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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)[▽]

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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 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

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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-

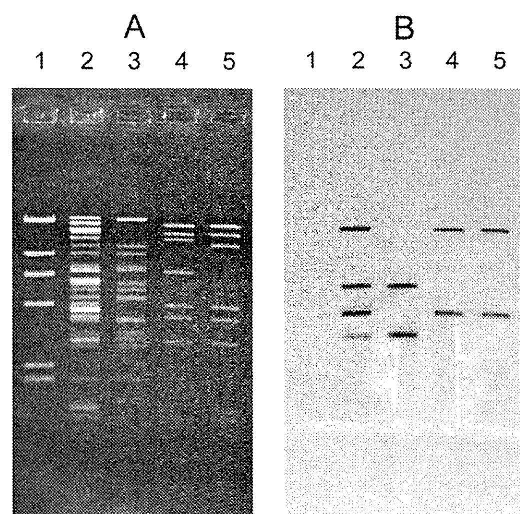

FIG. 1. Agarose gel electrophoresis of restriction endonuclease-digested DNA of pMG2200 and pMG2201 and Southern hybridization with genes specific from the pheromone-responsive plasmid. Agarose gel electrophoresis of EcoRI-digested plasmid DNA (A) and Southern hybridization with pMG326 (58) (B). Lanes: 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*	DD-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

TABLE 4—*Continued*

ORF	Direction[a]	5′ residue no.	3′ residue no.	Gene (bp)/ protein (aa)	Homology	% Identity	% Similarity	Organism or plasmid	Function
37	CW	40740	40940	201/66	Xis-Tn*1549*	98	100	*Enterococcus faecalis* V583 Tn*1549*	Excisionase
38	CW	41024	42217	1,194/397	Int-Tn*1549*	100	100	*Enterococcus faecalis* V583 Tn1549	Integrase
39	CW	42392	42492	101/32					
40	CW	42522	42695	174/57					
41	CW	42702	43643	942/313	PCP43	22	44	*Clostridium perfringens* pCP13	
42	CW	44015	44296	282/93	PCP41	30	50	*Clostridium perfringens* pCP13	Putative transcriptional regulator
43	CW	44289	44681	393/130	Hypothetical protein	26	47	*Agrobacterium tumefaciens*	Transcriptional regulator, AraC family
44	CW	44674	46734	2,061/686	PCP39	32	52	*Clostridium perfringens* pCP13	Putative relaxase (nickase)
45	CW	46811	48469	1,659/552					
46	CCW	49999	48656	1,341/447	PcfJ, Orf63	86, 76	93, 86	*Enterococcus faecalis* pCF10, pAD1	
47	CCW	50772	49999	792/258	PcfK, ORF62	78, 77	88, 87	*Enterococcus faecalis* pCF10, pAD1	Phage related
48	CCW	51467	50916	552/182	PcfL	100	100	*Enterococcus faecalis* pCF10	
49	CCW	51664	51491	174/57	PcfM	98	100	*Enterococcus faecalis* pCF10	
50	CCW	51852	51667	186/61	PcfN	100	100	*Enterococcus faecalis* pCF10	
51	CW	52148	52348	201/66	PcfP	100	100	*Enterococcus faecalis* pCF10	
52	CCW	52680	52450	231/76	PcfQ	100	100	*Enterococcus faecalis* pCF10	
53	CW	52837	53229	393/130	PcfR	98	100	*Enterococcus faecalis* pCF10	
54	CW	53295	53747	453/150	ORF1, PcfS	98, 98	98, 98	*Enterococcus faecalis* pYI14, pCF10	Phage-related single-strand binding protein
55	CW	53761	53913	153/50	ORF2	88	92	*Enterococcus faecalis* pYI14	
56	CW	53925	54515	591/196	ORF3, PcfT	84, 81	91, 88	*Enterococcus faecalis* pYI14, pCF10	Thermonuclease precursor
57	CW	54521	54841	330/109	ORF4, PcfU	97, 95	100, 97	*Enterococcus faecalis* pYI14, pCF10	
58	CW	54949	55194	246/81					
59	CW	55248	55430	183/60	ORF5	100	100	*Enterococcus faecalis* pYI14	
60	CW	55625	56341	717/238	ORF6	100	100	*Enterococcus faecalis* pYI14	
61	CW	56479	58266	1,788/595	$BacL_1$	99	99	*Enterococcus faecalis* pYI14	Prebacteriocin for Bac41
62	CW	58452	59087	636/211	$BacL_2$	100	100	*Enterococcus faecalis* pYI14	Prebacteriocin secretion
63	CW	59110	59541	432/133	ORF9	100	100	*Enterococcus faecalis* pYI14	
64	CW	59544	60071	528/175	ORF10	97	99	*Enterococcus faecalis* pYI14	
65	CW	60114	62294	2,181/726	BacA	100	100	*Enterococcus faecalis* pYI14	Activator for Bac41
66	CW	62402	62944	543/180	BacI	99	100	*Enterococcus faecalis* pYI14	Provides immunity against Bac41
67	CW	63011	63586	576/191	ORF13	98		*Enterococcus faecalis* pYI14	
68	CW	63729	64061	333/110	ORF14	98	100	*Enterococcus faecalis* pYI14	
69	CCW	65564	64245	1,317/439	Tnp	75	85	*Lactococcus lactis* Tn*5721*	Transposase
70	CW	65797	65937	141/46					
71	CW	66024	66854	831/276	ORF130	45	68	*Lactobacillus sakei*	
72	CW	66917	67126	210/69					
73	CW	68042	68464	423/140	Ej97D	58	80	*Enterococcus faecalis*	Enterocin EJ97 plasmid, CsbD stress protein
74	CW	68628	69194	567/188	EF0053	65	82	*Enterococcus faecalis*	
75	CW	69211	69408	198/65	EF0054	61	77	*Enterococcus faecalis*	
76	CW	69435	69971	537/178	Gls24	88	95	*Enterococcus faecalis* V583	Stress response
77	CCW	71158	70100	1,059/352	ExigDRAFT 2618	45	69	*Exiguobacterium sibiricum*	EzrA, septation ring formation regulator
78	CCW	71425	71183	243/80	EF2708	72	87	*Enterococcus faecalis* V583	Putative membrane protein
79	CW	71630	71872	243/80					
80	CW	71958	72170	213/70	Tnp	53	71	*Lactococcus lactis*	Transposase

