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* pY114, pCF10	Phage-related single-strand binding protein
55	CW	53761	53913	153/50	ORF2	88	92	*Enterococcus faecalis* pY114	
56	CW	53925	54515	591/196	ORF3, PcfT	84, 81	91, 88	*Enterococcus faecalis* pY114, pCF10	Thermonuclease precursor
57	CW	54521	54841	330/109	ORF4, PcfU	97, 95	100, 97	*Enterococcus faecalis* pY114, pCF10	
58	CW	54949	55194	246/81					
59	CW	55248	55430	183/60	ORF5	100	100	*Enterococcus faecalis* pY114	
60	CW	55625	56341	717/238	ORF6	100	100	*Enterococcus faecalis* pY114	
61	CW	56479	58266	1,788/595	$BacL_1$	99	99	*Enterococcus faecalis* pY114	Prebacteriocin for Bac41
62	CW	58452	59087	636/211	$BacL_2$	100	100	*Enterococcus faecalis* pY114	Prebacteriocin secretion
63	CW	59110	59541	432/133	ORF9	100	100	*Enterococcus faecalis* pY114	
64	CW	59544	60071	528/175	ORF10	97	99	*Enterococcus faecalis* pY114	
65	CW	60114	62294	2,181/726	BacA	100	100	*Enterococcus faecalis* pY114	Activator for Bac41
66	CW	62402	62944	543/180	BacI	99	100	*Enterococcus faecalis* pY114	Provides immunity against Bac41
67	CW	63011	63586	576/191	ORF13	98		*Enterococcus faecalis* pY114	
68	CW	63729	64061	333/110	ORF14	98	100	*Enterococcus faecalis* pY114	
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

Continued on following page

TABLE 4—*Continued*

ORF	Direction*	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, Tn3 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	Sea1	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

* 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

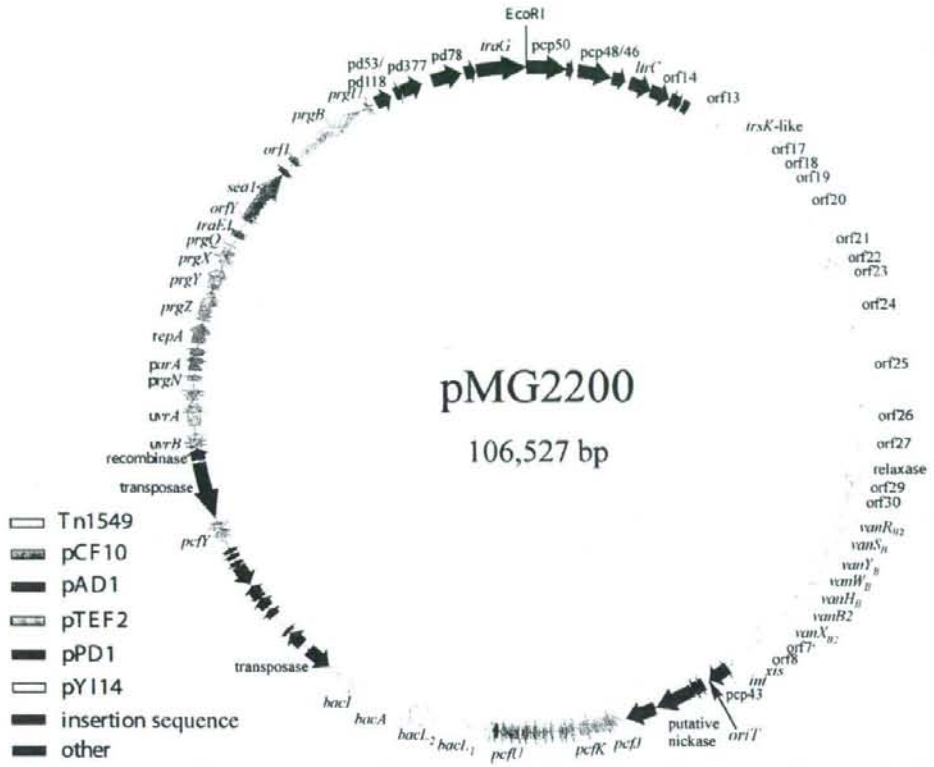

FIG. 2. Genetic map of pMG2200. The open arrows show the ORFs and the direction of transcription. Each color indicates significant homology with a reported plasmid or mobile element. Representative homologous genes are indicated on the ORFs (Table 2). The first G residue within an EcoRI site (GAATTC)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

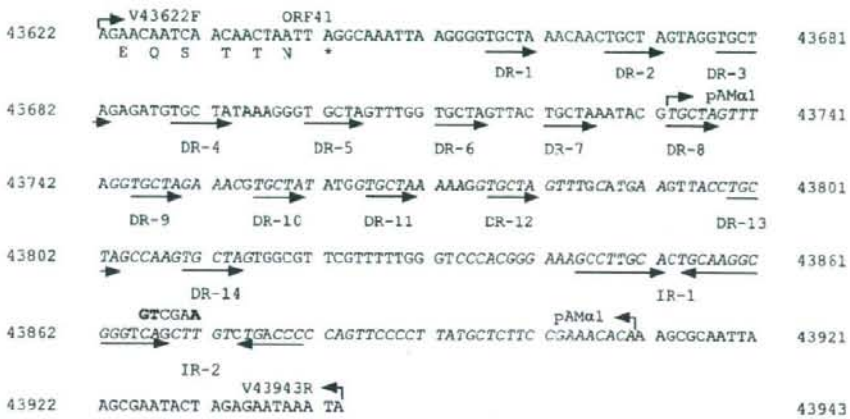

FIG. 3. Nucleotide sequence of the *oriT* region of plasmid pMG2200. The 332-bp noncoding DNA region between ORF41 and ORF42 is shown. The horizontal arrows under the sequences indicated the direct repeats TGCTA (DR-1 to DR-14) and inverted repeats (IR-1 and IR-2) in the *oriT* region. The names and 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*

