TABLE 2. Isolation frequency of carbapenem-resistant mutants of *P. aeruginosa* strains in broth culture with and without ciprofloxacin or ofloxacin

Strain no.[a]	Drug	MIC (μg/ml) for parent	Drug concn in selective agar (μg/ml)	Isolation frequency[b] (10^{-7})					
				Ciprofloxacin (0.03 μg/ml) in broth culture			Ofloxacin (0.25 μg/ml) in broth culture		
				−	+	Factor[c]	−	+	Factor[c]
PAO1	DRPM	0.2	0.4	<0.053	<0.053		<0.053	<0.053	
	MEPM	0.2	0.4	3.8 ± 0.70	26 ± 5.0	6.8	2.5 ± 0.50	15 ± 1.2	6.0
	IPM	0.8	1.6	0.18 ± 0.07	1.0 ± 0.20	5.6	0.43 ± 0.07	0.82 ± 0.01	1.9
GP4	DRPM	0.2	0.4	1.8 ± 0.31	5.6 ± 0.61	3.1	1.2 ± 0.22	3.8 ± 0.27	3.2
	MEPM	0.2	0.4	7.6 ± 0.51	29 ± 3.5	3.8	8.3 ± 0.54	41 ± 3.7	4.9
	IPM	0.8	1.6	2.0 ± 0.11	9.4 ± 0.81	4.7	1.8 ± 0.32	6.7 ± 0.49	3.7
GP20	DRPM	0.2	0.4	2.7 ± 0.13	7.0 ± 0.91	2.6	2.2 ± 0.15	5.9 ± 0.55	2.7
	MEPM	0.2	0.4	11 ± 1.8	65 ± 6.4	5.9	7.8 ± 0.95	25 ± 3.6	3.9
	IPM	0.8	1.6	4.0 ± 0.65	16 ± 2.5	4.0	3.6 ± 0.27	12 ± 4.0	3.3
GP37	DRPM	0.2	0.4	2.6 ± 0.21	5.0 ± 0.40	1.9	2.8 ± 0.19	6.7 ± 0.73	2.4
	MEPM	0.2	0.4	13 ± 1.9	31 ± 3.5	2.4	11 ± 1.7	40 ± 3.6	3.6
	IPM	0.8	1.6	4.6 ± 0.39	16 ± 2.1	3.5	4.7 ± 0.52	23 ± 2.6	4.9
GP62	DRPM	0.1	0.2	1.6 ± 0.21	4.2 ± 0.37	2.6	2.7 ± 0.26	11 ± 2.0	4.1
	MEPM	0.2	0.4	7.1 ± 0.68	24 ± 1.1	3.4	3.6 ± 0.24	18 ± 1.2	5.0
	IPM	0.8	1.6	1.8 ± 0.26	6.8 ± 0.56	3.8	1.7 ± 0.23	9.0 ± 0.79	5.3
PAO1	DRPM	0.2	0.8	<0.053	<0.053		<0.053	<0.053	
	MEPM	0.2	0.8	<0.053	<0.053		<0.053	<0.053	
	IPM	0.8	3.1	<0.053	<0.053		<0.053	<0.053	
GP4	DRPM	0.2	0.8	<0.053	<0.053		<0.020	<0.021	
	MEPM	0.2	0.8	3.1 ± 0.46	21 ± 2.6	6.8	5.4 ± 0.61	38 ± 3.2	7.0
	IPM	0.8	3.1	0.44 ± 0.030	1.6 ± 0.11	3.6	0.43 ± 0.051	2.2 ± 0.29	5.1
GP20	DRPM	0.2	0.8	<0.045	<0.14		<0.020	<0.020	
	MEPM	0.2	0.8	6.7 ± 0.40	37 ± 3.0	5.5	4.1 ± 0.41	14 ± 2.1	3.5
			1.6	0.59 ± 0.054	1.7 ± 0.23	2.9	0.12 ± 0.040	0.33 ± 0.056	2.8
	IPM	0.8	3.1	0.97 ± 0.061	4.0 ± 0.37	4.1	0.64 ± 0.043	1.7 ± 0.12	2.7
GP37	DRPM	0.2	0.8	<0.045	<0.057		<0.020	<0.021	
	MEPM	0.2	0.8	7.5 ± 0.70	23 ± 2.9	3.1	6.1 ± 0.50	28 ± 3.3	4.6
			1.6	<0.045	1.0 ± 0.08	≤10	<0.021	0.10 ± 0.018	4.8<
	IPM	0.8	3.1	0.35 ± 0.023	0.87 ± 0.041	2.5	0.35 ± 0.061	1.5 ± 0.11	4.3
GP62	DRPM	0.1	0.4	<0.084	<0.056		<0.021	<0.020	
	MEPM	0.2	0.8	4.7 ± 0.36	44 ± 4.6	9.4	1.9 ± 0.21	13 ± 1.5	6.8
	IPM	0.8	3.1	0.26 ± 0.027	0.70 ± 0.055	2.7	0.28 ± 0.031	1.5 ± 0.19	5.4
PAO1KTL	DRPM	0.8	1.6	<0.068	<0.109				
	MEPM	1.6	3.1	0.74 ± 0.12	6.12 ± 0.66	8.3			
	IPM	12.5	25	<0.068	<0.109				
GP4KT11	DRPM	1.6	3.1	<0.038	<0.049				
	MEPM	3.1	6.3	1.04 ± 0.26	4.44 ± 0.38	4.3			
	IPM	12.5	25	<0.038	<0.049				
GP61	DRPM	0.8	1.6	<0.099	<0.213				
	MEPM	1.6	3.1	0.20 ± 0.04	2.08 ± 0.22	10.4			
	IPM	6.3	12.5	<0.099	<0.213				
PAO1KTL	DRPM	0.8	3.1	<0.068	<0.109				
	MEPM	1.6	6.3	<0.068	<0.109				
	IPM	12.5	50	<0.068	<0.109				
GP4KT11	DRPM	1.6	6.3	<0.038	<0.049				
	MEPM	3.1	12.5	<0.038	<0.049				
	IPM	12.5	50	<0.038	<0.049				
GP61	DRPM	0.8	3.1	<0.099	<0.213				
	MEPM	1.6	6.3	<0.099	<0.213				
	IPM	6.3	25	<0.099	<0.213				

[a] Note that for comparison, strains are grouped by 1× and 2× the drug concentration in selective agar.

[b] The isolation frequency of the carbapenem-resistant mutant was determined by the number of colonies grown on a selective agar plate per number of inoculated cells, and the averages of the results of 10 independent experiments ± standard deviations are shown here.

[c] The factor indicates the ratio of the number of strain mutants in broth culture with fluoroquinolone to the number obtained without fluoroquinolone.

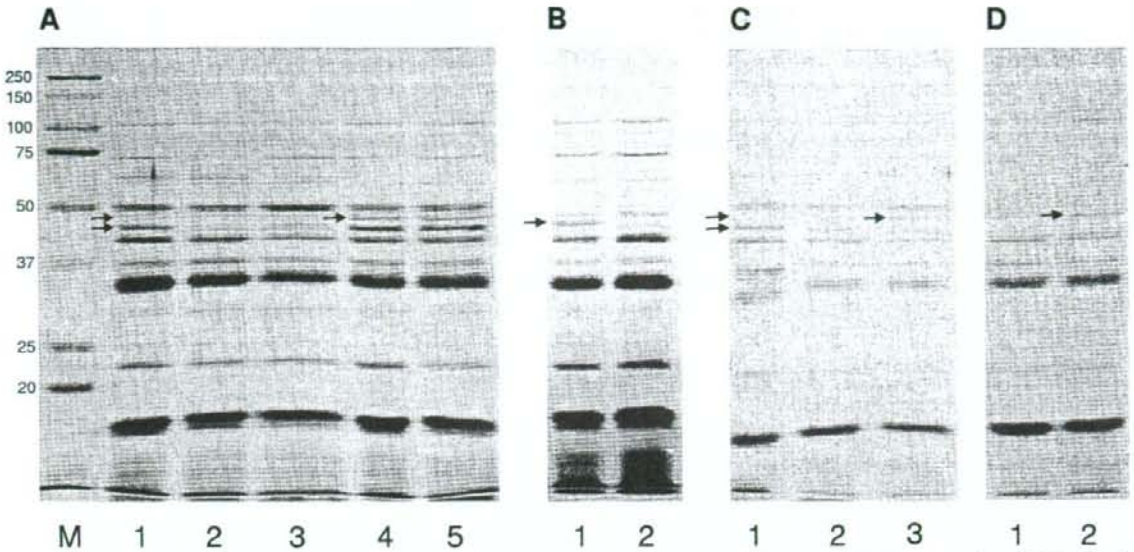

FIG. 1. SDS-PAGE of OMPs from carbapenem-susceptible *P. aeruginosa* strains and the carbapenem-resistant mutants. The OMPs of each strain were prepared as the Sarkosyl-insoluble fraction of the membrane preparation (29), and samples were subjected to SDS-PAGE (11% polyacrylamide) at a constant current of 25 mA (12). (A) *P. aeruginosa* PAO1 and its mutants. Lane M, protein molecular mass markers purchased from Bio-Rad (Hercules, CA); lane 1, PAO1; lane 2, PAO1IPM46 (*oprD*); lane 3, carbapenem-resistant mutant of PAO1 (PAO1KTL); lane 4, PAO1SO20 [*mexAB*(Hy)-*oprM*(Hy)]; lane 5, carbapenem-resistant mutant of PAO1 (PAO1KTS). (B) *P. aeruginosa* GP20 and its mutant. Lane 1, carbapenem-susceptible GP20; lane 2, carbapenem-resistant mutant of GP20KT21 isolated from selective agar plate containing MEPM. (C) *P. aeruginosa* PAO1 and its mutants. Lane 1, PAO1; lane 2, PAO1KTL; lane 3, PAO1KTL1. (D) *P. aeruginosa* GP61 and its mutant. Lane 1, IPM-resistant GP61 (i.e., higher IPM MIC); lane 2, MEPM-resistant mutant of GP61 isolated from selective agar plate containing MEPM. The numbers on the left side of panel A indicate the molecular mass of each marker in kilodaltons. The arrows in lanes 1 of panels A and C indicate the positions of proteins of about 46 kDa and 48 kDa, respectively. The arrows on the left side of lane 4 of panel A, lane 1 of panel B, lane 3 of panel C, and lane 2 of panel D indicate the positions of proteins of about 48 kDa, 46 kDa, 48 kDa, and 48 kDa, respectively.

lates in Japan is about 40%. The MICs of meropenem (MEPM) are ≧16 μg/ml (i.e., full resistance) and 8 μg/ml (i.e., intermediate resistance) for about 20% and 20% of isolates, respectively (22, 27), and about 0.4% of the fully resistant isolates produce metallo-β-lactamase (21).