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TABLE 4—*Continued*

ORF	Direction[a]	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	*Enterococcus faecalis* pCF10, pAD1	DNA invertase
83	CW	73331	73618	288/95	PcfZ, Orf87	90, 89	97, 96	*Enterococcus faecalis* pCF10, pAD1	
84	CW	73612	73716	105/35	UvrC-N (truncated)	97, 98	98, 100	*Enterococcus faecalis* pCF10, pAD1	
85	CCW	76852	73880	2,973/990	EP0008	99	100	*Enterococcus faecalis* pAM373	Transposase, Tn*3* family
86	CW	76983	77588	606/201	EP0007	63	78	*Enterococcus faecalis* pAM373	Recombinase
87	CW	77651	77775	125/40	UvrC-C (truncated)	97, 98	98, 100	*Enterococcus faecalis* pCF10, pAD1	
88	CW	77836	78045	210/69	UvrB	92, 92	97, 97	*Enterococcus faecalis* pCF10, pAD1	
89	CW	78057	78359	303/100	UvaB	94, 94	100, 94	*Enterococcus faecalis* pCF10, pAD1	
90	CW	78786	80114	1,329/442	UvrA	99, 97	99, 98	*Enterococcus faecalis* pCF10, pAD1	UV resistance
91	CW	80111	80461	351/116	UvaE, OrfB	95, 94	98, 95	*Enterococcus faecalis* pCF10, pAD1	
92	CW	80418	80630	213/70	UvaF, OrfC	97, 92	98, 97	*Enterococcus faecalis* pCF10, pAD1	
93	CW	81062	81358	294/98	PrgN, OrfE	98, 91	100, 98	*Enterococcus faecalis* pCF10, pAD1	Replication control
94	CW	81612	82394	783/260	ParA	99	99	*Enterococcus faecalis* pTEF2	Plasmid partitioning
95	CW	82387	82743	354/118	EF B0065	100	100	*Enterococcus faecalis* pTEF2	
96	CW	83003	84010	1,008/335	RepA, PrgW	97, 64	98, 77	*Enterococcus faecalis* pTEF2, pCF10	Plasmid replication
97	CW	84169	85806	1,638/545	PrgZ	99	99	*Enterococcus faecalis* pCF10	Pheromone uptake
98	CW	85817	86971	1,155/384	PrgY	100	100	*Enterococcus faecalis* pCF10	pheromone shutdown
99	CCW	87957	87004	954/319	PrgX	100	100	*Enterococcus faecalis* pCF10	cCF10 pheromone receptor, negative regulator(DNA binding protein)
100	CW	88166	88237	72/23	PrgQ	100	100	*Enterococcus faecalis* pCF10	iCF10 precursor, cCF10-inhibitor
101	CW	88726	89082	357/118	TraE1	100	100	*Enterococcus faecalis* pAD1	Positive regulator for conjugation
102	CW	89664	89969	306/100	OrfY	97	100	*Enterococcus faecalis* pAD1	
103	CW	89980	92655	2,676/891	Seal	89	94	*Enterococcus faecalis* pAD1	Surface exclusion protein
104	CW	92675	92953	279/92	EF2120	66	86	*Enterococcus faecalis* V583	
105	CW	93413	93748	336/111	Orf1	100	100	*Enterococcus faecalis* pAD1	
106	CW	94055	97981	3,927/1,308	PrgB, Asa1	95, 82	97, 87	*Enterococcus faecalis* pCF10, pAD1	Aggregation substance
107	CW	98078	98398	321/106	PrgU, Orf3	97, 98	99, 99	*Enterococcus faecalis* pCF10, pAD1	
108	CW	98614	99444	831/276	Pd53/Pd113 (combined)	81/95	95/87	*Enterococcus faecalis* pPD1	
109	CW	99690	100025	336/111	Pd96	56	71	*Enterococcus faecalis* pPD1	
110	CW	100033	101160	1,128/375	Pd377	85	89	*Enterococcus faecalis* pPD1	
111	CW	101325	101468	144/47					
112	CW	101688	103247	1,560/519	Pd78	88	91	*Enterococcus faecalis* pPD1	Pheromone-inducible surface protein
113	CW	103417	103950	534/177	RUMTOR 00674	20	45	*Ruminococcus torques*	Permease
114	CW	104015	19	2,532/843	TraG/VirD4	25	43	*Bacillus subtilis* p19	coupling protein

[a] 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

FIG. 2. Genetic map of pMG2200. The open arrows show the ORFs and the direction of transcription. Each color indicates significant homology with a reported plasmid or mobile element. Representative homologous genes are indicated on the ORFs (Table 2). The first G residue within an EcoRI site (GAATTC) on the map indicates the first nucleotide of pMG2200, as shown in Table 4.

vancomycin resistance and bacteriocin production on the strain, and the bacteriocin was active only against *E. faecalis* strains. Bacteriocin 41 (Bac41), which was encoded on pheromone-responsive plasmid pYI14 isolated from bacteriocinogenic strain *E. faecalis* YI714, was also active only against *E. faecalis* (61, 64). Strain OG1S carrying pMG2200 did not exhibit bacteriocin activity against *E. faecalis* FA2-2 carrying pYI14, and vice versa. These results indicated that the bacteriocin encoded on pMG2200 was identical to Bac41 with respect to its immunity characteristics. pMG2201 conferred erythromycin resistance and cytolysin activity (β-hemolysin/bacteriocin). Plasmid pMG2201 gave rise to the expected PCR product for the cytolysin genes (*cylL*$_L$, *cylL*$_S$, *cylA*, and *cylB*) of pAD1 by PCR analysis with primers specific for the genes, indicating that pMG2201 encoded the cytolysin (data not shown) (20, 36, 57).

Pheromone responses of pMG2200 and pMG2201. Donor cells of *E. faecalis* JH2SS carrying pMG2200 or pMG2201 were exposed for 2 h to either FA2-2 culture filtrate (i.e., pheromone) or synthetic pheromone cAD1, cPD1, cCF10, cOB1, or cAM373 (at a concentration of 100 ng/ml) for pheromone-responsive plasmids pAD1 (59.3 kb), pPD1 (58.9 kb), pCF10 (67.7 kb), pOB1 (64.7 kb), and pAM373 (36.7 kb), respectively, to induce the aggregation-mating function before a short (10-min) mating period (9). The short mating was carried out between the induced or noninduced donor cells and the plasmid-free recipient *E. faecalis* FA2-2. Plasmids pMG2200 and pMG2201 both responded to the FA2-2 filtrate and they responded to cCF10 and cAD1, respectively, indicating that pMG2200 and pMG2201 are identical to the pheromone-responsive plasmids pCF10 and pAD1, respectively, with respect to their pheromone responses (data not shown). Southern hybridization analysis with the 7-kbp probe specific for the conserved pheromone-responding genes of pPD1 (i.e., *traC*, *traB*, *traA*, *ipd*, *traE*, *traF*, *orfY*, *sea1*, and a part of *asp1*) indicated that both pMG2200 and pMG2201 contained the homologous genes shown in Fig. 1B (28, 58).