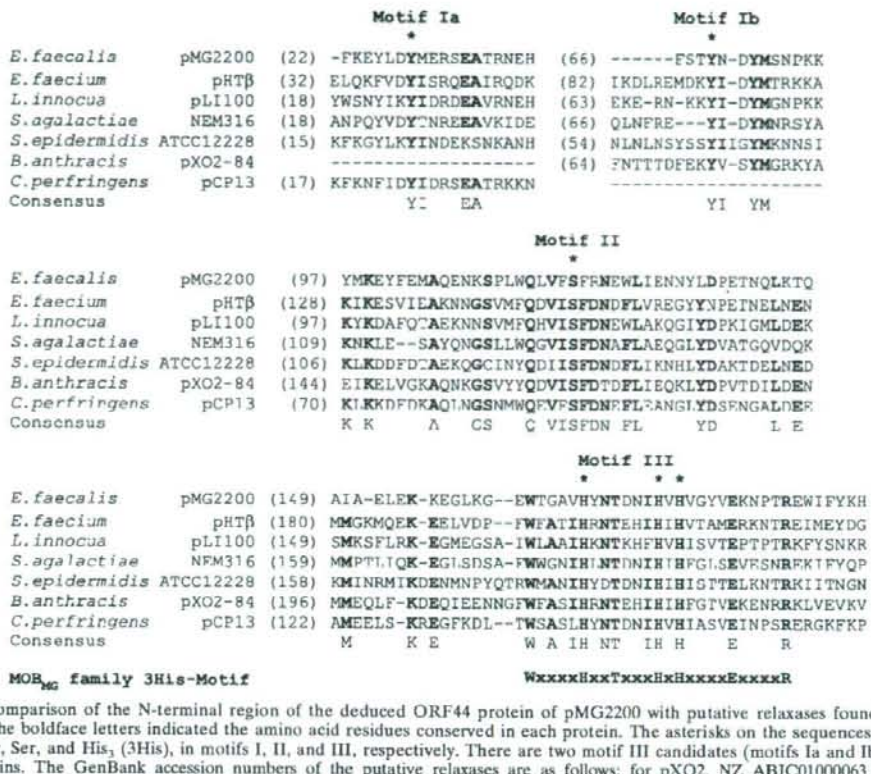

FIG. 4. Comparison of the N-terminal region of the deduced ORF44 protein of pMG2200 with putative relaxases found in sequence databases. The boldface letters indicated the amino acid residues conserved in each protein. The asterisks on the sequences show the key residues, Tyr, Ser, and His_3 (3His), 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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REFERENCES