Many cancer centers administer prophylactic fluoroquinolone to neutropenic patients after chemotherapy (2, 9, 30). Fluoroquinolones are mutagenic in bacteria, and their usage might enhance the frequency of mutations resulting in bacterial drug resistance (4, 10, 16, 28). We have shown that fluoroquinolone enhanced the carbapenem resistance mutation rate in *P. aeruginosa* and that carbapenem-resistant mutants were selected in the presence of carbapenems. We have also shown that the highest frequency of mutant isolation occurred during selection with MEPM, while doripenem (DRPM) inhibited mutant growth.

We used *P. aeruginosa* PAO1 and four other *P. aeruginosa* clinical isolates susceptible to carbapenems (Table 1). The agar dilution method was used to determine MICs according to CSLI guidelines. All cultures were incubated for 18 h at 37°C. The carbapenem-resistant mutants were isolated on AB3 agar plates containing each carbapenem (26). A culture of each strain was diluted 10^6-fold with fresh AB3 broth in either the presence or absence of the representative fluoroquinolones ciprofloxacin and ofloxacin (16). After incubation, 0.1 ml of each culture or its 10^{-1} dilution was spread onto an agar plate containing a carbapenem. Colony growth was then examined. Ten colonies selected at random from each selective agar plate were examined for drug resistance (MIC), and representative mutant strains were used for further analysis. In this study, "resistance" was defined as an increased MIC for the antibiotic in the mutant strain compared to its parent strain, as the mutant strains showed reduced susceptibility or resistance to carbapenems by CSLI criteria.

Carbapenem-resistant PAO1 mutants were obtained on agar containing drug concentrations equivalent to twice the MIC of MEPM and IPM (Table 2). The highest frequencies of mutant isolation were obtained by selection on MEPM. The presence of ciprofloxacin or ofloxacin at a subinhibitory concentration increased the number of mutants obtained by about seven- or sixfold, respectively, during MEPM selection. The results indicated that the highest frequency of mutant cells was obtained in the presence of MEPM, while DRPM inhibited mutant growth (25, 26).

Of the cultures grown with a concentration equivalent to 2× the MIC of MEPM, 55 of the 57 colonies (96.5%) obtained from cultures grown in the absence of ciprofloxacin and 151 of the 156 colonies (96.8%) grown in the presence of ciprofloxacin only showed resistance to carbapenems, as illustrated by the representative strain PAO1KTL (Table 1). The remaining colonies from both groups showed identical drug resistance

TABLE 3. Primers for real-time PCR and PCR amplification

Gene and primer	Sequence (5′→3′)	Position (5′→3′)[a]	Product size (bp)	Source or reference
ampC				
ampC1	CGGCTCGGTGAGCAAGACCTTC	264–285	218	5
ampC2	AGTCGCGGATCTGTGCCTGGTC	481–460		
bla_{OXA}				
OXA-F	CGMGCAAAWAMAGMWTAT		457	20
OXA-R1bio	ARABCCATTSCCCADCCA			
bla_{OXA-51}				
Oxa-51/F	CIYTIISIMGIGCIAAYAMIGARTAYG		498	1
Oxa-51/R	CAICCIGTIARCCAICCIACYTG			
mexA				
mexA/F	GTTCCCCAACCCGAACAAC	792–810	68	This study
mexA/R	TGACGCCTTCCTGCAACTG	859–841		
oprD				
oprD/F	CTACGGCTACGGCGAGGAT	1143–1161	58	This study
oprD/R	CACGTACTTGGCTTCGAGGTT	1200–1130		
oprD/F1	CACCTACGCAGATGCGAC	1045629–1045646[b]	1,640	This study
oprD/R1	CAGAGTAATGAGGAAGAC	1047268–1047251[b]		
rpoD				
rpoD/F	CCTGCCGGAGGATATTTCC	96–114	70	This study
rpoD/R	GATCCCCATGTCGTTGATCAT	165–145		

[a] The positions given are from the first base of the coding sequences of the genes.
[b] The positions given are the map coordinates of the *P. aeruginosa* PAO1 genome sequence.

patterns to a range of antibiotics, as seen in the representative strain PAO1KTS (Table 1).

When the concentration of the selective drug was increased by successive doubling from 2× the MIC to 16× the MIC for each drug, MEPM produced the highest frequency of carbapenem-resistant mutations in the four clinical strains (Table 2). DRPM inhibited growth of the mutants at the concentration used (26). The mutants only showed resistance to carbapenem-type antibiotics when grown in the presence of carbapenem drugs (Table 1).

The presence of fluoroquinolones in the cultures enhanced the mutation rate in all strains (Table 2).

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) of the outer membrane proteins (OMPs) from each strain indicated that the carbapenem-resistant mutant had a marked reduction in the concentration of OMP with a molecular mass of about 46 kDa, indicating that the mutant resulted from the reduced production of OprD (3, 24, 32, 33). The PAO1KTS mutant showed an increase in the concentration of the 48-kDa OMP, indicating that the mutant resulted from an increased production of the MexAB-OprM protein (11, 13, 15, 23). These results indicated that in *P. aeruginosa*, carbapenem predominantly selects for the carbapenem-resistant mutant with a reduced production of OprD. Figure 1 shows the results obtained with representative strains.

DNA sequence analysis of the *oprD* gene showed that the mutants had one of the following: an insertion of one nucleotide, an IS*407* insertion, a one-nucleotide substitution, or a one-nucleotide or multiple-nucleotide deletions (Table 3 and 4). The mutants resulted in a frameshift mutation resulting in either premature termination of translation or translation beyond the original stop codon (Table 4). There is no mutation within *oprD* of GP62KT41, which implies that there might be a mutation in the regulation of the *oprD* expression that has not yet been elucidated.

Compared to the parent strains, the PAO1KTS and PAO1SO20 mutants had almost identical levels of *oprD* transcript, but the level was reduced to one-tenth in the mutant GP62KT41. The level of *mexA* transcript was increased by about 10-fold in the mutants PAO1KTS and PAO1SO20 (18) compared to their parent strain, PAO1, indicating that the increased expression of the 48-kDa OMP resulted from the increased expression of *mexA* (Table 4). *ampC* expression by the mutants was similar to that of their parent strains (Table 4). It is known that *ampC* expression plays a role in carbapenem resistance when OprD is lost (14). PCR analysis did not detect the bla_{OXA} genes in any strain (Table 4).

Highly resistant mutants to MEPM were also obtained from cultures of *oprD* mutants of PAO1KTL, GP4KT11, and GP61 grown on selective agar containing 2× the MIC of MEPM, but were not selected with DRPM and IPM. The fluoroquinolones in the cultures enhanced the mutation rate (Table 2). These mutant strains showed high levels of resistance to MEPM, ceftazidime, piperacillin, and fluoroquinolones (Table 1) and increased production of both the 48-kDa OMP (Fig. 1) and the *mexA* transcript (Table 4), indicating that these mutants resulted from the increased expression of *mexAB-oprM* in addition to an *oprD* mutation. These highly resistant MEPM-resistant mutants were predominantly obtained by selection with MEPM. Thus, MEPM was highly effective in selecting carbapenem-resistant mutants that had either lost *oprD* from the