DNA sequence and genetic organization of plasmid pMG2200. The complete nucleotide sequence of pMG2200 was determined, and its molecular size was confirmed to be 106,527 bp (Table 4 and Fig. 2). The first G residue within an EcoRI site (GAATTC) was chosen as the first nucleotide of pMG2200, as shown in Fig. 2. All ORFs listed in Table 4 are numbered in relation to this nucleotide. The pMG2200 plasmid carries the conjugative transposon Tn*1549*-like element (33,812 bp) encoding VanB2-type vancomycin resistance, and it is located

between 8,580 bp and 42,391 bp in the clockwise orientation of the plasmid map (Fig. 2). The Tn*1549*-like element of pMG2200 contained 29 ORFs which were almost identical to the 29 ORFs of Tn*1549* (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 Tn*1549* (30), with the exception of ORF16 (99% amino acids identity). The Tn*1549*-like element contained the $vanR_B$, $vanS_B$, $vanY_B$, $vanW_B$, $vanH_B$, *vanB2*, and $vanX_B$ genes, which correspond to the seven equivalent genes in the VanB2 operon of Tn*1549* (30). The deduced amid acid sequence of VanB2 of the Tn*1549*-like element was almost identical to the deduced amid acid sequence of VanB2 of Tn*1549* 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 *pcfJ*, *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, Sea1, 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 $bacL_1$, $bacL_2$, *bacA*, and *bacI*, which are encoded on pheromone-responsive plasmid pYI14, which has been reported to be a novel bacteriocin 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/CTG ACCC (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^r), and the resultant plasmid was designated pMG2210 (Fig. 3). pMG2210 (Cm^r) containing the 322-bp region was mobilized by pMG2200 (Vm^r) 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 pAMα1 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 pAMα1 (9.8 kb) and pS86 (5.2 kb) are nonconjugative but mobilizable plasmids found in *E. faecalis* (17, 42). Plasmids pMG2200, pAMα1, 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 pHTβ 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_3 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^{2+} ions, and direct the activation of the active Tyr. These

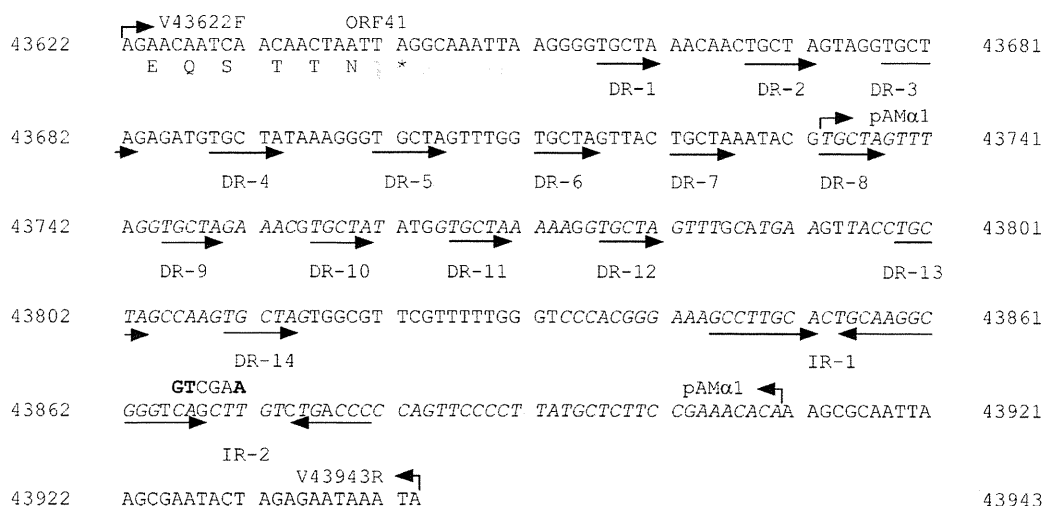

FIG. 3. Nucleotide sequence of the *oriT* region of plasmid pMG2200. The 332-bp noncoding DNA region between ORF41 and ORF42 is shown. The horizontal arrows under the sequences indicated the direct repeats TGCTA (DR-1 to DR-14) and inverted repeats (IR-1 and IR-2) in the *oriT* region. The names and 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 pAMα1 (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_3 sequence in motif III of the ORF44 of pMG2200 belonged to the MOB_{MG} family [$W(x_4)H(x_2)T(x_3)$ $HxH(x_4)E(x_4)R$, where uppercase letters represent conserved amino acids and "x" indicates the variable residues] found in TraI of pHTβ (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^r) and pAM714 (pAD1::Tn*917* [Em^r], 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 (2.6×10^{-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 (2.4×10^{-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 Tn*917-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 Tn*917-lac*

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                                  Motif Ia                   Motif Ib
                                    *                           *
E.faecalis      pMG2200 (22) -FKEYLDYMERSEATRNEH  (66) ------FSTYN-DYMSNPKK
E.faecium          pHTβ (32) ELQKFVDYISRQEAIRQDK  (82) IKDLREMDKYI-DYMTRKKA
L.innocua        pLI100 (18) YWSNYIKYIDRDEAVRNEH  (63) EKE-RN-KKYI-DYMGNPKK
S.agalactiae     NEM316 (18) ANPQYVDYTNREEAVKIDE  (66) QLNFRE---YI-DYMNRSYA
S.epidermidis ATCC12228 (15) KFKGYLKYINDEKSNKANH  (54) NLNLNSYSSYIIGYMKNNSI
B.anthracis     pXO2-84      -------------------  (64) FNTTTDFEKYV-SYMGRKYA
C.perfringens     pCP13 (17) KFKNFIDYIDRSEATRKKN       --------------------
Consensus                           YI   EA                     YI  YM

                                                Motif II
                                                   *
E.faecalis      pMG2200  (97) YMKEYFEMAQENKSPLWQLVFSFRNEWLIENNYLDPETNQLKTQ
E.faecium          pHTβ (128) KIKESVIEAKNNGSVMFQDVISFDNDFLVREGYYNPETNELNEN
L.innocua        pLI100  (97) KYKDAFQTAEKNNSVMFQHVISFDNEWLAKQGIYDPKIGMLDEK
S.agalactiae     NEM316 (109) KNKLE--SAYQNGSLLWQGVISFDNAFLAEQGLYDVATGQVDQK
S.epidermidis ATCC12228 (106) KLKDDFDTAEKQGCINYQDIISFDNDFLIKNHLYDAKTDELNED
B.anthracis     pXO2-84 (144) EIKELVGKAQNKGSVYYQDVISFDTDFLIEQKLYDPVTDILDEN
C.perfringens     pCP13  (70) KLKKDFDKAQLNGSNMWQEVFSFDNEFLEANGLYDSENGALDEE
Consensus                     K K     A   GS   Q VISFDN FL     YD     L E

                                                  Motif III
                                                     *      * *
E.faecalis      pMG2200 (149) AIA-ELEK-KEGLKG--EWTGAVHYNTDNIHVHVGYVEKNPTREWIFYKH
E.faecium          pHTβ (180) MMGKMQEK-EELVDP--FWFATIHRNTEHIHIHVTAMERKNTREIMEYDG
L.innocua        pLI100 (149) SMKSFLRK-EGMEGSA-IWLAAIHKNTKHFHVHISVTEPTPTRKFYSNKR
S.agalactiae     NEM316 (159) MMPTLIQK-EGLSDSA-FWWGNIHLNTDNIHIHFGLSEVESNREKIFYQP
S.epidermidis ATCC12228 (158) KMINRMIKDENMNPYQTRWMANIHYDTDNIHIHISTTELKNTRKIITNGN
B.anthracis     pXO2-84 (196) MMEQLF-KDEQIEENNGFWFASIHRNTEHIHIHFGTVEKENRRKLVEVKV
C.perfringens     pCP13 (122) AMEELS-KREGFKDL--TWSASLHYNTDNIHVHIASVEINPSRERGKFKP
Consensus                      M     K E        W A IH NT  IH H    E    R