1. **Altschul, S. F., W. Gish, W. Miller, E. W. Myers, and D. J. Lipman.** 1990. Basic local alignment search tool. J. Mol. Biol. **215**:403–410.
2. **Arthur, M., C. Molinas, F. Depardieu, and P. Courvalin.** 1993. Characterization of Tn*1546*, a Tn*3*-related transposon conferring glycopeptide resistance by synthesis of depsipeptide peptidoglycan precursors in *Enterococcus faecium* BM4147. J. Bacteriol. **175**:117–127.
3. **Carias, L. L., S. D. Rudin, C. J. Donskey, and L. B. Rice.** 1998. Genetic linkage and cotransfer of a novel, *vanB*-containing transposon (Tn*5382*) and a low-affinity penicillin-binding protein 5 gene in a clinical vancomycin-resistant *Enterococcus faecium* isolate. J. Bacteriol. **180**:4426–4434.
4. **Cetinkaya, Y., P. Falk, and C. G. Mayhall.** 2000. Vancomycin-resistant enterococci. Clin. Microbiol. Rev. **13**:686–707.
5. **Christie, P. J., and G. M. Dunny.** 1986. Identification of regions of the *Streptococcus faecalis* plasmid pCF-10 that encode antibiotic resistance and pheromone response functions. Plasmid **15**:230–241.
6. **Chung, J. W., and G. M. Dunny.** 1992. *Cis*-acting, orientation-dependent, positive control system activates pheromone-inducible conjugation functions at distances greater than 10 kilobases upstream from its target in *Enterococcus faecalis*. Proc. Natl. Acad. Sci. USA **89**:9020–9024.
7. **Clewell, D. B.** 1981. Plasmids, drug resistance, and gene transfer in the genus *Streptococcus*. Microbiol. Rev. **45**:409–436.
8. **Clewell, D. B.** 2007. Properties of *Enterococcus faecalis* plasmid pAD1, a member of a widely disseminated family of pheromone-responding, conjugative, virulence elements encoding cytolysin. Plasmid **58**:205–227.
9. **Clewell, D. B., and G. M. Dunny.** 2002. Conjugation and genetic exchange in enterococci, p. 265–300. *In* M. S. Gilmore et al. (ed.), The enterococci: pathogenesis, molecular biology, and antibiotic resistance. American Society for Microbiology, Washington, DC.
10. **Clewell, D. B., S. E. Flannagan, and D. D. Jaworski.** 1995. Unconstrained bacterial promiscuity: the Tn*916*-Tn*1545* family of conjugative transposon. Trends Microbiol. **3**:229–236.
11. **Clewell, D. B., P. K. Tomich, M. C. Gawron-Burke, A. E. Franke, Y. Yagi, and F. Y. An.** 1982. Mapping of *Streptococcus faecalis* plasmids pAD1 and pAD2 and studies relating to transposition of Tn*917*. J. Bacteriol. **152**:1220–1230.
12. **Courvalin, P.** 2006. Vancomycin-resistance in gram-positive cocci. Clin. Infect. Dis. **42**:S25–S34.
13. **Dahl, K. H., G. S. Simonsen, O. Olsvik, and A. Sundsfjord.** 1999. Heterogeneity in the *vanB* gene cluster of genomically diverse clinical strains of vancomycin-resistant enterococci. Antimicrob. Agents Chemother. **43**:1105–1110.
14. **De Boever, E. H., D. B. Clewell, and C. M. Fracer.** 2000. *Enterococcus faecalis* conjugative plasmid pAM373: complete nucleotide sequence and genetic analyses of sex pheromone response. Mol. Microbiol. **37**:1327–1341.
15. **Diekema, D. J., B. J. Boots Miller, T. E. Vaughn, R. F. Woolson, J. W. Vankey, E. J. Ernst, S. D. Flach, M. M. Ward, C. L. J. Franciscus, M. A. Pfaller, and B. N. Doebberling.** 2004. Antimicrobial resistance trends and outbreak frequency in United States hospitals. Clin. Infect. Dis. **38**:78–85.
16. **Dunny, G. M., B. L. Brown, and D. B. Clewell.** 1978. Induced cell aggregation and mating in *Streptococcus faecalis*: evidence for a bacterial sex pheromone. Proc. Natl. Acad. Sci. USA **75**:3479–3483.
17. **Dunny, G. M., and D. B. Clewell.** 1975. Transmissible toxin (hemolysin) plasmid in *Streptococcus faecalis* and its mobilization of noninfectious drug resistance plasmid. J. Bacteriol. **124**:784–790.
18. **Dunny, G. M., R. A. Craig, R. Carron, and D. B. Clewell.** 1979. Plasmid transfer in *Streptococcus faecalis*. Production of multiple sex pheromones by recipients. Plasmid **2**:454–465.
19. **Dunny, G. M., and B. A. Leonard.** 1997. Cell-cell communication in gram-positive bacteria. Annu. Rev. Microbiol. **51**:527–564.
20. **Eaton, T. J., and M. J. Gasson.** 2001. Molecular screening of *Enterococcus* virulence determinants and potential for genetic exchange between food and medical isolates. Appl. Environ. Microbiol. **67**:1628–1635.
21. **Ehrenfeld, E. E., and D. B. Clewell.** 1987. Transfer functions of the *Streptococcus faecalis* plasmid pAD1: organization of plasmid DNA encoding response to sex pheromone. J. Bacteriol. **169**:3473–3481.