TABLE 4. DNA sequence and genetic analysis of the carbapenem-resistant mutants

Strain[a]	Genotype or phenotype	Characteristic of DNA sequence of *oprD* gene[b]			Transcript for[c]:			Detection of bla_{OXA}[d]
		Parent gene/ protein size (bp/aa)	Mutant					
			Insertion or deletion	Deduced peptide size (aa)	*oprD*	*mexA*	*ampC*	
PAO1	Parent	1,332/443			1	1	1	ND
PAO1KTL	*oprD*		Deletion of ^{129}CCTG^{132} and ^{134}TGCTCCGCA^{142}	89	NT[e]	1.14	1.22	NT
PAO1KTL1	*oprD mexAB*(Hy)-*oprM*(Hy)		NT		NT	7.21	1.06	NT
PAO1KTS	*mexAB*(Hy)-*oprM*(Hy)				0.914	10.26	0.81	NT
PAO1IPM46	*oprD*		Substitution of C^{199} to T	66	NT	1.2	1.15	NT
PAO1SO20	*mexAB*(Hy)-*oprM*(Hy)				0.964	8.19	0.75	NT
GP4	Wild type	1,329/442			NT	1.00	1.00	ND
GP4KT11	*oprD*		Insertion of (T) between bp 927 (C) and 928 (G)	308	NT	0.95	1.20	NT
GP4KT111	*oprD mexAB*(Hy)-*oprM*(Hy)		NT		NT	14.16	0.76	NT
GP20	Wild type	1,323/440			NT	1.00	1.00	ND
GP20KT21	*oprD*		Insertion of IS*407* (1,236 bp) between bp 1216 and 1217	416	NT	0.90	0.96	NT
GP37	Wild type	1,329/442			NT	1.00	1.00	ND
GP37KT31	*oprD*		Insertion of C between bp 1205 and 1206	>442	NT	0.92	0.94	NT
GP37KT32	*oprD*		Insertion of C between bp 1205 and 1206	>442	NT	0.98	1.10	NT
GP62	Wild type	1,332/443			1.00	1.00	1.00	ND
GP62KT41	OprD$^-$		No mutation in open reading frame of *oprD*		0.095	0.98	1.20	NT
GP62KT43	*oprD*		Insertion of C between bp 147 and 148	52	NT	0.86	0.97	NT
GP62KT44	*oprD*		Deletion of A at bp 902	343	NT	1.10	1.17	NT
GP62KT45	*oprD*		Deletion of A at bp 902	343	NT	0.96	1.10	NT
GP61	Wild type, *oprD*	NT	NT		NT	1.00	1.00	ND
GP61KT51	*oprD mexAB*(Hy)-*oprM*(Hy)	NT	NT		NT	5.75	0.83	NT

[a] GP37KT32, GP62KT44, and GP62KT45 were isolated on selective agar containing MEPM after broth culture with ciprofloxacin. Other mutant strains including PAOKTL and PAO1KTS were selected on agar containing MEPM after broth culture without fluoroquinolone.

[b] DNA fragments 1,640 bp long containing the *oprD* gene were amplified by PCR using total DNA from each strain with specific primers (Table 3) and were sequenced.

[c] cDNA synthesis and real-time PCR were performed according to the manufacturer's protocols. The amount of *rpoD* (σ^{70}) transcript was monitored as an endogenous control to normalize the level of the transcript of interest (31). Specific primers for *ampC*, *mexA*, *oprD*, *rpoD*, and bla_{OXA} are listed in Table 3.

[d] The presence of bla_{OXA} was examined by PCR analysis with a specific primer (Table 3). ND, not detected.

[e] NT, not tested.

sensitive strain or had increased expression of *mexAB-oprM* in the *oprD-deficient* strain.

This work was supported by the grants from the Japanese Ministry of Education, Culture, Sport, Science and Technology [Tokutei-ryoiki (Matrix of Infection Phenomena), Kiban (B), Kiban (C)] and the Japanese Ministry of Health, Labor and Welfare (H15-Shinko-9 and H18-Shinko-11).

We thank Elizabeth Kamei for helpful advice and discussions.

REFERENCES

1. **Brown, S., H. K. Young, and S. G. Amyes.** 2005. Characterisation of OXA-51, a novel class D carbapenemase found in genetically unrelated clinical strains of *Acinetobacter baumannii* from Argentina. Clin. Microbiol. Infect. **11**:15–23.
2. **Bucaneve, G., A. Micozzi, F. Menichetti, P. Martino, M. S. Dionisi, G. Martinelli, B. Allione, D. D'Antonio, M. Buelli, A. M. Nosari, D. Cilloni, E. Zuffa, R. Cantaffa, G. Specchia, S. Amadori, F. Fabbiano, G. L. Deliliers, F. Lauria, R. Foa, and A. D. Favero.** 2005. Levofloxacin to prevent bacterial infection in patients with cancer and neutropenia. N. Engl. J. Med. **353**:977–987.
3. **Büscher, K.-H., W. Cullmann, W. Dick, and W. Opferkuch.** 1987. Imipenem resistance in *Pseudomonas aeruginosa* resulting from diminished expression of an outer membrane protein. Antimicrob. Agents Chemother. **31**:703–708.
4. **Carratala, J., A. Fernandez-Sevilla, F. Tubau, M. Callis, and F. Gudiol.** 1995. Emergence of quinolone-resistant *Escherichia coli* bacteremia in neutropenic patients with cancer who have received prophylactic norfloxacin. Clin. Infect. Dis. **20**:557–560.
5. **Dumas, J. L., C. van Delden, K. Perron, and T. Köhler.** 2006. Analysis of antibiotic resistance gene expression in *Pseudomonas aeruginosa* by quantitative real-time-PCR. FEMS Microbiol. Lett. **254**:217–225.
6. **Evans, K., L. Adewoye, and K. Poole.** 2001. MexR repressor of the *mexAB-oprM* multidrug efflux operon of *Pseudomonas aeruginosa*: identification of MexR binding sites in the *mexA-mexR* intergenic region. J. Bacteriol. **183**: 807–812.
7. **Ge, Y., M. A. Wikler, D. F. Sahm, R. S. Blosser-Middleton, and J. A. Karlowsky.** 2004. In vitro antimicrobial activity of doripenem, a new carbapenem. Antimicrob. Agents Chemother. **48**:1384–1396.
8. **Iso, Y., T. Irie, T. Iwaki, M. Kii, Y. Sendo, K. Motokawa, and Y. Nishitani.** 1996. Synthesis and modification of a novel 1 β-methyl carbapenem antibiotic, S-4661. J. Antibiot. **49**:478–484.
9. **Karp, J. E., W. G. Merz, C. Hendricksen, B. Laughon, T. Redden, B. J. Bamberger, J. G. Bartlett, R. Saral, and P. J. Burke.** 1987. Oral norfloxacin for prevention of gram-negative bacterial infections in patients with acute leukemia and granulocytopenia. Ann. Intern. Med. **106**:1–7.
10. **Kern, W. V., E. Andriof, M. Oethinger, P. Kern, J. Hacker, and R. Marre.**

1994. Emergence of fluoroquinolone-resistant *Escherichia coli* at a cancer center. Antimicrob. Agents Chemother. **38:**681–687.

11. **Köhler, T., M. Michea-Hamzehpour, S. F. Epp, and J.-C. Pechere.** 1999. Carbapenem activities against *Pseudomonas aeruginosa*: respective contributions of OprD and efflux systems. Antimicrob. Agents Chemother. **43:**424–427.
12. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) **227:**680–685.
13. **Li, X.-Z., D. Ma, D. M. Livermore, and H. Nikaido.** 1994. Role of efflux pump(s) in intrinsic resistance of *Pseudomonas aeruginosa*: active efflux as a contributing factor to β-lactam resistance. Antimicrob. Agents Chemother. **38:**1742–1752.
14. **Livermore, D. M.** 1992. Interplay of impermeability and chromosomal β-lactamase activity in imipenem-resistant *Pseudomonas aeruginosa*. Antimicrob. Agents Chemother. **36:**2046–2048.
15. **Livermore, D. M.** 2001. Of *Pseudomonas*, porins, pumps, and carbapenems. J. Antimicrob. Chemother. **47:**247–250.
16. **Mamber, S. W., B. Kolek, K. W. Brookshire, D. P. Bonner, and J. Fung-Tomc.** 1993. Activity of quinolones in the Ames *Salmonella* TA102 mutagenicity test and other bacterial genotoxicity assays. Antimicrob. Agents Chemother. **37:**213–217.
17. **Masuda, N., E. Sakagawa, and S. Ohya.** 1995. Outer membrane proteins responsible for multiple drug resistance in *Pseudomonas aeruginosa*. Antimicrob. Agents Chemother. **39:**645–649.
18. **Miwa, H., Y. Kimura, Y. Jinushi, T. Fujimura, T. Nishikawa, T. Munekage, N. Kuroda, Y. Yamano, M. Tsuji, K. Okazaki, T. Sato, and H. Matsuda.** 2005. Antipseudomonal activety of doripenem, a novel parenteral carbapenem antibiotic. Jpn. J. Chemother. **53(S-1):**80–91.
19. **Mushtaq, S., Y. Ge, and D. M. Livermore.** 2004. Doripenem versus *Pseudomonas aeruginosa* in vitro: activity against characterized isolates, mutants, and transconjugants and resistance selection potential. Antimicrob. Agents Chemother. **48:**3086–3092.
20. **Naas, T., L. Poirel, and P. Nordmann.** 2006. Pyrosequencing for rapid identification of carbapenem-hydrolysing OXA-type β-lactamases in *Acinetobacter baumannii*. Clin. Microbiol. Infect. **12:**1236–1240.
21. **Nishio, H., M. Komatsu, N. Shibata, K. Shimakawa, N. Sueyoshi, T. Ura, K. Satoh, M. Toyokawa, T. Nakamura, Y. Wada, T. Orita, T. Kofuku, K. Yamasaki, M. Sakamoto, S. Kinoshita, M. Aihara, and Y. Arakawa.** 2004. Metallo-β-lactamase-producing gram-negative bacilli: laboratory-based surveillance in cooperation with 13 clinical laboratories in the Kinki region of Japan. J. Clin. Microbiol. **42:**5256–5263.
22. **Ozaki, S., M. Nagasawa, T. Sato, Y. Mure, and K. Inuzuka.** 2007. Nation wide survey of bacterial drug susceptibilities of clinical isolates between 2004 and 2006 in Japan. *In* Y. Arakawa (ed.), Report of Research Project on Bacterial Drug Resistance. Japanese Ministry of Health Labor and Welfare, Tokyo, Japan.
23. **Pai, H., J.-W. Kim, J. Kim, J. H. Lee, K. W. Choe, and N. Gotoh.** 2001. Carbapenem resistance mechanisms in *Pseudomonas aeruginosa* clinical isolates. Antimicrob. Agents Chemother. **45:**480–484.
24. **Quinn, J. P., E. J. Dudek, C. A. di Vincenzo, D. A. Lucks, and S. A. Lerner.** 1986. Emergence of resistance to imipenem during therapy for *Pseudomonas aeruginosa* infections. J. Infect. Dis. **154:**289–294.
25. **Sakyo, S., K. Tanimoto, S. Fujimoto, and Y. Ike.** 1997. Carbapenem-induced resistance in *Pseudomonas aeruginosa* clinical strains to carbapenems including S4661, meropenem and imipenem, abstr. F-215, p. 183. Abstr. 37th Intersci. Conf. Antimicrob. Agents Chemother.
26. **Sakyo, S., H. Tomita, K. Tanimoto, S. Fujimoto, and Y. Ike.** 2006. Potency of carbapenems for the prevention of carbapenem-resistant mutants of *Pseudomonas aeruginosa*. J. Antibiot. **59:**220–228.
27. **Shimojima, M.** 2007. Bacterial drug susceptibilities of clinical isolates between April 2006 and March 2007 in Japan. Med. Consult. New Remedies **43:**697–753. (In Japanese.)
28. **Somolinos, N., R. Arranz, M. C. Del Rey, and M. L. Jimenez.** 1992. Superinfections by *Escherichia coli* resistant to fluoroquinolones in immunocompromised patients. J. Antimicrob. Chermother. **30:**730–731.
29. **Spratt, B. G.** 1977. Properties of the penicillin-binding proteins of *Escherichia coli* K12. Eur. J. Biochem. **72:**341–352.
30. **Talbot, G. H., P. A. Cassileth, L. Paradiso, R. Correa-Coronas, L. Bond, and The Enoxacin Prophylaxis Study Group.** 1993. Oral enoxacin for infection prevention in adults with acute nonlymphocytic leukemia. Antimicrob. Agents Chemother. **37:**474–482.
31. **Tanaka, K., and H. Takahashi.** 1991. Cloning and analysis of the gene (*rpoDA*) for the principal σ factor of *Pseudomonas aeruginosa*. Biochim. Biophys. Acta **1089:**113–119.
32. **Trias, J., J. Dufresne, R. C. Levesque, and H. Nikaido.** 1989. Decreased outer membrane permeability in imipenem-resistant mutants of *Pseudomonas aeruginosa*. Antimicrob. Agents Chemother. **33:**1201–1206.
33. **Trias, J., and H. Nikaido.** 1990. Outer membrane protein D2 catalyzes facilitated diffusion of carbapenems and penems through the outer membrane of *Pseudomonas aeruginosa*. Antimicrob. Agents Chemother. **34:**52–57.