MOB_MG family 3His-Motif                        WxxxxHxxTxxxHxHxxxxExxxxR
```

FIG. 4. Comparison of the N-terminal region of the deduced ORF44 protein of pMG2200 with putative relaxases found in sequence databases. The boldface letters indicated the amino acid residues conserved in each protein. The asterisks on the sequences show the key residues, Tyr, Ser, and His_3 (3His), in motifs I, II, and III, respectively. There are two motif III candidates (motifs Ia and Ib) in the most of the proteins. The GenBank accession numbers of the putative relaxases are as follows: for pXO2, NZ_ABJC01000063.1; for pCP13, NC_003042.1; for ATCC 12228, NC_004461.1; for NEM316, NC_004368.1; for pLI100, NC_003383.1; for pHTβ, NC_007594.1.

insertion mutants (i.e., pAM7314, pAM7330, and pAM2125) of the 3′ noncoding region in the E region (52). The 3′ terminal border of the E region is mapped by the Tn*917-lac* insertion of pAM2125 and is located 371 bp from the stop codon of *traE1* (unpublished data). Further analyses of pMG2200 might provide clues that will allow elucidation of the regulation of the pheromone-responsive plasmids.

Concluding comments. The two pheromone-responsive conjugative plasmids pMG2200 (106.5 kbp) and pMG2201 (65.7 kbp) were isolated from VanB2-type *E. faecalis* isolates. This report describes the first case of the isolation and characterization of pheromone-responsive conjugative plasmid pMG2200 encoding the *vanB* resistance determinant. pMG2200 encoded vancomycin resistance and bacteriocin and responded to pheromone cCF10, and pMG2201 encoded erythromycin resistance and cytolysin (Hly/Bac) and responded to pheromone cAD1. Our results show that an *E. faecalis* strain can acquire these characteristics and that these characteristics provide a selective advantage for the organism by allowing it to obtain the pheromone-responsive plasmids encoding drug resistance or bacteriocins by conjugation with plasmid-bearing bacteria in patients in the clinical setting. The plasmid also conferred the cytolysin (Hly/Bac) function for pathogenesis (38, 39). The complete nucleotide sequence of pMG2200 showed that pMG2200 consists of five major segments: (i) conjugative transposon Tn*1549*-like elements (33,812 bp) encoding the *vanB2*-type determinant, (ii) genes that regulate the pheromone response of pheromone-responsive plasmids, (iii) genes for UV resistance, (iv) the bacteriocin determinant, and (v) the origin of plasmid transfer. The genes corresponding to the pheromone-responsive regulatory genes, with the exception of the positive regulator *traE1* of plasmid pAD1, showed high levels of homology (100% amino acid identity) to those of pCF10. The data indicated that pMG2200 is a new type of pheromone-responsive plasmid which is a naturally occurring chimeric plasmid with regard to the negative regulatory gene *prgX* (*prgQ*) of pCF10 and the positive regulatory gene *traE1* of pAD1, resulting in a *prgX-prgQ-traE1* genetic organization. Using the chimeric plasmid, we showed that *traE1* is *cis* acting. The nucleotide sequence of the plasmid origin showed a high level of homology to a region within plasmid pAMα1 of *E. faecalis* that is unrelated to the *oriT* region of pAMα1, and the ORF corresponding to the putative relaxase showed homology to that of *E. faecium* conjugative plasmid pHTβ (61, 64). These results indicate that the diversity within the genetic organization of housekeeping genes, such as the regulatory regions, origin of transfer, and plasmid replication in the pheromone-

responsive plasmids, could result from genetic recombination between different pheromone-responsive plasmids or between a pheromone-responsive and a non-pheromone-responsive plasmid.

To our knowledge, there has been only one report on sequence analysis of the conjugative transposon Tn*1549* encoding the VanB gene cluster (30). The conjugative transposon Tn*1549*-like element encodes a *vanB2*-type resistance determinant that is almost completely identical to the Tn*1546* transposon residing on pIP834 of *E. faecalis* E93/268 (30). There has been no report to date of a pheromone-responsive highly conjugative plasmid carrying the Tn*1549*-like element encoding the VanB gene cluster. Our report is the first to describe a pheromone-responsive plasmid carrying the Tn*1549*-like element encoding the VanB2 gene cluster.

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ORIGINAL ARTICLE

Molecular characterization of *erm*(B)- and *mef*(E)-mediated erythromycin-resistant *Streptococcus pneumoniae* in China and complete DNA sequence of Tn*2010*

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Keywords
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Abstract

Aims: To characterize the *erm*(B)- and *mef*(E)-mediated erythromycin-resistant *Streptococcus pneumoniae* clinical isolates obtained from ten hospitals located different cities in China.

Methods and Results: Totally 83 *S. pneumoniae* were collected, and eighteen representative strains of 66 strains that exhibited erythromycin resistance were used for further characterization by antibiograms, serotyping, PFGE, MLST, DNA sequencing of the macrolide-resistance elements and mapping of the elements on the chromosome. Twelve isolates showed a high-level resistance to erythromycin, and six other isolates showed a low-level resistance to erythromycin. Thirteen isolates harboured a Tn*2010* transposon (26·4 kbp) encoding the *erm*(B), *tet*(M) and *mef*(E) genes and were classified into three types by Tn*2010* structures. The remaining five isolates harboured a Tn*6002* transposon (20·9 kbp) encoding the *erm*(B) and *tet*(M) genes and were classified into three types by Tn*6002* locations on the chromosome. Three of the Tn*6002* elements were located within the Tn*5252*-like element, implying that these composed a large mobile element. The MLST analyses showed that several clones had been disseminated and that the CC271 strains carrying the Tn*2010* element expressing the high-level resistance to erythromycin were predominant in China. Four new MLST strains, which were designated as ST3262, ST3263, ST3397 and ST3398 were also identified.