22. **Evers, S., and P. Courvalin.** 1996. Regulation of VanB-type vancomycin resistance gene expression by $VanS_B$-$VanS_R$ two-component regulatory system in *Enterococcus faecalis* V583. J. Bacterol. **178**:1302–1309.
23. **Francia, M. V., and D. B. Clewell.** 2002. Amplification of the tetracycline resistance determinant of pAMα1 in *Enterococcus faecalis* requires a site-specific recombination event involving relaxase. J. Bacteriol. **184**:5187–5193.
24. **Francia, M. V., W. Haas, R. Wirth, E. Samberger, A. Muscholl-Silberhorn, M. S. Gilmore, Y. Ike, K. E. Weaver, F. Y. An, and D. B. Clewell.** 2001. Completion of the nucleotide sequence of the *Enterococcus faecalis* conjugative virulence plasmid pAD1 and identification of a second transfer origin. Plasmid **46**:117–127.
25. **Francia, M. V., A. Varsaki, M. P. Garcillan-Barcia, A. Latorre, C. Drainas, and F. de la Cruz.** 2004. A classification scheme for mobilization regions of bacterial plasmids. FEMS Microbiol. Rev. **28**:79–100.
26. **Franke, A. E., and D. B. Clewell.** 1981. Evidence for a chromosome-borne resistance transposon (Tn*916*) in *Streptococcus faecalis* that is capable of "conjugal" transfer in the absence of a conjugative plasmid. J. Bacteriol. **145**:494–502.
27. **Fujimoto, S., H. Hashimoto, and Y. Ike.** 1991. Low cost device for electrotransformation and its application to the highly efficient transformation of *Escherichia coli* and *Enterococcus faecalis*. Plasmid **26**:131–135.
28. **Fujimoto, S., H. Tomita, E. Wakamatsu, K. Tanimoto, and Y. Ike.** 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.
29. **Fujita, N., M. Yoshimura, T. Komori, K. Tanimoto, and Y. Ike.** 1998. First report of the isolation of high-level vancomycin-resistant *Enterococcus faecium* from a patient in Japan. Antimicrob. Agents Chemother. **42**:2150.
30. **Garnier, F., S. Taourit, P. Glaser, P. Courvalin, and M. Galimand.** 2000. Characterization of transposon Tn*1549*, conferring VanB-type resistance in *Enterococcus* spp. Microbiology **146**:1481–1489.
31. **Gilmore, M. S., R. A. Segarra, M. C. Booth, C. P. Bogie, L. R. Hall, and D. B. Clewell.** 1994. Genetic structure of the *Enterococcus faecalis* plasmid pAD1-encoded cytolytic toxin system and its relationship to lantibiotic determinants. J. Bacteriol. **176**:7335–7344.
32. **Haas, W., B. D. Shepard, and M. S. Gilmore.** 2002. Two-component regulator of *Enterococcus faecalis* cytolysin responds to quorum-sensing autoinduction. Nature **415**:84–87.
33. **Hirt, H., D. A. Manias, E. M. Bryan, J. R. Klein, J. K. Marklund, J. H. Staddon, M. L. Paustian, W. Kapur, and G. M. Dunny.** 2005. Characterization of the pheromone response of the *Enterococcus faecalis* conjugative plasmid pCF10: complete sequence and comparative analysis of the transcriptional and phenotypic responses of pCF10-containing cells to pheromone induction. J. Bacteriol. **187**:1044–1054.
34. **Ike, Y., and D. B. Clewell.** 1984. Genetic analysis of the pAD1 pheromone response in *Streptococcus faecalis*, using transposon Tn*917* as an insertional mutagen. J. Bacteriol. **158**:777–783.
35. **Ike, Y., and D. B. Clewell.** 1992. Evidence that the hemolysin/bacteriocin phenotype of *Enterococcus faecalis* subsp. *zymogenes* can be determined by plasmids in different incompatibility groups as well as by the chromosome. J. Bacteriol. **174**:8172–8177.
36. **Ike, Y., D. B. Clewell, R. A. Segarra, and M. S. Gilmore.** 1990. Genetic analysis of the pAD1 hemolysin/bacteriocin determinant in *Enterococcus faecalis*: Tn*917* insertional mutagenesis and cloning. J. Bacteriol. **172**:155–163.
37. **Ike, Y., R. C. Craig, B. A. White, Y. Yagi, and D. B. Clewell.** 1983. Modification of *Streptococcus faecalis* sex pheromones after acquisition of plasmid DNA. Proc. Natl. Acad. Sci. USA **8**:5369–5373.
38. **Ike, Y., H. Hashimoto, and D. B. Clewell.** 1984. Hemolysin of *Streptococcus faecalis* subspecies *zymogenes* contributes to virulence in mice. Infect. Immun. **45**:528–530.
39. **Ike, Y., H. Hashimoto, and D. B. Clewell.** 1987. High incidence of hemolysin production by *Enterococcus* (*Streptococcus*) *faecalis* strains associated with human parenteral infection. J. Clin. Microbiol. **25**:1524–1528.
40. **Lauderdale, T. L., L. C. McDonald, Y. R. Shiau, P. C. Chen, H. Y. Wang, J. F. Lai, and M. Ho.** 2002. Vancomycin-resistant enterococci from humans and retail chickens in Taiwan with unique VanB phenotype-*vanA* genotype incongruence. Antimicrob. Agents Chemother. **46**:525–527.