RESEARCH LETTER

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

Koichi Tanimoto[1] & Yasuyoshi Ike[1,2]

[1]Laboratory of Bacterial Drug Resistance, Gunma University Graduate School of Medicine, Maebashi, Gunma, Japan; and [2]Department of Bacteriology, Gunma University Graduate School of Medicine, Maebashi, Gunma, Japan

Correspondence: Yasuyoshi Ike, Department of Bacteriology and Bacterial Infection Control, Gunma University Graduate School of Medicine, 3-39-22 Showa-Machi, Maebashi, Gunma 371-8511, Japan. Tel.: +81 27 220 7990; fax: +81 27 220 7996; e-mail: yasuike@med.gunma-u.ac.jp

Received 28 April 2008; accepted 14 August 2008.
First published online 15 September 2008.

DOI:10.1111/j.1574-6968.2008.01342.x

Editor: David Studholme

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

Abstract

pMG1 (65.1 kb) is a highly conjugative, pheromone-independent *Enterococcus faecium* plasmid that carries a Tn*4001*-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 pHTβ plasmids. Like the pHTβ 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 Tn*917* 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.

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.

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^r; 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 pHTα (65.9 kbp), pHTβ (63.7 kbp) and pHTγ (66.5 kbp), are highly conjugative plasmids carrying Tn*1546*-like transposons that encode vancomycin resistance. DNA

sequencing and genetic analysis of pHTβ, 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 pHTβ 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 $\mu g\,mL^{-1}$; chloramphenicol, 10 $\mu g\,mL^{-1}$; erythromycin, 10 $\mu g\,mL^{-1}$; gentamicin, 500 $\mu g\,mL^{-1}$; kanamycin, 20 $\mu g\,mL^{-1}$ for *E. coli* and 500 $\mu g\,mL^{-1}$ for *E. faecalis*; rifampin, 25 $\mu g\,mL^{-1}$; fusidic acid, 25 $\mu g\,mL^{-1}$; spectinomycin, 40 $\mu g\,mL^{-1}$ for *E. coli* and 100 $\mu g\,mL^{-1}$ for *E. faecalis*.

Plasmid DNA preparation

The Qiaprep Spin Miniprep Kit (Qiagen Sciences, MD) was used for plasmid DNA preparation from *E. coli*. The alkali lysis method was modified (Sambrook *et al.*, 1989) for DNA preparation from *E. faecalis* to include lysozyme treatment (10 $\mu g\,mL^{-1}$, 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
Bacterial strains			
E. faecalis			
FA2-2	*rif fus*		Franke & Clewell (1981)
OG1X	*str*	Protease-negative derivative of OG1-10	Ike *et al.* (1983)
E. coli			
DH5α	*recA1 endA1 gyrA96 thi-1 relA1 hsdR17 supE44 DlacU169 f80 lacZDM15*		Invitrogen
Plasmids			
pMG1	Gm^r, Km^r	65.1-kb conjugative plasmid from *E. faecium* strain	Ike *et al.* (1998)
pTV1Ts	Em^r, Cm^r	Transposon delivery vector, temperature sensitive, pE194Ts(Cm^r)::Tn*917*(Em^r)	Youngman (1987)
pBluescriptSK	*amp*	*E. coli* cloning vector	Stratagene
pMW119	*amp*	*E. coli* cloning vector	Nippon Gene
pDL278	*spc*	*E. coli–E. faecalis* shuttle vector	LeBlanc & Lee (1991)
pKO101	*amp spc*	Spc^r gene of pDL278 was cloned in pMW119	This study
pMG230	Gm^r, Km^r, Em^r, Tra	pMG1::Tn*917*, Tn*917* was inserted at the position of 13 575*	This study
pMG231	Gm^r, Km^r, Em^r, Tra	pMG1::Tn*917*, Tn*917* was inserted at the position of 16 167*	This study
pMG232	Gm^r, Km^r, Em^r, Tra	pMG1::Tn*917*, Tn*917* was inserted at the position of 16 656*	This study
pMG233	Gm^r, Km^r, Em^r, Tra	pMG1::Tn*917*, Tn*917* was inserted at the position of 27 284*	This study
pMG234	Gm^r, Km^r, Em^r, Tra	pMG1::Tn*917*, Tn*917* was inserted at the position of 29 138*	This study
pMG235	Gm^r, Km^r, Em^r, Tra	pMG1::Tn*917*, Tn*917* was inserted at the position of 36 393*	This study
pMG236	Gm^r, Km^r, Em^r, Tra	pMG1::Tn*917*, Tn*917* was inserted at the position of 40 579*	This study
pMG237	Gm^r, Km^r, Em^r, Tra	pMG1::Tn*917*, Tn*917* was inserted at the position of 41 528*	This study
pRep	*amp spc*	5.9-kb HindIII fragment of pMG1 was cloned in pKO101, Rep^+ in *E. faecalis*	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 Tn*7*-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 σ^{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 Tn*917* and isolation of *tra* mutants

Tn*917* 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 $\mu g\,mL^{-1}$) and erythromycin (0.5 $\mu g\,mL^{-1}$). 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 Tn*917* 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 (Tn*917* 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 Tn*917* insertion