Conclusions: The erythromycin resistance determinant of *S. pneumoniae* that had been isolated in China was located in Tn*2010* or the Tn*6002* element and several clones had been disseminated, and the CC271 strains carrying the Tn*2010* element expressing the high-level resistance to erythromycin were predominant in China.

Significance and Impact of the Study: This is the first molecular analysis of erythromycin-resistant *Streptococcus pneumoniae* clinical isolates in China, and the first report of the complete nucleotide sequence of Tn*2010* (26 390 bp).

Introduction

Streptococcus pneumoniae is an important human pathogen associated with respiratory tract infections. Antibiotic treatment of these infections has become a growing problem because of the emergence of resistance to both penicillin and nonbeta-lactam antibiotics (Fuller *et al.* 2005; Daneman *et al.* 2006). During the last decade, a rapid increase in the resistance of *S. pneumoniae* to macrolides has been observed in China. Data from the China Bacterial Resistance Surveillance Study Group (BRSSG) and Ministry of Health National Antibacterial Resistance Investigation Net (MOHNARIN) showed that the rate of erythromycin resistance increased from 40% in 1999 to

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91·9% in 2008 (Li *et al.* 2001; Y. Li & Y. Lv, unpublished results).

Macrolide resistance in *S. pneumoniae* is mediated by two main mechanisms: target modification caused by a ribosomal methylase encoded mainly by the *erm*(B) gene is related to the MLS_B phenotype (resistance to macrolide-lincosamide-streptogram B) and an efflux pump system that is associated with the *mef* gene and related to the M phenotype and is resistant to 14- and 15-membered macrolides only. The Prospective Resistant Organism Tracking and Epidemiology for the Ketolide Telithromycin (PROTEKT) Surveillance Study has shown that the most common macrolide resistance mechanism is ribosomal methylation mediated by *erm*(B) (55·0% of erythromycin-resistant *S. pneumoniae*), except in Canada, Greece and the United States where the drug efflux mediated by *mef*(A) is predominant (Felmingham *et al.* 2007; Farrell *et al.* 2008). In addition, isolates carrying both resistance determinants are becoming increasingly common in several areas, such as some Asian countries, South Africa and the United States, since their isolation was first described in a South African study (Mcgee *et al.*, 2001; Farrell *et al.* 2005). These dual-gene isolates were mainly of serotype 19F or 19A, showed multi-drug resistance and were classed as clonal complex CC271. Recent studies have shown that both *erm*(B) and *mef* genetic elements have integrated into the Tn*916*-like transposon, which has been designated as Tn*2010* (Del Grosso *et al.* 2006, 2007).

Studies from China have shown that the MLS_B phenotype is dominant and 44·0–79·5% of erythromycin-resistant strains carry *erm*(B) as the sole resistance gene and that 17·8–44·1% had both *erm*(B) and *mef*(E) (Zhao *et al.* 2004; Yang *et al.* 2005; Wu *et al.* 2006). A detailed characterization of erythromycin-resistant *S. pneumoniae* has not been reported in China. The aim of this work was the genetic characterization of erythromycin-resistant *S. pneumoniae* and the genetic elements carrying *erm*(B) and/or *mef*(E).

Materials and methods

Bacterial strains and culture

Eighty-three *S. pneumoniae* clinical isolates were obtained from ten hospitals (i.e., hospital A, D, G, H, I, K, M, O, P and Q) in different cities throughout China. A, D, G, H, I, K, M, O, P and Q hospitals were located in Beijing, Shenyang, Nanjing, Shanghai, Hangzhou, Shenzhen, Changsha, Chengdu, Chongqing and Xian, respectively. These hospitals joined the Chinese Ministry of Health National Antibacterial Resistance Investigation Net (MOHNARIN) in the period 2004–2005 (Table 1). MOHNARIN is a National Surveillance Program aimed at providing geographically relevant data on the resistance trends of key pathogens and an alert mechanism for new emerging resistance threats. Representative isolates were selected from patients hospitalized in respiratory ward or intensive care unit (ICU). Each strain corresponded to one individual patient, and most of them were isolated from community-acquired infections. The number of strains obtained from A, D, G, H, I, K, M, O, P and Q hospitals was 4, 3, 4, 4, 4, 5, 5, 10, 31 and 13, respectively. Hospital P has isolated many strains from hospital-acquired infections; then, we obtained over-represented number of isolates from hospital P and included the isolates in this study. The 83 isolates were obtained from sputum (68 (82%)), nasopharynx (2 (2·4%)), blood (4 (5%)) and unrecorded sources. *S. pneumoniae* strains were grown in Brain Heart Infusion (BHI) broth (Difco, Detroit, MI) or on BHI agar plate at 37°C in the anaerobic jar of GasPak System (BBL, Fisher Scientific, Tokyo, Japan) using AnaeroPack-Anaero (Mitsubishi Gas Chemical Co., Tokyo, Japan), which is a disposable oxygen-absorbing and carbon dioxide-generating agent.

Antimicrobials and susceptibility test

The following antimicrobials were tested: penicillin, amoxicillin, ceftriaxone, cefotaxime, cefepime, imipenem, panipenem, erythromycin, clarithromycin, azithromycin, clindamycin, tetracycline, levofloxacin and vancomycin. All antimicrobials were purchased from the National Institute for the Control of Pharmaceutical and Biological Products (NICPBP) of China. Broth dilution MICs (minimum inhibitary concentrations) were determined according to the recommended method of the Clinical and Laboratory Standards Institute (CLSI). The results were interpreted according to the CLSI criteria, using penicillin breakpoints in place before they were changed in 2008 (susceptible, $\leq$0·06 mg l^{-1}; intermediate, 0·12 to 1 mg l^{-1}; and resistant, $\geq$2 mg l^{-1}) (CLSI, 2006). *S. pneumoniae* ATCC49619 was used as the control strain.

Genomic DNA isolation

The genomic DNAs were prepared from *S. pneumoniae* using an ISOPLANT kit according to the manufacturer's instructions (Nippon Gene Co., Toyama, Japan).

Serotyping

Serotyping for *S. pneumoniae* was performed by PCR with primers specific for the genes responsible for the biosynthesis of type 1, 3, 4, 6B, 14, 18C, 19A, 19F and 23F and serogroups 6, 19, and 23 of the capsular polysaccharide (Brito *et al.* 2003; Lawrence *et al.* 2003).