41. **Leclercq, R., E. Derlot, J. Duval, and P. Courvalin.** 1988. Plasmid-mediated resistance to vancomycin and teicoplanin in *Enterococcus faecium*. N. Engl. J. Med. **319:**157–161.
42. **Martinez-Bueno, M., E. Valdivia, A. Galvez, and M. Maqueda.** 2000. pS86, a new theta-replicating plasmid from *Enterococcus faecalis*. Curr. Microbiol. **41:**257–261.
43. **Martone, W. J.** 1998. Spread of vancomycin resistant enterococci: why did it happen in the United States? Infect. Control Hosp. Epidemiol. **19:**539–545.
44. **Morrison, D., N. Woodford, and B. Cookson.** 1997. Enterococci as emerging pathogens of humans. J. Appl. Microbiol. **83**(Suppl.):89S–99S.
45. **Muscholl, A., D. Galli, G. Wanner, and R. Wirth.** 1993. Sex pheromone plasmid pAD1-encoded aggregation substance of *Enterococcus faecalis* is positively regulated in *trans* by *traE1*. Eur. J. Biochem. **214:**333–338.
46. **Muscholl-Shilberhorn, A. B.** 2000. Pheromone-regulated expression of sex pheromone plasmid pAD1-encoded aggregation substance depends on at least six upstream genes and a *cis*-acting, orientation-dependent factor. J. Bacteriol. **182:**3816–3825.
47. **Oana, K., Y. Kawakami, M. Ohnishi, M. Ishikawa, M. Hirota, M. Tozuka, K. Atarashi, K. Baba, K. Fujiki, M. Okazaki, T. Honda, and T. Hayashi.** 2001. Molecular and epidemiological study of the first outbreak of *vanB* type vancomycin-resistant *Enterococcus faecalis* in Japan. Jpn. J. Infect. Dis. **54:** 17–22.
48. **Ozawa, Y., K. Tanimoto, S. Fujimoto, H. Tomita, and Y. Ike.** 1997. Cloning and genetic analysis of the UV resistance determinant (*uvr*) encoded on the *Enterococcus faecalis* pheromone-responsive conjugative plasmid pAD1. J. Bacteriol. **179:**7468–7475.
49. **Ozawa, Y., K. Tanimoto, T. Nomura, M. Yoshinaga, Y. Arakawam, and Y. Ike.** 2002. Vancomycin-resistant enterococci in humans and imported chickens in Japan. Appl. Environ. Microbiol. **68:**6457–6461.
50. **Patel, R., J. R. Uhl, P. Kohner, M. K. Hopkins, J. M. Steckelberg, B. Kline, and F. R. Cockerill III.** 1998. DNA sequence variation within *vanA*, vanB, *vanC-1*, and *vanC-2/3* genes of clinical *Enterococcus* isolates. Antimicrob. Agents Chemother. **42:**202–205.
51. **Paulsen, I. T., L. Banerjei, G. S. A. Myers, K. E. Nelson, R. Sechadri, T. D. Read, D. E. Fouts, J. A. Eisen, S. R. Gill, J. F. Heidelberg, H. Tettelin, R. J. Dobson, L. Umayam, L. Brinkac, M. Beanan, S. Daugherty, R. T. DeBoy, S. Durkin, J. Kolonay, R. Madupu, W. Nelson, J. Vamathevan, B. Tran, J. Upton, T. Hansen, J. Shetty, H. Khouri, T. Utterback, D. Radune, K. A. Kethum, B. A. Dougherty, and C. M. Fraser.** 2003. Role of mobile DNA in the evolution of vancomycin-resistant *Enterococcus faecalis*. Science **299:** 2071–2074.
52. **Pontius, L. T., and D. B. Clewell.** 1992. Conjugative transfer of *Enterococcus faecalis* plasmid pAD1: nucleotide sequence and transcriptional fusion analysis of a region involved in positive regulation. J. Bacteriol. **174:**3152–3160.
53. **Quintiliani, R., and P. Courvalin.** 1994. Conjugal transfer of the vancomycin resistance determinant *vanB* between enterococci involves the movement of large genetic elements from chromosome to chromosome. FEMS Microbiol. Lett. **119:**359–364.
54. **Rice, L. B., L. L. Carias, C. L. Donskey, and S. D. Rudin.** 1998. Transferable, plasmid-dedicated VanB-type glycopeptide resistance in *Enterococcus faecium*. Antimicrob. Agents Chemother. **42:**963–964.
55. **Sahm, D. F., J. Kissinger, M. S. Gilmore, P. R. Murray, R. Mulder, J. Solliday, and B. Clarke.** 1989. In vitro susceptibility studies of vancomycin-resistant *Enterococcus faecalis*. Antimicrob. Agents Chemother. **33:**1588–1591.
56. **Sambrook, J., and D. W. Russell.** 2001. Molecular cloning: a laboratory manual, 3rd ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
57. **Semedo, T., M. A. Santos, P. Martins, M. F. S. Lopes, J. J. F. Marques, R. Tenreiro, and M. T. B. Crespo.** 2003. Comparative study using type strains and clinical and food isolates to examine hemolytic activity and occurrence of the *cyl* operon in enterococci. J. Clin. Microbiol. **41:**2569–2576.
58. **Shiojima, M., H. Tomita, K. Tanimoto, S. Fujimoto, and Y. Ike.** 1997. High-level plasmid-mediated gentamicin resistance and pheromone response of plasmids present in clinical isolates of *Enterococcus faecalis*. Antimicrob. Agents Chemother. **41:**702–705.
59. **Tomich, P. K., F. Y. An, and D. B. Clewell.** 1980. Properties of erythromycin-inducible transposon Tn*917* in *Streptococcus faecalis*. J. Bacteriol. **141:**1366–1374.
60. **Tomita, H., and D. B. Clewell.** 2000. A pAD1-encoded small RNA molecule, mD, negatively regulates *Enterococcus faecalis* pheromone response by enhancing transcription termination. J. Bacteriol. **182:**1062–1073.
61. **Tomita, H., S. Fujimoto, K. Tanimoto, and Y. Ike.** 1996. Cloning and genetic organization of the bacteriocin 31 determinant encoded on the *Enterococcus faecalis* pheromone-responsive conjugative plasmid pYI17. J. Bacteriol. **178:** 3585–3593.
62. **Tomita, H., S. Fujimoto, K. Tanimoto, and Y. Ike.** 1997. Cloning and genetic and sequence analysis of the bacteriocin 21 determinant encoded on the *Enterococcus faecalis* pheromone-responsive conjugative plasmid pPD1. J. Bacteriol. **179:**7843–7855.
63. **Tomita, H., and Y. Ike.** 2005. Genetic analysis of transfer-related regions of the vancomycin resistance *Enterococcus* conjugative pHTβ: identification of *oriT* and a putative relaxase gene. J. Bacteriol. **187:**7727–7737.
64. **Tomita, H., E. Kamei, and Y. Ike.** 2008. Cloning and genetic analyses of the bacteriocin 41 determinant encoded on the *Enterococcus faecalis* pheromone-responsive conjugative plasmid pYI14: a novel bacteriocin complemented by two extracellular components (lysine and activator). J. Bacteriol. **190:**2075–2085.
65. **Tomita, H., C. Pierson, S. K. Lim, D. B. Clewell, and Y. Ike.** 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.
66. **Tomita, H., K. Tanimoto, S. Hayakawa, K. Morinaga, K. Ezaki, H. Oshima, and Y. Ike.** 2003. Highly conjugative pMG1-like plasmids carrying Tn*1546*-like transposons that encode vancomycin resistance in *Enterococcus faecium*. J. Bacteriol. **185:**7024–7028.
67. **Uttley, A. H., C. H. Collins, J. Naidoo, and R. C. George.** 1988. Vancomycin-resistant enterococci. Lancet **i:**57–58.
68. **Weaver, K. E., and D. B. Clewell.** 1988. Regulation of the pAD1 sex pheromone response in *Enterococcus faecalis*: construction and characterization of *lacZ* transcriptional fusions in a key control region of the plasmid. J. Bacteriol. **170:**4343–4352.
69. **Weaver, K. E., and D. B. Clewell.** 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.
70. **Wirth, R., F. Y. An, and D. B. Clewell.** 1986. Highly efficient protoplast transformation system for *Streptococcus faecalis* and a new *Escherichia coli-S. faecalis* shuttle vector. J. Bacteriol. **165:**831–836.
71. **Yagi, Y., and D. B. Clewell.** 1980. Recombination-deficient mutant of *Streptococcus faecalis*. J. Bacteriol. **143:**966–970.
72. **Yu, H. S., S. Y. Seol, and D. T. Cho.** 2003. Diversity of Tn*1546*-like elements in vancomycin-resistant enterococci isolated from humans and poultry in Korea. J. Clin. Microbiol. **41:**2641–2643.
73. **Zechner, E. L., F. de la Cruz, R. Eisenbrandt, A. M. Grahn, G. Koraimann, E. Lanka, G. Muth, W. Pansegrau, C. M. Thomas, B. M. Wilkins, and M. Zatyka.** 2000. Conjugative-DNA transfer processes, p. 87–174. *In* C. M. Thomas (ed.), The horizontal gene pool. Bacterial plasmids and gene spread, Harwood Academic Publishers, The Netherlands.
74. **Zheng, B., H. Tomita, Y. H. Xiao, S. Wang, Y. Li, Y., and Y. Ike.** 2007. Molecular characterization of vancomycin-resistant *Enterococcus faecium* isolates from mainland China. J. Clin. Microbiol. **45:**2813–2818.