The insertion sites of Tn*917* 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 Tn*917*, the region flanking the left end of Tn*917* was amplified. The Tn*917* insertion site was determined by sequencing the PCR product purified from an agarose block after agarose gel electrophoresis. The three nested primers (Tn*917*L, Tn*917*L1 and Tn*917*L2) were designed based on sequences close to the left inverted repeat of Tn*917*. The sequences of the three nested primers and random primer are as follows: Tn*917*L (5′-GAGAGATGTC ACCGTCAAGT-3′), Tn*917*L1 (5′-TTAAAACGGTTGAAAA CTG-3′), Tn*917*L2 (5′-TGTACCACTAATAACTCACAATAG-3′) and random primer (5′-NNNNNNNNNGATC-3′). Total DNA from the mutant strains was prepared with ISOPLANT2 (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 spc/F (5′-TCCCCCCGGGCGATTTTCGTTCGTG AATACATG-3′) and spc/B (5′-TCCCCCCGGGCCAATTAG 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 pHTβ plasmids (Tomita & Ike, 2005). The degree of identity between the deduced amino acid residues of the majority of the 61 homologous ORFs and the pHTβ residues ranged from 90% to 100%. Like the pHTβ 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 pHTβ and other elements

	pMG1			pHTβ			Other elements		
ORF	5′	3′	Amino acid size	Matching ORF (pHTβ)	Amino acid size	Identity† (%)	Identification (organism)/protein family	Identity† (%)	*E*-value
1	268	831	187	56	187	100	Hypothetical protein		
2	842	1702	286	57	286	98	*traA* (pMG1, *E. faecium*)	100	
3	2096	2593	165				Dehydrofolate reductase (*S. haemolyticus, L. monocytogenes*)	73	5e-64
4	3830	3955	41				Hypothetical protein		
5	3996	4286	96				Transposase, IS*1485* (*E. faecium*)	96	2e-37
6	4514	5158	214				Transposase, IS*1485* (*E. faecium*)	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 (*E. faecalis*), pXO2-38 (*B. anthracis*)	32	2e-71
11	9345	9644	99	60	99	98	PrgN (*E. faecalis*, 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	TraI topoisomerase (*L. lactis*), pXO2-77/76 (*B. anthracis*)	34	2e-98
14	12886	13485	199	2	199	99	AbiQ (*L. lactis*)	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 (*B. anthracis*)	29	8e-28
18	15356	15793	145	6	145	100	YvfU (*B. subtilis*), 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 (*B. anthracis*)	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 (*B. anthracis*)	26	3e-9
26	22161	23576	471	14	471	99	VirB11 family sec protein, pXO2-25 (*B. anthracis*)	40	1e-99
27	23614	24408	264	15	264	99	pXO2-23/24 (*B. anthracis*)	50	6e-77
28	24408	25205	265	16	265	99	pXO2-22 (*B. anthracis*)	43	8e-51
29	25223	25585	120	17	120	95	pXO2-21(*B. anthracis*)	51	6e-30
30	25599	25745	48	18	48	100	Hypothetical protein		

Table 2. Continued.

	pMG1			pHTβ			Other elements		
ORF	5′	3′	Amino acid size	Matching ORF (pHTβ)	Amino acid size	Identity† (%)	Identification (organism)/protein family	Identity† (%)	*E*-value
31	25997	26272	91	19	91	100	pXO2-18 (*B. anthracis*)	47	5e-15
32	26381	28207	608	20	608	97	pXO2-17 (*B. anthracis*)	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 (*B. anthracis*)	54*/52*	1e-50/1e-170
35	31858	34494	878	23	913	95	pXO2-14 (*B. anthracis*)	25*	1e-30
36	34542	34853	103	24	103	98	pXO2-11 (*B. anthracis*)	35	2e-12
37	34850	35467	205	25	205	100	pXO2-10 (*B. anthracis*)	35	9e-24
38	35483	35950	155	26	155	99	Hypothetical protein		
39	35966	37921	651	27	651	100	pXO2-9 (*B. anthracis*)/TraE (*L. lactis*)	48/22	1e-168/7e-12
40	37943	39079	378	28	378	100	pXO2-8 (*B. anthracis*)/ORF14(Tn*916*: *E. faecalis*), NLP/P60 family cell wall-associated hydrase	43/32	7e-78/2e-50
41	39094	39744	216	29	216	98	Hypothetical protein		
42	39758	40654	298	30	298	99	pXO2-5 (*B. anthracis*)	25	4e-19
43	40690	40920	76	31	76	100	Hypothetical protein		
44	41271	41567	98	32	98	98	pXO2-4 (*B. anthracis*)	22	0.013
45	41564	42031	155	33	155	96	Hypothetical protein		
46	42113	43633	506	34	506	99	pXO2-84 (*B. anthracis*), 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 (*B. anthracis*)/LtrC(pMRC01: *L. lactis*)	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 (*B. anthracis*)/LtrC-like protein (*S. epidermidis*)	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 (*Clostridium acetobutylicum*)	36*	2e-17
61	52480	53667	395	49	395	87	Hypothetical protein		
62	55007	55549	180	51	135	75	Transcriptional regulator, Cro/CI family (bacteriophage bIL311, *L. lactis* prophage ps3)	45*	2e-11
63	55744	56640	298				Transcriptional regulator, Cro/CI family (bacteriophage bIL311, *L. lactis* prophage ps3)	41*	3e-11
64	56689	58071	460	51	135	72	Transcriptional regulator, Cro/CI family (bacteriophage bIL311, *L. lactis* prophage ps3)	40*	9e-8
65	58085	58867	260	51	135	95	Transcriptional regulator, Cro/CI family (bacteriophage bIL311, *L. lactis* prophage ps3)	29*	8e-9
66	58907	59518	203	52	203	100	Sin recombinase (pSK1: *S. aureus*)	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, IS*256*	100	
71	61724	62170	148				Hypothetical protein (Tn*4001*: *S. aureus*)	100	
72	62171	63610	479				Bifunctional AAC/APH (Tn*4001*: *S. aureus*)	100	
73	63748	64920	390				Transposase, IS*256*	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 pHTβ, the matching gene in the BLAST search and the name of a similar gene in another organism.

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

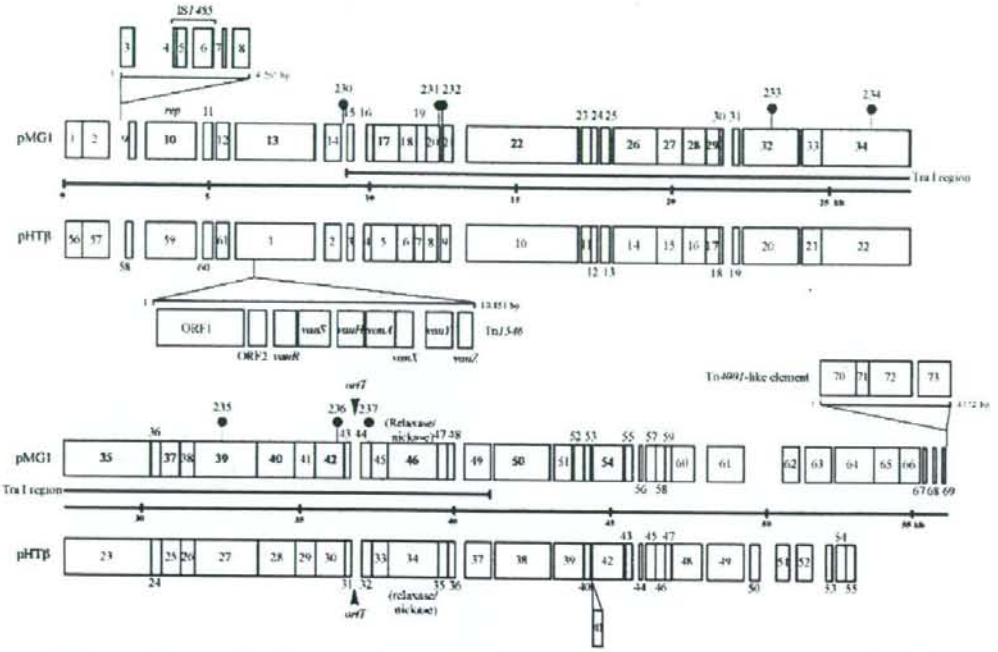

Fig. 1. Genetic map and ORFs deduced from the complete plasmid sequence of pMG1 plasmid and the pHTβ plasmid. Boxes indicate the ORFs identified on pMG1 and pHTβ. All ORFs are transcribed from left to right, except ORF1 of Tn*1546* on pHTβ. The locations of the Tn*917* insertions in pMG1 (vertical bar with black circle) are shown on the map. The number on the insert indicates the pMG plasmid construct (pMG1::Tn*917*), 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* multiresistance 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*), pAMβ1 (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 pHTβ, 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 between ORF31 and −32 of pHTβ, 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 IS*1485*, which was originally identified in *E. faecium*, and ORF5 and ORF6 corresponded to orfA and orfB of IS*1485*, respectively (Cheng *et al.*, 1999). IS*1485* 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 IS*256*. The regions between 60 448 and 61 771 bp, and 63 647 and 64 970 bp of the plasmid map were identical to IS*256* and these two IS*256* sequences were located in the same orientation. ORF72 was located between these two IS*256* sequences and was identical to the aminoglycoside-resistance gene found in Tn*4001* encoding the bifunctional aminoglycoside-modifying enzyme, AAC (*aac6′*)/APH (*aph2″*) conferring kanamycin and gentamicin resistance; however, the corresponding gene in Tn*4001* is flanked by two IS*256* sequences located in different orientations (Rouch *et al.*, 1987). The DNA

Table 3. Transcripts of ORFs produced in pMG1::Tn*917* mutant plasmids

Plasmid	ORF15	ORF17	ORF20	ORF21	ORF22	ORF26	ORF32	ORF34	ORF39	ORF40	ORF42	ORF44	ORF49	ORF50
pMG230	Tn*917**	+	+	+	+	+	+	+	+	+	+	+	NT	+
pMG231	+	+	Tn*917*	+	+	+	+	+	+	+	+	+	NT	NT
pMG232	+	+	+	Tn*917*	−	−	−	−	−	−	−	+	+	+
pMG233	NT	NT	NT	+	NT	+	Tn*917*	−	−	−	−	+	NT	NT
pMG234	NT	NT	NT	NT	NT	NT	+	Tn*917*	−	−	−	+	NT	NT
pMG235	+	NT	NT	NT	NT	NT	NT	+	Tn*917*	−	−	+	NT	NT
pMG236	NT	NT	NT	+	+	+	+	+	+	+	Tn*917*	+	NT	NT
pMG237	NT	NT	NT	+	+	NT	NT	+	NT	NT	+	Tn*917*	−	+

