
100	CW	88166	88237	72/23	PrgQ	100	100	<i>Enterococcus faecalis</i> pCF10	iCF10 precursor, cCF10-inhibitor
101	CW	88726	89082	357/118	TruE1	100	100	<i>Enterococcus faecalis</i> pAD1	Positive regulator for conjugation
102	CW	89664	89969	306/100	OrfY	97	100	<i>Enterococcus faecalis</i> pAD1	
103	CW	89980	92655	2,676/891	Seal	89	94	<i>Enterococcus faecalis</i> pAD1	Surface exclusion protein
104	CW	92675	92953	279/92	EF2120	66	86	<i>Enterococcus faecalis</i> V583	
105	CW	93413	93748	336/111	OrfI	100	100	<i>Enterococcus faecalis</i> pAD1	
106	CW	94055	97981	3,927/1,308	PrgB, AsaI	95, 82	97, 87	<i>Enterococcus faecalis</i> pCF10, pAD1	Aggregation substance
107	CW	98078	98398	321/106	PrgU, Orf3	97, 98	99, 99	<i>Enterococcus faecalis</i> pCF10, pAD1	
108	CW	98614	99444	831/276	Pd53/Pd113 (combined)	81/95	95/87	<i>Enterococcus faecalis</i> pPD1	
109	CW	99690	100025	336/111	Pd96	56	71	<i>Enterococcus faecalis</i> pPD1	
110	CW	100033	101160	1,128/375	Pd377	85	89	<i>Enterococcus faecalis</i> pPD1	
111	CW	101325	101468	144/47					
112	CW	101688	103247	1,560/519	Pd78	88	91	<i>Enterococcus faecalis</i> pPD1	Pheromone-inducible surface protein
113	CW	103417	103950	534/177	RUMTOR 00674	20	45	<i>Ruminococcus torques</i>	Permease
114	CW	104015	19	2,532/843	TruG/VirD4	25	43	<i>Bacillus subtilis</i> p19	coupling protein

<sup>a</sup> CW, clockwise; CCW, counterclockwise.

resistant transconjugants indicated that the plasmid found in each particular group was identical. Further plasmid analysis showed that the vancomycin-resistant transconjugants carried a 106.5-kbp plasmid and the erythromycin resistant transconjugants carried a 65.7-kbp plasmid (Fig. 1A).

The 106.5-kbp plasmid harbored by the vancomycin-resistant transconjugant and the 65.7-kbp plasmid harbored by the erythromycin-resistant transconjugant derived from the representative *E. faecalis* NKH15 strain were designated pMG2200 and pMG2201, respectively (Fig. 1A). pMG2200 conferred