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Cloning and Genetic Analyses of the Bacteriocin 41 Determinant Encoded on the *Enterococcus faecalis* Pheromone-Responsive Conjugative Plasmid pYI14: a Novel Bacteriocin Complemented by Two Extracellular Components (Lysin and Activator)▽†

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The conjugative plasmid pYI14 (61 kbp) was isolated from *Enterococcus faecalis* YI714, a clinical isolate. pYI14 conferred a pheromone response on its host and encoded bacteriocin 41 (*bac41*). Bacteriocin 41 (Bac41) only showed activity against *E. faecalis*. Physical mapping of pYI14 showed that it consisted of EcoRI fragments A to P. The clone pHT1100, containing EcoRI fragments A (12.6 kbp) and H (3.5 kbp), conferred the bacteriocin activity on *E. faecalis* strains. Genetic analysis showed that the determinant was located in a 6.6-kbp region within the EcoRI AH fragments. Six open reading frames (ORFs) were identified in this region and designated ORF7 (*bacL*$_1$) ORF8 (*bacL*$_2$), ORF9, ORF10, ORF11 (*bacA*), and ORF12 (*bacI*). They were aligned in this order and oriented in the same direction. ORFs *bacL*$_1$, *bacL*$_2$, *bacA*, and *bacI* were essential for expression of the bacteriocin in *E. faecalis*. Extracellular complementation of bacteriocin expression was possible for *bacL*$_1$ and -*L*$_2$ and *bacA* mutants. *bacL*$_1$ and -*L*$_2$ and *bacA* encoded bacteriocin component L and activator component A, respectively. The products of these genes are secreted into the culture medium and extracellularly complement bacteriocin expression. *bacI* encoded immunity, providing the host with resistance to its own bacteriocin activity. The *bacL*$_1$-encoded protein had significant homology with lytic enzymes that attack the gram-positive bacterial cell wall. Sequence data for the deduced *bacL*$_1$-encoded protein suggested that it has a domain structure consisting of an N-terminal signal peptide, a second domain with the enzymatic activity, and a third domain with a three-repeat structure directing the proenzyme to its cell surface receptor.

Bacteriocins are bacterial proteins or peptides which inhibit the growth of other bacteria that are closely related to the producer strain. They usually exhibit a relatively narrow spectrum of activity and are produced by a wide variety of gram-positive and gram-negative bacteria (27). Bacteriocin production is thought to provide the host strain with an ecological or other selective advantage over other strains.

Many *Enterococcus faecalis* clinical isolates produce a bacteriocin (3, 5), and the bacteriocin is frequently encoded on the *E. faecalis* pheromone-responding conjugative plasmid (6, 14, 21, 46). Several *E. faecalis* bacteriocins have been genetically and biochemically characterized (15, 35), including the β-hemolysin/bacteriocin (cytolysin) (6, 7, 18, 20, 22) and the peptide antibiotics AS-48 (33), bacteriocin 21 (47), and bacteriocin 31 (46), which are encoded by the *E. faecalis* conjugative plasmids pAD1 (58 kbp), pMB2 (58 kbp), pPD1 (59 kbp), and pYI17 (57.5 kbp), respectively.

A significant number of *E. faecalis* clinical isolates produce hemolysin/bacteriocin (10, 26), and more than 50% of the hemolytic clinical isolates carry transferable hemolysin/bacteriocin determinants (21, 26). The hemolysin/bacteriocin of pAD1 is associated with virulence in animal models (4, 25, 29), and this plasmid is considered to be a typical *E. faecalis* hemolysin/bacteriocin plasmid (21, 31). The mechanism of hemolysin/bacteriocin production in *E. faecalis* has been studied in detail with the hemolysin/bacteriocin determinant on this plasmid (16, 17, 18, 22, 39). The active hemolysin/bacteriocin is produced by extracellular complementation of the two CylL factors (i.e., $CylL_L$ and $cylL_S$) and CylA.

Previously, we have shown that bacteriocins or bacteriocinogenic *E. faecalis* clinical isolates can be classified into five groups on the basis of their bacteriocin activity against *E. faecalis* FA2-2 and OG1-10, *Enterococcus hirae* 9790, *Streptococcus pyogenes*, *Streptococcus agalactiae*, *Streptococcus sanguinis*, *Streptococcus pneumoniae*, *Staphylococcus aureus*, and *Staphylococcus epidermidis* (46). *E. faecalis* FA-2-2 and OG1-10 and *E. hirae* have been chosen as representative enterococcal strains for the examination and classification of the bacteriocins produced by the clinical isolates in this study. Class 1 types produce the β-hemolysin/bacteriocin (cytolysin) and are active against a wide variety of gram-positive bacteria, including *S. aureus* (2, 15, 17, 24, 46). The β-hemolysin/bacteriocin (cytolysin) of pAD1 belongs to class 1. Class 2 is active against a broad spectrum of bacteria, including *E. faecalis*, other *Streptococcus* spp., and *S. aureus*. AS-48 and bacteriocin 21 belong to class 2. Class 3 is active against *E. faecalis* and *E. hirae*. Class 4 is active against *E. faecalis*, and class 5 is active against *E. hirae*. The YI717, YI718, and YI719 strains belong to class 3 and harbor plasmids pYI17 (57.5 kb), pYI18,

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and pYI19, respectively (46). These plasmids encode the same bacteriocin with respect to immunity to the bacteriocin activity. Bacteriocin 31 (Bac31), encoded on pYI17, is representative of the class 3 bacteriocins and is active against *E. faecalis* and *E. hirae*, as is the membrane-active class II bacteriocin of lactic acid bacteria (46). The Bac31 determinant consists of the structural gene *bacA* and the immunity gene *bacB*.