*Tn*917* insertions were mapped in the ORFs listed above, except that the Tn*917* 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 Tn*4001*, with the exception of the orientation of IS*256*R and a 43-bp deletion upstream from the left end of IS256R. This Tn*4001*-like transposon was flanked by an 8-bp direct repeat (TAGAATTA) at both ends. The direct repeats at both ends of the Tn*4001*-like transposon and IS*1485* 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 Tn*917* 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 σ^{70}-dependent promoters (−10 and −35) upstream of ORF15, ORF16, ORF21, ORF22 and ORF44 in the 27.9-kb region that lies between the Tn*917* 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 Tn*917* 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 pHTβ plasmid (Tomita & Ike, 2005). The minimum Tra I region of the pHTβ plasmid consisted of ORFs 8–34 of the pHTβ 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

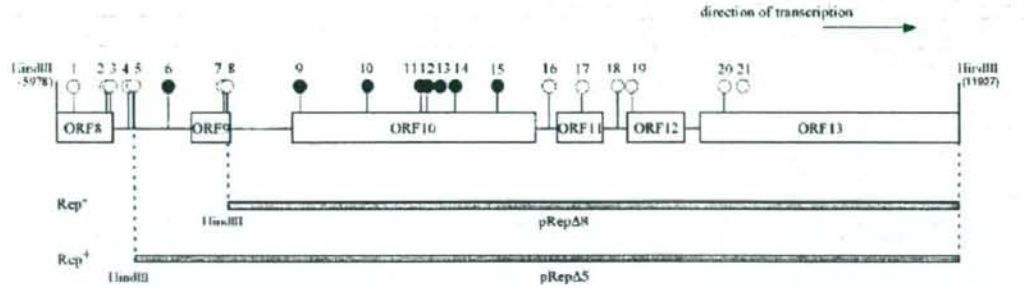

Fig. 2. Autonomously replicating 6-kb HindIII fragment of pMG1 in *Enterococcus faecalis*. Open boxes indicate ORFs. ORF8 and ORF12 are truncated; ORF8 does not have the N-terminal region and ORF12 does not have the C-terminal region. The locations of Transprimer-5 insertions in the pRep plasmid (vertical 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 pRepΔ5 and pRepΔ8. The HindIII site at the left end of each gray line is located in Transprimer-5. The Rep$^+$ or Rep$^-$ 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 multiresistance 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 pHTβ 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, pRepΔ5 and pRepΔ8, were constructed from mutant plasmids #5 and #8, respectively, to confirm the requirement for the region located between OFR8 and ORF9 (Fig. 2) by partial digestion by HindIII, followed by self-ligation. These were then used to transform *E. coli* DH5α. 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 pRepΔ8. A deletion mutant lacking ORF8 was obtained

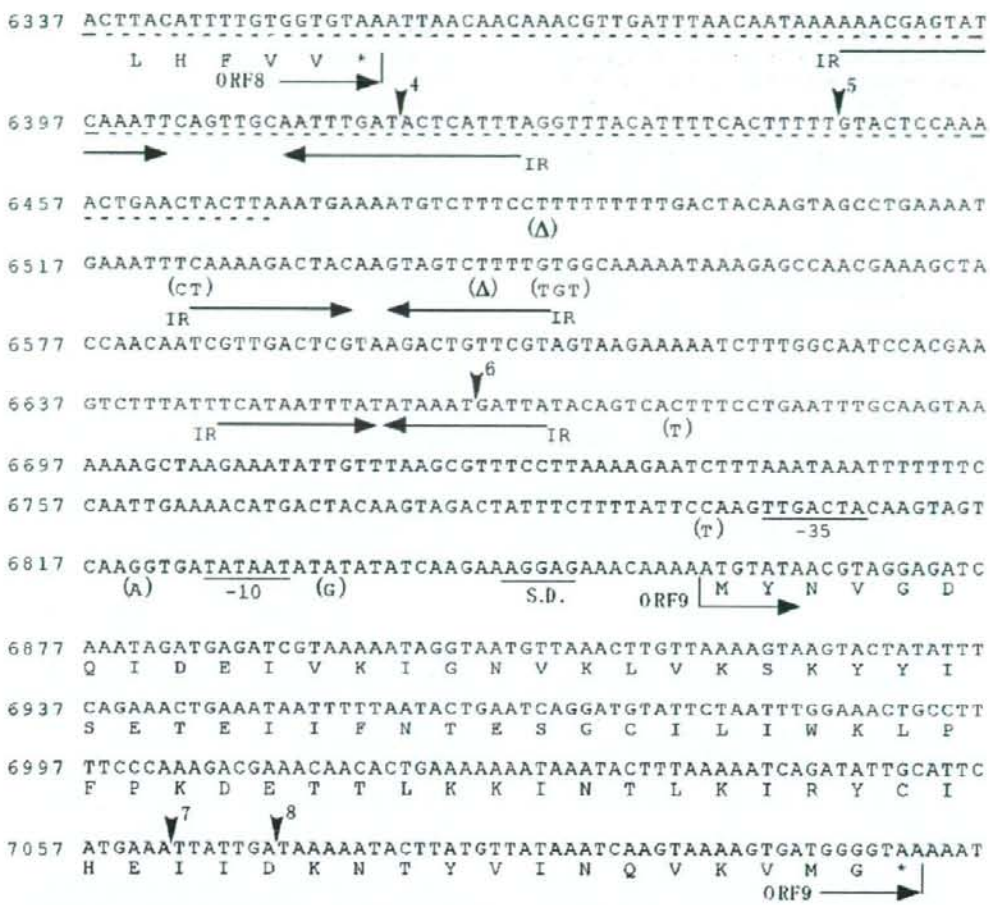

Fig. 3. Nucleotide sequences of the region between ORF8 and ORF9 of the pMG1 plasmid. The 780-bp DNA sequence of the region between ORF8 and ORF9 is shown. The horizontal arrows labeled with IR indicate the inverted repeats. The numbered arrowheads facing downwards indicate the locations of the Transprimer-5 insertion shown in Fig. 2. The nucleotide substitutions in pHTβ are shown in parentheses below the sequences. Δ, indicates deletion. pMG1 and pHTβ 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 pRepΔ5. The two deletion-mutant plasmids were used to transform *E. faecalis* OG1X to examine their ability to replicate. Spectinomycin-resistant transformants were obtained with pRepΔ5, but not with pRepΔ8. 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).

Acknowledgements

This work was supported by grants from the Japanese Ministry of Education, Culture, Sport, Science and Technology [Tokutei-ryoiki (Matrix of Infection Phenomena), Kiban (B), Kiban (C)], and the Japanese Ministry of Health, Labor and Welfare (H18-Shinko-11).

We thank E. Kamei for helpful advice on the manuscript.

References

Ausubel FM, Brent R, Kingston RW, Moore DD, Seidman JG, Smith JA & Struhl K (1987) *Current Protocols in Molecular Biology*. John Wiley & Sons Inc., New York.

Cheng SQ, Musso RE, Liu R, Niemeyer DM, Shaw JJ, McCleskey FK & DelVecchio VG (1999) Cloning of insertion sequence IS*1485* from *Enterococcus* species. *Plasmid* **42**: 42–44.

Clewell DB & Dunny GM (2002) Conjugation and genetic exchange in enterococci. *The Enterococci: Pathogenesis, Molecular Biology, and Antibiotic Resistance* (Gilmore MS, ed), pp. 265–300. American Society for Microbiology, Washington, DC.

Dougherty BA, Hill C, Weidman JF, Richardson DR, Venter JC & Ross RP (1998) Sequence and analysis of the 60 kb conjugative, bacteriocin-producing plasmid pMRC01 from *Lactococcus lactis* DPC3147. *Mol Microbiol* **29**: 1029–1038.

Franke AE & Clewell DB (1981) Evidence for a chromosome-borne resistance transposon (Tn*916*) in *Streptococcus faecalis* that is capable of "conjugal" transfer in the absence of conjugative plasmid. *J Bacteriol* **145**: 494–502.

Fujimoto S, Hashimoto H & Ike Y (1991) Low cost device for electro-transformation and its application to the highly efficient transformation of *Escherichia coli* and *Enterococcus faecalis*. *Plasmid* **26**: 131–135.

Fujimoto S, Tomita H, Wakamatsu E, Tanimoto K & Ike Y (1995) Physical mapping of the conjugative bacteriocin plasmid pPD1 of *Enterococcus faecalis* and identification of the determinant related to the pheromone response. *J Bacteriol* **177**: 5574–5581.

Goodner B, Hinkle G, Gattung S *et al.* (2001) Genome sequence of the plant pathogen and biotechnology agent *Agrobacterium tumefaciens* C58. *Science* **294**: 2323–2328.

Harrison RW, Miller JC, D'Souza MJ & Kampo G (1997) Easy gene walking. *Biotechniques* **22**: 650–653.

Hedberg PJ, Leonard BA, Ruhfel RE & Dunny GM (1996) Identification and characterization of the genes of *Enterococcus faecalis* plasmid pCF10 involved in replication and in negative control of pheromone-inducible conjugation. *Plasmid* **35**: 46–57.

Ike Y, Craig RA, White BA, Yagi Y & Clewell DB (1983) Modification of *Streptococcus faecalis* sex pheromones after acquisition of plasmid DNA. *Proc Natl Acad Sci USA* **80**: 5369–5373.

Ike Y, Tanimoto K, Tomita H, Takeuchi K & Fujimoto S (1998) Efficient transfer of the pheromone-independent *Enterococcus faecium* plasmid pMG1 (Gm^r) (65.1 kilobases) to *Enterococcus* strains during broth mating. *J Bacteriol* **180**: 4886–4892.