between 8,580 bp and 42,391 bp in the clockwise orientation of the plasmid map (Fig. 2). The Tn1549-like element of pMG2200 contained 29 ORFs which were almost identical to the 29 ORFs of Tn1549 (34 kbp) in pIP834, which is found in *E. faecalis* E93/268 (10, 30). The 18 ORFs from ORF13 to ORF30 that are located at the left-end extremity and that are aligned in the order identified in pMG2200 were completely identical to the 18 ORFs of Tn1549 (30), with the exception of ORF16 (99% amino acids identity). The Tn1549-like element contained the *vanR<sub>B</sub>*, *vanS<sub>B</sub>*, *vanY<sub>B</sub>*, *vanW<sub>B</sub>*, *vanH<sub>B</sub>*, *vanB2*, and *vanX<sub>B</sub>* genes, which correspond to the seven equivalent genes in the VanB2 operon of Tn1549 (30). The deduced amid acid sequence of VanB2 of the Tn1549-like element was almost identical to the deduced amid acid sequence of VanB2 of Tn1549 at a level of 99% amino acid identity. The ORFs from ORF46 to ORF57 in an approximately 6.2-kbp region running from 48,656 bp to 54,841 bp of the map and ORF79 to ORF108 in the approximately 30.55-kbp region running from 72,694 bp to 103,247 bp of the map showed a level of homology of 80 to 100% amino acid identity with the genes or the ORFs found in the pheromone-responding plasmids (pAD1, pCF10, pPD1, pAM373, and pTEF2) (5, 9, 14, 24, 28, 51). These regions contained ORFs that correspond to the ORFs *pcfI*, *pcfK*, *pcfL*, *pcfM*, *pcfN*, *pcfP*, *pcfQ*, *pcfR*, *pcfS*, *pcfT*, *pcfU*, *pcfY*, and *pcfZ* of pCF10; the UV resistance genes *uvrC*, *uvaB*, *uvrB*, *uvrA*, *uvaE*, and *uvaF* (*orfB*, *orfC*) of pCF10 or pAD1; the plasmid maintenance genes (plasmid partition and replication) *par* and *rep* of pTEF2; and *prgN*, *prgZ*, *prgY*, *prgX*, and *prgQ* of pCF10 (33, 48, 51). Like the ORFs or genes in pCF10, these ORFs align in this order in pMG2200. ORF94 to ORF97 were identical to *prgZ*, *prgY*, *prgX*, and *prgQ*, respectively, which are the pheromone-responding regulatory genes that allow the cell surface receptor to take up exogenous pheromone, that shut down pheromone production or reduce endogenous pheromone levels, that act as the pheromone receptor and negative regulator for the downstream genes of *prgQ*, and that act as the pheromone inhibitor, respectively (19). The ORFs downstream of the regulatory genes, ORF98 to ORF108, were similar to those found in other pheromone-responsive plasmids, such as pAD1 and pPD1. ORFs 98, 99, 100, 102, and 103 were highly homologous to TraE1, OrfY, Seal, Orf1, and Asa1 of pAD1, respectively. The deduced ORF98 protein showed 100% amino acid identity with TraE1 of plasmid pAD1, which is a key positive regulator for the pheromone-responsive plasmid. In contrast, the other regulatory genes corresponding to *prgZ*, *prgY*, *prgX*, and *prgQ* of plasmid pCF10 showed a high level of homology with the equivalent genes in plasmid pCF10. Like the pheromone-responsive plasmids, there were two inverted repeat sequences (IRS1 and IRS2) in the noncoding region between ORF97 (*prgQ*) and ORF98 (*traE1*) that stopped the transcript from the promoter region of the pheromone inhibitor of *prgQ* in the case of pCF10 (60). The sequence of the upstream region of IRS2 was identical to the sequence in pCF10, and the sequence of the downstream region of IRS2 was identical to the sequence in pAD1. Ten ORFs from ORF59 to ORF68 showed a high level of homology with genes in the region of the Bac41 determinant, which is active against *E. faecalis*, and consisted of *bacl<sub>1</sub>*, *bacl<sub>2</sub>*, *bacl<sub>3</sub>*, *bacl<sub>4</sub>*, and *bacl<sub>5</sub>*, which are encoded on pheromone-responsive plasmid pYI14, which has been reported to be a novel bac-

terioin for cell wall lysis found in *E. faecalis* YI714 (64). *E. faecalis* OG1S harboring pMG2200 showed resistance (immunity) to Bac41, indicating that pMG2200 encodes a Bac41-like bacteriocin.

**Cloning and genetic analysis of *oriT* region of pMG2200.** The transfer origin (*oriT*) is thought to be characteristic of the conjugative plasmid and is essential for the transfer of the transferable or mobile element (25). Known *oriT* regions are classified into several groups on the basis of sequence similarities (73). No sequence that was identical or similar to the known *oriT* regions of conjugative plasmids in gram-positive bacteria was found in the pMG2200 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 (25, 73). In the noncoding region between ORF41 and ORF42, multiple direct repeats and two inverted repeats were found (Fig. 3). The direct repeats were composed of 14 copies of 5-bp TGCTA sequences. The 14 direct repeats (DR-1 to DR-14) were located several base pairs away (from 5 to 7 bp) from each other. The inverted repeats were composed of GCCTTGCA/TGCAAGGC (IR-1) and GGGTCAG/CTGACCC (IR-2).