In this report, we describe the cloning and genetic analysis of the bacteriocin 41 determinant encoded on *E. faecalis* pheromone-responsive conjugative plasmid pYI14, which is a representative class 4 bacteriocin. We also describe the identification of the two functional domains that are required to produce the active bacteriocin by extracellular complementation of the two factors.

MATERIALS AND METHODS

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

Conjugation experiments. Broth mating and solid-surface mating were performed as previously described (48, 49), with a donor/recipient ratio of 1:10. Broth matings (in THB) were carried out for 4 h, and solid-surface matings (on THB agar plates) were carried out overnight (16 h) at 37°C. Transfer frequencies were calculated as the number of transconjugants per donor cell (at the end of mating). Pheromone induction and detection of cell aggregation were performed as previously described (11, 12).

Soft-agar assay for bacteriocin production and immunity. The bacteriocin production assay was performed as described previously (22). The test for immunity to the bacteriocin was performed essentially as described previously (22).

Plasmid DNA methodology. Recombinant plasmids were generated in *E. coli* DH5α. Transformation of bacterial cells with plasmid DNA was achieved by electrotransformation as described previously (13). Plasmid DNA was purified from *E. coli* (38) or from *E. faecalis* as previously described (14). DNA fragments were purified from an agarose gel after electrophoresis with a Gene Clean II kit (Bio 101, Inc.). Recombinant DNA methodology, analyses of plasmid DNA with restriction enzymes, and agarose gel electrophoresis were carried out by standard methods (38). Restriction enzymes were purchased from New England BioLabs, Roche, Nippon Gene, and Takara Co., and reactions were carried out under the conditions recommended by the manufacturers. DNA ligations were performed with a DNA ligation kit from Takara. To end fill the endonuclease-digested DNA fragment for ligation, a DNA-blunting kit and Klenow enzyme were obtained from Takara and used according to the manufacturer's protocol (45).

Determination of the pYI14 restriction map. pYI14 plasmid DNA was digested with EcoRI, BamHI, KpnI, SphI, or XbaI or double digested with a combination of two of these restriction enzymes. Agarose gel electrophoresis analysis of the digested DNAs was performed to determine the cleavage sites within the plasmid. To determine the order of the EcoRI fragments of pYI14, a relational clone set was constructed as previously described (14, 46). After agarose gel electrophoresis of plasmid pYI14 DNA partially digested with EcoRI, fragments greater than 7 kb in size were eluted and used for cloning. The cloning vectors used were pBluescript-SK(+) and pAM401, and the host strain was *E. coli* DH5α.

DNA sequence analysis. Nucleotide sequence analysis was carried out as previously described (14). A deletion kit (Nippon Gene) was used. BamHI-E, BamHI-F, EcoRI-H, and the 2.1-kb fragment between BamHI-F and EcoRI-H were individually cloned into the pBluescript vector. The clones were used to construct a series of deletional clones. The resulting constructs were sequenced in both orientations with the *Taq* Dye primer and the *Taq* Big Dye terminator cycle sequencing kit (Applied Biosystems), a model 377 DNA sequencer, and a 310 gene analyzer (ABI Prism). A database search was performed with the BLASTn and tBLASTx programs of the National Center for Biotechnology Information, Bethesda, MD (1).

Generation of transposon (Tn*5*, mini-Tn*7*) insertion mutants. Insertion of Tn*5* (Km^r) into the cloned plasmid DNA was performed as described elsewhere (47). Target plasmid pHT1100(pAM401 containing EcoRI fragments A and H) was introduced into *E. coli* K-12 TH688 (with Tn*5* in the *thr* locus) (42) by electrotransformation. Transformants were spread onto selective plates containing kanamycin and chloramphenicol, and the plates were left at room temperature for 10 days. The bacteria that grew on the selective plates were pooled, and the plasmid DNA was then isolated and used to transform *E. coli* DH5α. The transformants were selected on plates containing kanamycin and chloramphenicol for the selection of Tn*5*-borne kanamycin resistance and plasmid-borne chloramphenicol resistance, respectively. The transformants were purified and examined to determine the location of Tn*5* within the plasmid. The precise locations of Tn*5* insertions were determined by DNA sequence analysis with a synthetic primer that hybridized to the end of Tn*5*. A GPS kit (NEB) was used to generate mini-Tn*7* insertion mutants with plasmid pHT1100 according to the manufacturer's instructions.

PCR amplification and primers. PCR amplification was performed with the thermostable DNA polymerase Takara *Taq* (Takara Bio Inc.) and a Perkin-Elmer 9600 thermal cycler. PCR conditions varied according to the primers used and the size of the anticipated product. The custom primers used in this study were obtained from Invitrogen (Tokyo, Japan) and are listed in Table 1. Each of the amplified PCR products was trimmed by the appropriate restriction enzyme, purified with a QIAquick-spin column (Qiagen), and cloned into plasmid pAM401.

Nucleotide sequence accession number. The nucleotide sequence reported in this article is available from the DDBJ, EMBL, and GenBank nucleotide sequence databases under accession number AB271686.