LeBlanc DJ & Lee LN (1991) Replication functions of pVA380-1. *Genetics and Molecular Biology of Streptococci, Enterococci, and Lactococci* (Dunny GM, Cleary PP & McKay LL, eds), pp. 224–227. American Society for Microbiology, Washington, DC.

Machida RJ, Miya MU, Nishida M & Nishida S (2002) Complete mitochondrial DNA sequence of *Tigriopus japonicus* (*Crustacea: Copepoda*). *Mar Biotechnol* **4**: 406–417.

Makino S, Uchida I, Terakado N, Sasakawa C & Yoshikawa M (1989) Molecular characterization and protein analysis of the *cap* region, which is essential for encapsulation in *Bacillus anthracis*. *J Bacteriol* **171**: 722–730.

Miller JH (1972) *Experiments in Molecular Genetics*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Nelson KE, Fouts DE, Mongodin EF *et al.* (2004) Whole genome comparisons of serotype 4b and 1/2a strains of the food-borne pathogen *Listeria monocytogenes* reveal new insights into the core genome components of this species. *Nucleic Acids Res* **32**: 2386–2395.

Rasko DA, Ravel J, Okstad OA *et al.* (2004) The genome sequence of *Bacillus cereus* ATCC 10987 reveals metabolic adaptations and a large plasmid related to *Bacillus anthracis* pXO1. *Nucleic Acids Res* **32**: 977–988.

Rouch DA, Byrne ME, Kong YC & Skurray RA (1987) The *aacA–aphD* gentamicin and kanamycin resistance determinant of Tn*4001* from *Staphylococcus aureus*: expression and nucleotide sequence analysis. *J Gen Microbiol* **133**: 3039–3052.

Sambrook J, Fritsch EF & Maniatis T (1989) *Molecular Cloning: A Laboratory Manual*, 2nd edn. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Schleifer KH (1986) Gram-positive cocci. *Bergey's Manual of Systematic Bacteriology, Vol. 2* (Sneath PHA, Mair NS, Sharpe ME & Holt JG, eds), pp. 999–1103. Williams & Wilkins, Baltimore, MD.

Schwarz FV, Perreten V & Teuber M (2001) Sequence of the 50-kb conjugative multi-resistance plasmid pRE25 from *Enterococcus faecalis* RE25. *Plasmid* **46**: 170–187.

Tanimoto K & Ike Y (2002) Analysis of the conjugal transfer system of the pheromone-independent highly transferable Enterococcus plasmid pMG1: identification of a *tra* gene (*traA*) up-regulated during conjugation. *J Bacteriol* **184**: 5800–5804.

Tomita H & Ike Y (2005) Genetic analysis of transfer-related regions of the vancomycin resistance *Enterococcus* conjugative plasmid pHTb: identification of *oriT* and a putative relaxase gene. *J Bacteriol* **187**: 7727–7737.

Tomita H, Pierson C, Lim SK, Clewell DB & Ike Y (2002) Possible connection between a widely disseminated conjugative gentamicin resistance (pMG1-like) plasmid and the emergence of vancomycin resistance in *Enterococcus faecium*. *J Clin Microbiol* **40**: 3326–3333.

Tomita H, Tanimoto K, Hayakawa S, Morinaga K, Ezaki K, Oshima H & Ike Y (2003) Highly conjugative pMG1-like plasmids carrying Tn*1546*-like transposons that encode vancomycin resistance in *Enterococcus faecium*. *J Bacteriol* **185**: 7024–7028.

Weaver KE & Clewell DB (1990) Regulation of the pAD1 sex pheromone response in *Enterococcus faecalis*: effects of host strain and *traA*, *traB*, and C region mutants on expression of an E region pheromone-inducible *lacZ* fusion. *J Bacteriol* **172**: 2633–2641.

Weaver KE, Rice LB & Churchwood G (2002) Plasmids and transposons. *The Enterococcus: Pathogenesis, Molecular Biology, and Antibiotic Resistance* (Gilmore MS, ed), pp. 219–263. ASM Press, Washington, DC.

Youngman P (1987) Plasmid vector for recovering and exploiting Tn*917* transposition in *Bacillus* and other gram-positive bacteria. *Plasmids: A Practical Approach* (Hardy KG, ed), pp. 79–104. IRL Press Ltd, Oxford.

ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, Feb. 2009, p. 735–747 Vol. 53, No. 2
0066-4804/09/$08.00+0 doi:10.1128/AAC.00754-08

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)[▽]

Bo Zheng,[1,3] Haruyoshi Tomita,[1]* Takako Inoue,[1] and Yasuyoshi Ike[1,2]

Department of Bacteriology[1] and Laboratory of Bacterial Drug Resistance,[2] Gunma University Graduate School of Medicine, Maebashi, Gunma 371-8511, Japan, and Institute of Clinical Pharmacology, Peking University First Hospital, Beijing 10034, China[3]

Received 9 June 2008/Returned for modification 18 July 2008/Accepted 18 November 2008

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 Tn*1549*-like element encoding *vanB2*-type resistance and the Bac41-like bacteriocin genes of pheromone-responsive plasmid pYI14. 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_{MG} 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 Tn*1546* transposon or a Tn*1546*-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 Tn*1549* (34 kb) (30) and the closely related transposon Tn*5382* (27 kb) (3), which have similarities with the Tn*916* family of conjugative transposons (10, 26). The transposable elements

* Corresponding author. Mailing address: Department of Bacteriology, Gunma University Graduate School of Medicine, Maebashi, Gunma 371-8511, Japan. Phone: 81-27-220-7990. Fax: 81-27-220-7996. E-mail: tomitaha@med.gunma-u.ac.jp.

▽ Published ahead of print on 24 November 2008.

TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant features	Reference or source
Strains		
E. faecalis		
FA2-2	*rif fus*	11
JH2SS	*spc str*	59
UV202	*rif fus*, recombination-deficient mutant of JH2-2	71
OG1S(OG1-10)	*str*, derivative of OG1	18
OG1X	*str*, protease-negative derivative of OG1-10	37
NKH15	Representative of vancomycin-resistant (*vanB2*-type) first hospital outbreak strain, pMG2200 (Van^r, Bac), pMG2201 (Em^r, Cyl)	This study; 47
E. faecium		
BM4105RF	*rif fus*, derivative of plasmid-free *E. faecium* BM4105	65
BM4105SS	*spc str*, derivative of plasmid-free *E. faecium* BM4105	65
E. coli DH5α	*endA1 recA1 gyrA96 thi-1 hsdR17 supE44 relA1* Δ*(argE-lacZYA)U169*	Bethesda Research Laboratories
Plasmids		
pMG2200	*vanB2*, Bac44, 106.5-kb pheromone (cCF10)-responsive conjugative plasmid from NKH15	This study
pMG2201	Em^r, cytolysin (Hly/Bac), 60-kb pheromone (cAD1)-responsive conjugative plasmid from NKH15	This study
pMG2210	pAM401 containing the 322-bp *oriT* region of pMG2200	This study
pAM401	*E. coli-E. faecalis* shuttle, *cat tet*	70
pUC18	*E. coli* cloning vector, Amp^r	Nippon gene
pMG326	pMW119 containing a 16.7-kbp EcoRI-SalI fragment of pPD1; pheromone regulatory region	28, 58
pYI14	Bac41, a 61-kb pheromone-responsive conjugative plasmid from YI714	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 cytolysin.

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 μg ml^{-1}; streptomycin, 250 μg ml^{-1}; spectinomycin, 250 μg ml^{-1}; chloramphenicol, 20 μg ml^{-1}; rifampin (rifampicin), 25 μg ml^{-1}; and fusidic acid, 25 μg ml^{-1}. The following antibiotics were used at the indicated concentrations for the selection of *E. coli*: ampicillin, 100 μg ml^{-1}, and chloramphenicol, 50 μg ml^{-1}. All antibiotics were obtained from Sigma Chemical Co. 5-Bromo-4-chloro-3 indolyl-β-D-galactopyranoside was used at 40 μg 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 5×10^5 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[a]	Date of isolation (yr/mo/day)	Source	Diagnosis or underlying disease	MIC (μg/ml)[b]										Transfer frequency[c] (per donor cell)						Plasmid contents[e]	
														Vancomycin resistance		Erythromycin resistance		Bacteriocin production[d]			
				VCM	TEIC	ABPC	CPFX	EM	GM	KM	SM	CP	TC	Broth mating (4 h)	Filter mating (16 h)	Broth mating (4 h)	Filter mating (16 h)	Bac	Cytolysin (Hly/Bac)	pMG2200-like	pMG2201-like
NKH1	1999/7/9	Sputum	Urinary tract infection	128	0.25	4	64	0.25	>1,024	>1,024	32	8	64	10^{-7}	10^{-3}	$<10^{-7}$	$<10^{-7}$	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^{-5}	10^{-4}	10^{-4}	10^{-2}	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^{-5}	10^{-4}	10^{-4}	10^{-2}	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^{-5}	10^{-2}	10^{-3}	10^{-2}	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^{-5}	10^{-4}	$<10^{-7}$	$<10^{-7}$	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^{-4}	10^{-2}	10^{-3}	10^{-2}	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^{-5}	10^{-3}	$<10^{-7}$	$<10^{-7}$	P	N	P	N
NKH8	1999	Rectal swab	Pneumonia	256	0.25	8	64	>1,024	>1,024	>1,024	32	8	64	10^{-3}	10^{-2}	10^{-3}	10^{-2}	P	P	P	P
NKH9	1999	Rectal swab	Mallory-Weiss syndrome	256	0.25	8	64	>1,024	1,024	>1,024	32	8	64	10^{-7}	10^{-3}	10^{-3}	10^{-2}	P	P	P	P
NKH10	1999	Rectal swab	Bedsore	256	0.25	4	64	>1,024	>1,024	>1,024	32	8	64	10^{-6}	10^{-2}	10^{-3}	10^{-2}	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^{-5}	10^{-2}	$<10^{-7}$	$<10^{-7}$	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^{-4}	10^{-2}	10^{-3}	10^{-2}	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^{-5}	10^{-2}	10^{-3}	10^{-2}	P	P	P	P
NKH14	1999	Sputum	Pneumonia	256	0.25	8	64	>1,024	>1,024	>1,024	32	8	64	10^{-6}	10^{-2}	10^{-3}	10^{-2}	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^{-4}	10^{-2}	10^{-3}	10^{-2}	P	P	P	P
NKH16	1999/8/1	Rectal swab	Choledocholithiasis	64	0.25	8	64	0.125	>1,024	>1,024	32	4	32	10^{-5}	10^{-2}	$<10^{-7}$	$<10^{-7}$	P	N	P	N
NKH17	1999/8/1	Rectal swab	Cholecystitis	32	0.125	8	64	0.125	>1,024	>1,024	32	4	32	10^{-3}	10^{-2}	$<10^{-7}$	$<10^{-7}$	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^{-3}	10^{-2}	10^{-4}	10^{-2}	P	P	P	P