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Table 1 Chinese clinical isolated erythromycin-resistant *Streptococcus pneumoniae* and characteristics

Strain	Hospital	City	Age	Date of isolation	Source	Diagnosis/underlying disease*	HAI/CAI†	Antibiotic(s)used before isolation‡	Serotype	PFGE§	ST	Genetic element¶	P/G**
05D058	D	Shenyang	37	2005/01/06	Sputum	Pneumonia	CAI	None	19A	a3	320	Tn*2010*	A
05M016	M	Changsha	3	2004/10/29	Blood	Leukemia	CAI	None	19F	a1	271	Tn*2010*	A
05O173	O	Chengdu	<1	2005/02/27	Sputum	Bronchitis	CAI	None	19F	a2	271	Tn*2010*	A
05P107	P	Chongqing	73	2004/12/14	Sputum	Pneumonia/diabetes	CAI	None	19F	a1	271	Tn*2010*	A
05P117	P	Chongqing	29	2004/12/20	Sputum	Pneumonia/postoperative	HAI	PEN	19F	a1	271	Tn*2010*	A
05P128	P	Chongqing	24	2005/01/04	Sputum	Pneumonia/Guillain–Barre Syndrome	HAI	CLI + LVX	19F	a1	271	Tn*2010*	A
05P286	P	Chongqing	58	2005/05/16	Sputum	Pneumonia/postoperative	HAI	PEN + MEZ/SBT	19F	a1	271	Tn*2010*	A
05P288	P	Chongqing	56	2005/05/24	Sputum	Pneumonia	CAI	None	19F	a1	271	Tn*2010*	A
05P294	P	Chongqing	56	2005/05/29	Sputum	Bronchitis/cardiopathy	HAI	None	19F	a1	271	Tn*2010*	A
05P311	P	Chongqing	75	2005/06/27	Sputum	Pneumonia/COPC	HAI	None	19F	a1	271	Tn*2010*	A
05P343	P	Chongqing	36	2005/08/23	Sputum	Pneumonia/postoperative	HAI	PEN	19F	a1	271	Tn*2010*	A
05O035	O	Chengdu	4	2004/10/18	Sputum	Bronchitis	CAI	None	19F	a4	236	Tn*2010*	B
05P006	P	Chongqing	44	2004/09/18	Sputum	Pneumonia	CAI	None	NT††	e	3398	Tn*2010*	C
05O071	O	Chengdu	4	2004/12/14	Sputum	Bronchitis	CAI	A/C + PEN	NT††	d	3397	Tn*6002*	D
05K022	K	Shenzhen	22	2004/11/03	Sputum	Pneumonia	CAI	None	23F	b	342	Tn*6002*‡‡	E
05Q056	Q	Xian	50	2004/12/02	Sputum	Pneumonia/COPC	HAI	AZM + LVX	23F	b	342	Tn*6002*‡‡	E
05P141	P	Chongqing	65	2005/01/12	Sputum	Bronchitis/COPD	CAI	None	6B	f	3263	Tn*6002*‡‡	E
05O070	O	Chengdu	1	2004/12/13	Eyes secretion	Conjunctivitis	CAI	None	6B	c	3262	Tn*6002*	F

*COPD, chronic obstructive pulmonary disease.

†HAI, hospital-acquired infection; CAI, community-acquired infection.

‡Drug abbreviations: A/C, amoxicillin–clavulanic acid; PEN, penicillin; CLI, clindamycin; MEZ, mezlocillin; SBT, sulbactam; AZM, azithromycin; LVX, levofloxacin.

§PFGE: Types designated with a lower-case letter and related clones designated with the same letter followed by a number as described in text.

¶Tn*2010* element encoded *erm*(B), *mef*(E) and *tet*(M). Tn*6002* element encoded *erm*(B) and *tet*(M).

**P/G: phenotype/genotype, combination types according to different phenotype and genotype of mobile genetic element (Table 3, Fig. 2).

††NT: nontypeable.

‡‡Tn*6002* element was inserted into downstream of *orf28* in Tn*5252* (Ayoubi *et al.* 1991).

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PFGE

Streptococcus pneumoniae DNA embedded in an agarose block was prepared using a modification of the method of Lefevre *et al.* (1993). The guidelines proposed by Tenover *et al.* were basically used for the interpretation of PFGE results in this study. With these guidelines, strains that differed by between one and six bands were considered to be related clones, and banding pattern difference of three fragments could have occurred as a result of a

Table 2 Oligonucleotide primer pairs used

Gene	Primer		Product size (bp)	Reference
	Designation	Sequence (5′-3′)		
Resistance and transposons related genes				
erm(A)	ermA0	TCT AAA AAG CAT GTA AAG GAA	472	Sutcliffe *et al.* (1996)
	ermA2	CAG AAT CTA CAT TAG GCT TAG GG		Trallero *et al.* (2007)
erm(B)	ermB1	GAA AAG GTA CTC AAC CAA ATA	639	Sutcliffe *et al.* (1996)
	ermB2	AGT AAC GGT ACT TAA ATT GTT TAC		
mef	mef1	AGT ATC ATT AAT CAC TAG TGC	345	Sutcliffe *et al.* (1996)
	mef2	TTC TTC TGG TAC TAA AAG TGG		
tet(M)	tetM1	GAA CTC GAA CAA GAG GAA AGC	740	Brenciani *et al.* (2007)
	tetM2	ATG GAA GCC CAG AAA GGA T		
aphA3	aphA1	TAA AAG ATA CGG AAG GAA TGT CTC	824	this study
	aphA2	TCG ACC GGA CGC AGA AGG CAA TGT		Caillaud *et al.* (1987)
int	int-for	GCG TGA TTG TAT CTC ACT	1046	Brenciani *et al.* (2007)
	int-rev	GAC GCT CCT GTT GCT TCT		
xis	xis-for	AAG CAG ACT GAG ATT CCT A	139	Brenciani *et al.* (2007)
	xis-rev	GCG TCC AAT GTA TCT ATA A		
tndX	tndX-for	ATG ATG GGT TGG ACA AAG A	610	Brenciani *et al.* (2007)
	tndX-rev	CTT TGC TCG ATA GGC TCT A		
tnpR	tnpR-for	CCA AGG AGC TAA AGA GGT CCC	1528	Brenciani *et al.* (2007)
	tnpR-rev	GTC CCG AGT CCC ATG GAA GC		
tnpA	tnpA-for	GCT TCC ATG GGA CTC GGG AC	2115	Brenciani *et al.* (2007)
	tnpA-rev	GCT CCC AAT TAA TAG GAG A		
Transposons mapping				
orf24	TN6-rev	CCA TCA AAC ATT CAT TCA GC	3356	Brenciani *et al.* (2007)
orf20	J13	GGT TTT GTG GTT AGT TTT		
orf20	J12	CCC ATT GAA GAC GCA GAA GT	4841/7689*	Brenciani *et al.* (2007)
orf15	J15-rev	AAA GGA AGC CGA TAG GAT AAA		
orf15	J15	TTT ATC CTA TCG GCT TCC TTT	3956	Brenciani *et al.* (2007)
tet(M)	O7	CGG TAG TTT TT CTG CAT CAA C		
orf12	L27	CCT ATG GTT ATG CAT AAA AAT CCC	1424	this study
tet(M)	tetM2	ATG GAA GCC CAG AAA GGA T		Brenciani *et al.* (2007)
tet(M)	tetM1	GAA CTC GAA CAA GAG GAA AGC	3835/9344†	Brenciani *et al.* (2007)
xis	xis-rev	GCG TCC AAT GTA TCT ATA A		
tet(M)	tetM1	GAA CTC GAA CAA GAG GAA AGC	4002	Brenciani *et al.* (2007)
mel	OM21	GGC AAA ATC ACT GAG TAT TGG		Del Grosso *et al.* (2004)
mel	msrA2	TTA ATT TCC GCA CCG ACT A	3621	Amezaga *et al.* (2002)
orf9	SG3	GAA TCT TTA GCC AGC GGT ATC		Del Grosso *et al.* (2004)
mef(E)	OM18	TGC TTG CCC TGC CCA TAT TG	3550	Del Grosso *et al.* (2004)
xis-rev	xis-rev	GCG TCC AAT GTA TCT ATA A		Brenciani *et al.* (2007)
Inverse PCR				
orf24	TN1	ATA AAG TGT GAT AAG TCC AG		This study
orf24	L7	GTA GAA GCT AAA GAT GGT AAA CTT		This study
int	TN4	AGG CTT TAC GAG CAT TTA AG		This study
int	N1	GCA GAA ATC AGT AGA ATT GCC C		This study

*The former product size was expected according to the reported sequence of Tn*916* (accession no.EFU09422), with the later according to the reported sequence of Tn*6002* (accession no AY898750).

†The former product size was expected according to the reported sequence of Tn*916* (accession no.EFU09422), with the later according to the reported sequence of Tn*2009* (accession no AF376746).