To identify the functional *oriT* region of pMG2200, a 322-bp segment containing a potential candidate for the *oriT* region was cloned into pAM401 (Cm<sup>r</sup>), and the resultant plasmid was designated pMG2210 (Fig. 3). pMG2210 (Cm<sup>r</sup>) containing the 322-bp region was mobilized by pMG2200 (Vm<sup>r</sup>) and transferred at the same frequency as the parent plasmid (data not shown). The results showed that the noncoding region between ORF41 and ORF42 is the functional *oriT* region of pMG2200.

The internal 178-bp segment within the *oriT* region located between 43,733 bp and 43,911 bp of the map showed significant homology (more than 80%) with the region from 3618 to 3795 bp of plasmid pAM $\alpha$ 1 and the region from 2078 to 1901 bp of plasmid pS86; however, these regions were not related to the *oriT* regions of these plasmids (Fig. 3) (23, 42). Both pAM $\alpha$ 1 (9.8 kb) and pS86 (5.2 kb) are nonconjugative but mobilizable plasmids found in *E. faecalis* (17, 42). Plasmids pMG2200, pAM $\alpha$ 1, and pS86 each had seven copies of the 5-bp direct repeat sequences (TGCTA) and two inverted repeat sequences in the homologous regions.

**Putative DNA relaxase/nickase gene ORF44.** In addition to the *oriT* sequence, the relaxase/nickase is an important feature of conjugative plasmids that is essential for the initiation of DNA transfer (25, 73). ORF44, which encoded a 686-amino-acid protein, showed a significant level of similarity to the predicted relaxase/nickase gene *traI* (ORF34) of plasmid pHTB isolated from a vancomycin-resistant *E. faecium* strain (63, 66). The three conserved motifs (motifs I to III) of the DNA relaxase were found in the N-terminal portion (i.e., about 377 amino acids) of the deduced ORF44 protein (Fig. 4). Motif I contained 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<sub>3</sub> motif. It has been suggested that the His residues aid with the nucleophilic activity of the Tyr residue in motif I, coordinate the required Mg<sup>2+</sup> ions, and direct the activation of the active Tyr. These



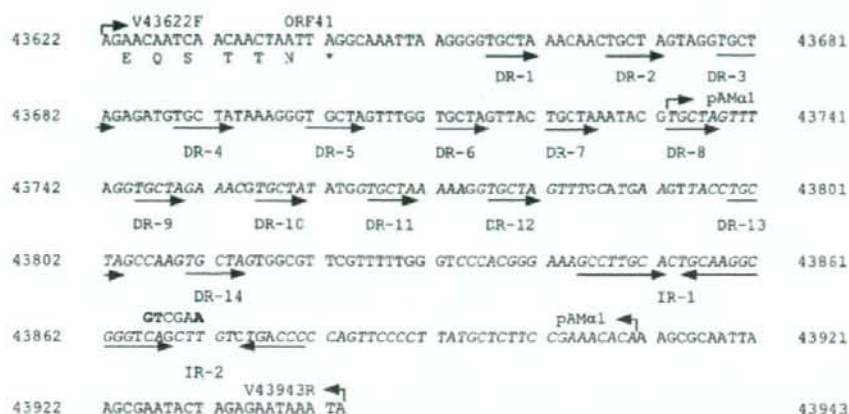


FIG. 3. Nucleotide sequence of the *oriT* region of plasmid pMG2200. The 332-bp noncoding DNA region between ORF41 and ORF42 is shown. The horizontal arrows under the sequences indicated the direct repeats TGCTA (DR-1 to DR-14) and inverted repeats (IR-1 and IR-2) in the *oriT* region. The names and the locations of the oligonucleotide primers used for the analysis of the *oriT* region are shown on the sequence with the right-angled arrows. The complementary sequence corresponding to 3'-GTCGAA-5' shows the possible nick site. The italicized characters in the 178-bp segment mapped between 43,733 bp and 43,910 bp indicate the sequences identical to the sequence found in plasmid pAMa1 (from positions 3618 to 3795 bp on the plasmid).

three motifs are thought to form part of the catalytic center of the relaxase (25, 72).