[a] The Tn *1549*-like element was located on plasmids in all strains.
[b] Abbreviations: VCM, vancomycin; TEIC, teicoplanin; ABPC, ampicillin; CPFX, ciprofloxacin; EM, erythromycin; GM, gentamicin; KM, kanamycin; SM, streptomycin; CP, chloramphenicol; TC, tetracycline.
[c] The wild-type strains and *E. faecalis* FA2-2 were used as the donor and recipient, respectively (11).
[d] The indicator strains used for bacteriocin activity were *Staphylococcus aureus* FDA209P, *E. faecalis* FA2-2 and OG1S, *E. faecium* BM4105RF, and *Enterococcus hirae* ATCC 9790 (61). P, positive; N, negative.
[e] The pMG2200-like plasmid is 106.5 kb, *vanB2*, Bac41, and cCF10 responsive. The pMG2201-like plasmid is 65.7 kb, EM^r, cytolysin (Hly/Bac) positive, and cAD1 responsive. P, positive; N, negative.

TABLE 3. Sequences of oligonucleotides used in the study

Oligonucleotide name	Sequence (5′–3′)[a]	Plasmid generated with the primer or use
V43622F	CCG GGA TCC AGA ACA ATC AAC AAC TAA TTA GGC	pMG2210
V43943R	GCG GGA TCC TAT TTA TTC TCT AGT ATT CGC	pMG2210
cylL$_L$1	GAT GGA GGG TAA GAA TTA TGG	57
cylL$_L$2	GCT TCA CCT CAC TAA GTT TTA TAG	57
cylL$_S$1	GAA GCA CAG TGC TAA ATA AGG	57
cylL$_S$2	GTA TAA GAG GGC TAG TTT CAC	57
cylB-TE15	ATT CCT ACC TAT GTT 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

[a] 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^r) 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 cytolysin (Hly/Bac) genes in the VRE isolates. To detect the cytolysin (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$_L$*, *cylL$_S$*, *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 cytolysin 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^{-3} to 10^{-5} 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^{-8} 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 cytolysin activities, the vancomycin-resistant transconjugants showed only bacteriocin activity, and the erythromycin-resistant transconjugant showed only cytolysin activity.

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

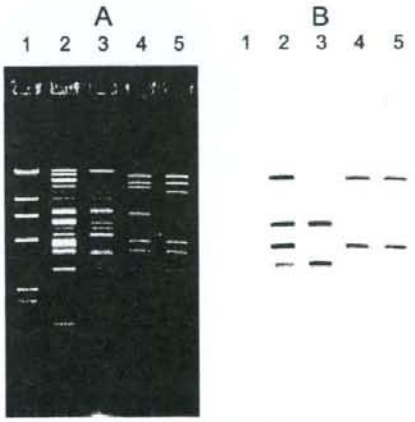

FIG. 1. Agarose gel electrophoresis of restriction endonuclease-digested DNA of pMG2200 and pMG2201 and Southern hybridization with genes specific from the pheromone-responsive plasmid. Agarose gel electrophoresis of EcoRI-digested plasmid DNA (A) and Southern hybridization with pMG326 (58) (B). Lanes: 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[a]	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	*Clostridium perfringens* pCP13	Transmembrane protein, ATPase
2	CW	2019	2399	381/126	PCP49	25	43	*Clostridium perfringens* pCP13	
3	CW	2636	4351	1,716/571	PCP48/46 (combined)	30	49	*Clostridium perfringens* pCP13	TraC-like transmembrane bound ATPase
4	CW	4404	5084	1,077/359	PCP46	30	53	*Clostridium perfringens* pCP13	
5	CW	5294	6445	1,149/383	LtrC-like	28	45	*Bacillus thuringiensis*	
6	CW	6442	7524	1,083/360	ORF14	48	66	*Enterococcus faecalis* Tn*916*	
7	CW	7579	7821	243/80	Hypothetical protein	42	58	*Lactobacillus plantarum*	
8	CW	7822	8148	327/108	PemK-like protein	47	68	*Enterococcus faecium*	Toxin of the ChpA-ChpR toxin-antitoxin system, endoribonuclease
9	CW	8240	8579	341/113					Interrupted by Tn*1549*-like insertion at 114th amino acid of the original ORF
10	CW	8831	10030	1,200/399	ORF13	100	100	*Enterococcus faecalis* V583 Tn*1549*	
11	CW	10052	10264	213/70	ORF14	100	100	*Enterococcus faecalis* V583 Tn*1549*	
12	CW	10339	10815	477/158	ORF15	100	100	*Enterococcus faecalis* V583 Tn*1549*	
13	CW	10812	12620	1,806/564	ORF16, TrsK-like protein	99	99	*Enterococcus faecalis* V583 Tn*1549*	TraG/TraD/VirD4 family involved in type IV secretion
14	CW	13004	13867	864/287	ORF17	100	100	*Enterococcus faecalis* V583 Tn*1549*	
15	CW	13898	14440	543/180	ORF18, MunI-like protein	100	100	*Enterococcus faecalis* V583 Tn*1549*	Methyltransferase
16	CW	14454	14876	423/140	ORF19	100	100	*Enterococcus faecalis* V583 Tn*1549*	
17	CW	14806	17205	2,400/799	ORF20, TrsE-like protein	100	100	*Enterococcus faecalis* V583 Tn*1549*	VirB4
18	CW	17237	19228	1,992/663	ORF21	100	100	*Enterococcus faecalis* V583 Tn*1549*	
19	CW	19251	19502	252/83	ORF22	100	100	*Enterococcus faecalis* V583 Tn*1549*	
20	CW	19492	20721	1,230/409	ORF23	100	100	*Enterococcus faecalis* V583 Tn*1549*	
21	CW	20718	22799	2,082/693	ORF24	100	100	*Enterococcus faecalis* V583 Tn*1549*	DNA topoisomerase III-like protein
22	CW	22948	26868	3,921/1,306	ORF25, LtrC-like protein	100	100	*Enterococcus faecalis* V583 Tn*1549*	
23	CW	26869	27813	945/314	ORF26	100	100	*Enterococcus faecalis* V583 Tn*1549*	
24	CCW	28766	28320	447/148	ORF27	100	100	*Enterococcus faecalis* V583 Tn*1549*	
25	CCW	30435	29107	1,329/442	ORF28, relaxase	100	100	*Enterococcus faecalis* V583 Tn*1549*	
26	CCW	30725	30396	330/109	ORF29	100	100	*Enterococcus faecalis* V583 Tn*1549*	
27	CCW	31354	30983	372/123	ORF30	100	100	*Enterococcus faecalis* V583 Tn*1549*	
28	CW	32627	33286	660/219	$VanR_B$	100	100	*Enterococcus faecalis* V583 Tn*1549*	Two-component regulatory system, regulator protein
29	CW	33289	34632	1,344/447	$VanS_B$	99	99	*Enterococcus faecalis* V583 Tn*1549*	Two-component regulatory system, sensor protein
30	CW	34803	35609	807/268	$VanY_B$	100	100	*Enterococcus faecalis* V583 Tn*1549*	D-Carboxypeptidase
31	CW	35627	36454	828/275	VanW	100	100	*Enterococcus faecalis* V583 Tn*1549*	
32	CW	36451	37422	972/323	$VanH_B$	94	97	*Enterococcus faecalis* V583 Tn*1549*	D-2-Hydroxyacid dehydrogenase
33	CW	37415	38443	1,029/342	VanB2	99	99	*Enterococcus faecalis* V583 Tn*1549*	D-Ala:D-Lac ligase
34	CW	38449	39057	609/202	$VanX_B$	95	97	*Enterococcus faecalis* V583 Tn*1549*	DD-Dipeptidase
35	CW	39645	40076	432/143	ORF7	99	100	*Enterococcus faecalis* V583 Tn*1549*	
36	CW	40083	40316	234/77	ORF8	85	88	*Enterococcus faecalis* V583 Tn*1549*	

Continued on following page