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single genetic event. Types were designated with a lowercase letter, and related clones were designated with the same letter based on the visual comparison of patterns. The followed number showed the differences of one to three restriction fragments that were likely because of a single genetic event (Singh *et al.* 2006).

MLST analyses

Internal fragments of the *aroE*, *gdh*, *gki*, *recp*, *spi*, *xpt* and *ddl* genes, which are the seven housekeeping genes of *S. pneumoniae*, were amplified by PCR from chromosomal DNA using the primer pairs described in the MLST database (http://spneumoniae.mlst.net) (Enright and Spratt 1998). The allele numbers of the seven housekeeping loci and the resulting sequence types were also obtained from the MLST website.

Detection of resistance genes

Detection of the macrolide-resistant genes *erm*(A), *erm*(B) and *mef*, the tetracycline-resistant gene *tet*(M), the kanamycin-resistant gene *aphA3* and the *int* (integrase), *xis* (excisase) and *tndX* (resolvase) genes of the Tn*916* transposon family, and the *tnpA* (transposase) and *tnpR* (resolvase) genes related to the Tn*917* transposon was carried out by PCR using the primers described in Table 2 (Sutcliffe *et al.* 1996; Brenciani *et al.* 2007; Trallero *et al.* 2007). DNA sequence analysis for each of the determinants, and *orf20* and P0 was also performed as necessary (Warburton *et al.* 2007). The drug-resistant determinants within the Tn*6002* or Tn*2010* elements are located in the order *erm*(B) and *tet*(M), and *erm*(B), *tet*(M) and *mef*(E), respectively. To determine a linkage between the *erm*(B) and *tet*(M) pair, and the *tet*(M) and *mef* pair, respectively, six primer combinations were used to analyze the reciprocal orientation of the three genes, and the PCR products were analyzed.

Structure of the composite elements and detection of chromosomal insertion sites

Based on the results of PCR of transposon-related genes and the linkage between resistance genes, the structure of the resistance gene-carrying elements was examined by a series of PCRs with the primers designed by Brenciani *et al.* (2007) and Del Grosso *et al.* (2004) (Table 2). The sizes of the fragments obtained by PCR were compared with those reported previously (Del Grosso *et al.* 2006; Brenciani *et al.* 2007). One strain chosen as representative for phenotype/genotype A and the transposon was sequenced completely. Complete DNA sequence analysis of the Tn*2010*-like element was performed in the representative strain 05P294.

orf24 and *int* are located at each end of the transposable element of the Tn*916* derivatives (Flannagan *et al.* 1994). The DNA regions flanking both ends of the transposable elements were investigated using inverse PCR (iPCR) assays. Chromosomal DNA was digested with NdeI, and the fragments were ligated. PCR were performed with primer pairs for *orf24* end and the *int* end, respectively (Table 2). The PCR products were purified and sequenced and submitted to DNA sequencing analysis. If one of the terminal junctions of the element could not be obtained by iPCR, it was obtained by direct PCR amplification of the region spanning the end of the elements and the chromosomal DNA downstream/upstream of the insertion site based on the published genome database for the *S. pneumoniae* R6 strain (Hoskins *et al.* 2001). A serial of reverse primers were designed on the basis of the genomic sequence at an approximately 1·5-kb interval from the insertion point to approximately 15 kb downstream or upstream.

Nucleotide sequence accession number

The complete nucleotide sequence of transposon Tn*2010* of strain 05P294 was assigned GenBank accession no. AB426620. The partial DNA sequences of Tn*2010* from strains 05D058, 05O035 and 05P006 containing the *erm*(B) elements and its junctions into Tn*916* were assigned GenBank accession nos. AB426621, AB426622 and AB426623, respectively. The partial DNA sequences for Tn*2010* from the three strains containing the mega elements and its junctions into Tn*916* were assigned GenBank accession nos. AB426624, AB426625 and AB426626, respectively.

Results and discussion

Drug susceptibilities of the isolates

Of the 83 isolates, 66 (80%) exhibited resistance to the macrolides examined (i.e., erythromycin, clarithromycin, azithromycin and clindamycin). Erythromycin resistance level (MIC mg l^{-1}) ranged between 1 and >256 mg l^{-1}, and 36 (43%) strains exhibited a high level of erythromycin resistance of >256 mg l^{-1}, and six (7%) strains exhibited low level of erythromycin resistance between 1 and 8 mg l^{-1}. Susceptibility (MIC) to erythromycin by the 17 erythromycin-sensitive strains was ≤0·25 mg l^{-1}. MICs of penicillin for the 83 isolates were distributed between 0·016 and 4 mg l^{-1}, and 33 (40%) of 83 isolates exhibited penicillin resistance (i.e., MIC > 0·016 mg l^{-1}) according to the previous criteria of CLSI when this surveillance study was performed. The criteria was changed in 2008, and the susceptibility breakpoint for nonmeningitis

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isolates is >8 mg L^{-1} now. These data indicated a high incidence of erythromycin-resistant strains, which is consistent with the results of other reports (Zhao *et al.* 2004; Yang *et al.* 2005). Of the 83 clinical isolates, a total of eighteen erythromycin-resistant strains from six hospitals were selected as representative strains for further analysis of their erythromycin-resistant determinants (Table 1). We focused on these eighteen isolates including six isolates showing low-level resistance to erythromycin (MICs were between 1 and 8 mg l^{-1}) and twelve showing high-level resistance to erythromycin (>256 mg l^{-1}) and intermediate resistance (or resistance) to penicillin according to the previous criteria (MICs were between 1 and 2 mg l^{-1}). The reason was that macrolide and penicillin resistances are significant in the clinical setting and that the low-level erythromycin-resistant *S. pneumoniae* isolate was not common in China and little was known about the strains.

Phenotypic and genotypic characterization of isolates