The conserved His<sub>3</sub> sequence in motif III of the ORF44 of pMG2200 belonged to the MOB<sub>MG</sub> family [W(x<sub>4</sub>)H(x<sub>2</sub>)T(x<sub>2</sub>)HxH(x<sub>4</sub>)E(x<sub>4</sub>)R, where uppercase letters represent conserved amino acids and "x" indicates the variable residues] found in TraI of pHTB (Fig. 4) (63).

**Pheromone-specific plasmid transfers.** The specific pheromone induces transfer of the corresponding plasmid. The transfer of pMG2200 was induced by the pheromone cCF10. Nucleotide sequence analysis of pMG2200 revealed that the deduced pheromone receptor and negative regulatory gene (i.e., ORF99 [*prgX*]) was identical to the *prgX* gene, a key negative regulator of pCF10, which is responsive to cCF10. pMG2200 encoded the deduced positive regulatory gene (i.e., ORF101 [*traE1*]) that is identical to *traE1* of pAD1, which is derepressed by cAD1. The *traE1* gene product, the E-region product(s), positively regulates the structural transfer genes, including the aggregation substance gene (*asa1*), downstream of *traE1*. The molecular mechanism of the regulation by *traE1* has not been elucidated. There are reports that *traE1* acts in *trans* (8, 45). Another report shows that *traE1* acts as a *cis* element in gene regulation (46). All of the analyses were performed under artificial conditions with cloned elements of the regions on multicopy vector plasmids; thus, these activities might differ from the activity in the wild-type plasmid. pMG2200 was a naturally occurring chimeric plasmid, as described above. Its pheromone receptor was identical to PrgX of pCF10, and its positive regulator was identical to TraE1 of pAD1. Using plasmid pMG2200, we examined whether the *traE1* gene regulated the plasmid transfer in a *trans* or a *cis* manner. We constructed a donor strain harboring two plasmids, pMG2200 (Vam<sup>+</sup>) and pAM714 (pAD1::Tn917 [Em<sup>r</sup>], a derivative of pAD1 showing the wild-type pheromone-response and transfer). Plasmids pMG2200 and pAM714 had

different pheromone receptors, and the pheromones were PrgX for cCF10 and TraA for cAD1, but both plasmids encoded an identical positive regulator, the *traE1* gene (E region). If the *traE1* gene product regulates the expression of structural genes in a *trans* manner, either cCF10 or cAD1 would induce the expression of the *traE1* gene encoded on a plasmid, and TraE1 would then positively regulate the expression of both structural genes encoded on the two plasmids, which would result in the transfer of both pMG2200 and pAM714 in the cell.

After pheromone induction by either cCF10 or cAD1, a short mating experiment between JH2SS(pMG2200, pAM714) and FA2-2 was performed, as described in Materials and Methods. After incubation with the pheromones, cell aggregates (clumping) were observed, indicating that the aggregation substance gene(s) was expressed in the cell. cCF10 induced only the transfer of pMG2200 ( $2.6 \times 10^{-6}$  per donor cell) and did not induce the transfer of pAM714 ( $<1.1 \times 10^{-8}$  per donor cell). cAD1 induced only the transfer of pAM714 ( $2.4 \times 10^{-5}$  per donor cell) and not that of pMG2200 ( $<1.1 \times 10^{-8}$  per donor cell). This result implies that TraE1 acted in a *cis* manner for plasmid transfer. In the case of pCF10, it is considered that the small gene products (RNA molecules; i.e., PrgR and PrgS) of pCF10, which are located on the equivalent region of *traE1* in pAD1, regulate the downstream structural genes in a *cis* manner by an unknown mechanism (6). A similar mechanism might exist in pAD1 and pMG2200, which both contain the *traE1* gene. It is notable that the previous data showed that Tn917-*lac* insertion mutants in the noncoding downstream region of *traE1* of pAD1 resulted in a defect in plasmid transfer and mating aggregates and that this region was genetically determined and mapped as the positive regulatory E region on pAD1 prior to the determination of the *traE1* gene by nucleotide sequence analysis (21, 52, 69). There is no current explanation for the transfer-deficient Tn917-*lac*