Of the 18 strains, twelve were highly resistant to erythromycin and clindamycin (MIC ≥ 128 mg l^{-1}) and showed intermediate resistance or full resistance to penicillin (MIC ≥ 1 mg l^{-1}) and tetracycline (MIC 4–16 mg l^{-1}). Among the other six low-level erythromycin-resistant strains (MIC 1–8 mg l^{-1}), five were susceptible to penicillin (MIC ≤ 0·25 mg l^{-1}), and one susceptible to both penicillin and clindamycin (Table 3). All tested strains were susceptible to vancomycin, and two strains were resistant to levofloxacin.

erm(B) and *tet*(M) were identified in all 18 isolates, but *erm*(A) and *aphA3* were not identified in any isolate (Table 3). *erm*(A) confer the low-level resistance to macrolides in *S. pneumoniae* and is found in other countries (Sutcliffe *et al.* 1996; Trallero *et al.* 2007). *aphA3*-encoding aminoglycoside resistance is encoded on the pneumococcal conjugative transposon Tn*1545* that belongs to the Tn*916* family and spread in pneumococci (Caillaud *et al.* 1987). Of the 18 isolates, thirteen strains carried *mef*(E) in addition to *erm*(B) and *tet*(M). In these 13 strains, eleven strains showed high resistance to erythromycin, and belonged to serotype 19, and the other two strains had low resistance to erythromycin (Table 1). One of the two strains belonged to serotype 19, and the other strain was nontypeable by PCR. Of the five strains that had *erm*(B) and *tet*(M), but did not carry *mef*(E), one strain showed high resistance to erythromycin, and four strains showed low resistance to erythromycin, two of these four strains belonged to serotype 23F and the other two belonged to serotype 6B.

PFGE analysis showed that of the thirteen strains carrying both *erm*(B) and *mef*(E), twelve showed similar profiles: with nine isolates having an identical profile (typed as a1), which were isolated from hospitals P and M, and the other three isolates differed by one or two bands, indicating that the strains were related (i.e., type a2, a3 and a4) (Table 1, data not shown) (Tenover *et al.* 1995). These results indicated that the 'type a' strains were clonally spread in China. Strain 05P006 also carried both *erm*(B) and *mef*(E), but showed a different pattern. Five strains shared four different patterns (type b, c, d and f) (Table 1). Two 'type b' strains (serotype 23F), 05K022 and 05Q056, were isolated from hospitals K and Q, respectively.

MLST analysis was performed. Of the 13 strains carrying both erythromycin-resistant genes, 10 belonged to ST271, 1 belonged to ST320 and 1 belonged to ST236. These are included in the clonal complex CC271, which is common in erythromycin-resistant *S. pneumoniae* (Ko and Song 2004; Del Grosso *et al.* 2007). In strain 05P006, *xpt* and *ddl* belonged to new alleles, and the concatenation was designated as ST3398 (Table 1). Among the five strains carrying the single erythromycin-resistant gene (*ermB*), two (i.e., 05K022 and 05Q056) belonged to ST342, which is included in CC271. In strain 05O071, *gdh* and *ddl* of the seven housekeeping genes belonged to new alleles and the strain was designated as ST3397. In strain 05O070 and 05P141, new concatenations were found and were designated as ST3262 and ST3263, respectively.

The twelve strains that were isolated from four hospitals (D, M, O and P), belonged to serotype 19F except one strain of 19A, clonal complex 271, and were clonally related by PFGE analysis (Farrell *et al.* 2005, 2008). These strains were identical to one of the internationally spread multidrug-resistant pneumococcal clone Taiwan^{19F}-14 (Del Grosso *et al.* 2007). Taiwan^{19F}-14 belongs to clonal complex CC271 and is resistant to penicillin, erythromycin and tetracycline, carries Tn*2010* element encoded *erm(B)*, *mef(E)* and *tet(M)*.

Composite elements and insertion sites

The results of sequence analysis of the region carrying the drug-resistant genes showed that the erythromycin-resistant genes were carried on the Tn*2010* or Tn*6002* transposons (Table 1). Tn*6002* (20·9 kbp) is a result of the insertion of the *erm*(B)-containing DNA fragment (2·8 kb) into Tn*916* (18 kb), and Tn*2010* (26·4 kb) is the result of the insertion of mega (macrolide efflux genetic element) (5·5 kbp), which includes the *mef*(E)-*msr*(D) operon encoding macrolide resistance, into *orf6* of Tn*916* in Tn*6002* (Fig. 1). The *erm*(B) element, which is composed of P0, P1, *erm*(B), P3 and P4, lies just before nucleotide T of the stop codon TAA of ORF20 of Tn*916*, which is located upstream of P0 in both Tn*2010* and

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Table 3 Drug susceptibilities and the drug resistance gene or transposon-related gene of *Streptococcus pneumoniae* strains

Drug susceptibilities (MIC mg l^{-1})													Drug resistance gene or transposon-related gene									
Strain	ERY	CLI	CLR	AZM	TET	LVX	VAN	PEN	AMX	A/C	CXM	CTR	*erm*(A)	*erm*(B)	*mef*(E)	*tet*(M)	*aphA3*	*int*	*xis*	*tndX*	*tnpA*	*tnpR*
05D058	>256	>256	256	>256	4	1	1	2	4	2	4	0·5	−	+	+	+	−	+	+	−	−	−
05M016	>256	>256	>256	>256	8	1	1	2	4	4	8	2	−	+	+	+	−	+	+	−	−	−
05O173	>256	>256	>256	>256	8	1	1	1	1	1	2	0·5	−	+	+	+	−	+	+	−	−	−
05P107	>256	>256	>256	>256	8	1	1	2	8	4	8	2	−	+	+	+	−	+	+	−	−	−
05P117	>256	>256	>256	>256	8	16	1	2	8	4	8	2	−	+	+	+	−	+	+	−	−	−
05P128	>256	>256	>256	>256	8	8	1	1	4	2	8	2	−	+	+	+	−	+	+	−	−	−
05P286	>256	>256	>256	>256	8	1	1	1	4	4	8	1	−	+	+	+	−	+	+	−	−	−
05P288	>256	>256	>256	>256	8	1	1	1	4	4	8	2	−	+	+	+	−	+	+	−	−	−
05P294	>256	256	256	>256	16	1	1	2	4	2	8	1	−	+	+	+	−	+	+	−	−	−
05P311	256	256	128	>256	8	1	1	2	2	2	8	1	−	+	+	+	−	+	+	−	−	−
05P343	>256	>256	256	>256	8	1	0·5	1	4	2	8	2	−	+	+	+	−	+	+	−	−	−
05O035	1	8	2	16	2	1	0·5	0·25	0·5	0·25	0·5	0·125	−	+	+	+	−	+	+	−	−	−
05P006	1	0·125	2	64	8	1	1	0·016	0·016	0·016	0·016	0·016	−	+	+	+	−	+	+	−	−	−
05O071	>256	>256	>256	>256	8	1	1	1	2	1	2	0·5	−	+	−	+	−	+	+	−	−	−
05K022	8	32	16	128	8	1	1	0·062	0·016	0·016	0·125	0·031	−	+	−	+	−	+	+	−	−	−
05Q056	8	256	16	128	16	1	1	0·062	0·031	0·031	0·062	0·031	−	+	−	+	−	+	+	−	−	−
05P141	8	8	8	64	2	1	1	0·031	0·016	0·062	0·062	0·016	−	+	−	+	−	+	+	−	−	−
05O070	8	256	8	32	2	1	1	0·016	0·016	0·016	0·016	0·008	−	+	−	+	−	+	+	−	−	−

ERY, erythromycin; CLI, clindamycin; CLR, clarithromycin; AZM, azithromycin; TET, tetracycline; LVX, levofloxacin; VAN, vancomycin; PEN, penicillin; AMX, amoxicillin; A/C, amoxicillin–clavulanic acid; CXM, cefuroxime; CTR, ceftriaxone.

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