RESULTS

Bacteriocinogenic *E. faecalis* strain and the pheromone-responsive bacteriocin plasmid. Four strains that were active against *E. faecalis* and were classified as class 4 bacteriocinogenic strains were isolated from clinical urine samples and were designated YI712, YI714, YI715, and YI716. YI712 harbored plasmid pYI12 (72 kb). YI714 and YI715 harbored plasmids pYI14 (61 kb) and pYI141 (48 kb) and plasmids pYI15 (61 kb) and pYI151 (48 kb), respectively. YI716 did not carry any plasmid. Each strain was used as a donor in mating experiments with plasmid-free recipient strain *E. faecalis* FA2-2 (Rif^r Fus^r) to determine whether these plasmids conferred bacteriocinogenic activity on the host. After incubating the broth mating cultures for 4 h, appropriately diluted mixtures were plated on an agar plate containing rifampin (25 μg/ml) and fusidic acid (25 μg/ml) to select for the recipient strains. After overnight incubation of the plates, a total of approximately 500 *E. faecalis* FA2-2 colonies were obtained from each mating and examined for bacteriocin production. Approximately 1 in 500 cells obtained from the mating experiments with each of the strains described above expressed bacteriocin activity against *E. faecalis* FA2-2. The bacteriocinogenic transconjugants of YI712, YI714, and YI715 harbored pYI12 (72 kb), pYI14 (61 kb), and pYI15 (61 kb), respectively. The same EcoRI restriction profiles were obtained for pYI14 and pYI15, implying that the two plasmids were identical. Each plasmid transferred between *E. faecalis* FA2-2 and *E. faecalis* OG1-10 at a frequency of about 10^{-3} per donor cell by broth mating. *E. faecalis* FA2-2(pYI12), FA2-2(pYI14), and FA2-2(pYI15) did not exhibit bacteriocin activity against *E. faecalis* OG1-10(pYI12), OG1-10(pYI14), or OG1-10(pYI15). These results imply that plasmids pYI12, pYI14, and pYI15 encoded the same bacteriocin with respect to the immunity character-

TABLE 1. Bacterial strains, plasmids, and oligonucleotides used in this study

Strain, plasmid, or oligonucleotide	Relevant features or sequence (5′–3′)[a]	Reference(s), source, or generated plasmid(s)
Strains		
E. faecalis		
FA2-2	*rif fus*	7
JH2SS	*spc str*	44
OG1-10	*str*; derivative of OG1	12
OG1X	*str*; protease-negative derivative of OG1-10	23
YI712	pYI12(Bac)	This study
YI714	pYI14(Bac), pYI141 (48 kb); clinical isolate	This study
YI715	pYI15(Bac), pYI151 (48 kb); clinical isolate	This study
E. coli DH5α	*endA1 recA1 gyrA96 thi-1 hsdR17 supE44 relA1* Δ(*argE-lacZYA*)*U169*	Bethesda Research Laboratories
Plasmids		
pAM401	*E. coli-E. faecalis* shuttle plasmid; *cat tet*	50
pLZ12-Km	*E. coli-Streptococcus* shuttle plasmid; *aphA*	19
pBlueScript SKII(+)	*E. coli* cloning vector; Amp^r	Stratagene
pPD1	Bac21, 59-kb conjugative plasmid from strain 39-5	14, 47
pMG326	pMW119 containing a 16.7-kbp EcoRI-SalI fragment of pPD1; pheromone-regulatory region	14, 41
pYI12	Bac41, 72-kb conjugation plasmid from YI712	This study
pYI14	Bac41, 61-kb conjugative plasmid from YI714	This study
pYI15	Bac41, 61-kb conjugative plasmid from YI715	This study
pHT1100	EcoRI-relational clone of pYI14; pAM401 containing EcoRI fragments A and H	This study
pHT1101	EcoRI-relational clone of pYI14; pAM401 containing EcoRI fragment A	This study
pHT1102	EcoRI-relational clone of pYI14; pAM401 containing EcoRI fragments H and M	This study
pMG1103	Derivative of pHT1100 with BamHI E fragment deleted	This study
pMG1104	Derivative of pHT1100 with BamHI F fragment deleted	This study
pMG1106	Derivative of pHT1100; BamHI site at 4.1 kbp blunted with Klenow enzyme	This study
pMG1108	Derivative of pHT1100; BamHI site at 6.3 kbp blunted with Klenow enzyme	This study
pMG1109	Derivative of pHT1100; KpnI site at 4.6 kbp blunted with DNA-blunting kit (Takara)	This study
pMG1105-*n*	Tn*5* insertional derivatives of pHT1100	This study
pMG1107-*n*	Mini-Tn*7* insertional derivatives of pHT1100 created with GPS kit (New England BioLabs)	This study
pMG1110	$bacL_1$ and $bacL_2$; pAM401 containing 2,932-bp EcoRI fragment amplified by PCR	This study
pMG1111	*bacA*; pAM401 containing 2,836-bp SalI fragment amplified by PCR	This study
pMG1112	*bacI*; pAM401 containing 777-bp BamHI fragment amplified by PCR	This study
pMG1113	*bacI* and ORF13; pAM401 containing 1,513-bp BamHI fragment amplified by PCR	This study
pMG1114	pLZ12-Km containing 10-kbp BglII fragment mapped from 1.7 kbp to 11.7 kbp	This study
pMG1115	Derivative of pMG1114; EcoRI fragment (8.5 kbp to vector region) deleted	This study
pMG1116	Derivative of pMG1114; three HindIII fragments (4.6- to 6.6-kbp region) deleted	This study
Oligonucleotides		
B9P2842F	ccg gaa tTC TAG CAA CCG AAA ACC ACG TTG G	pMG1110
B9P5773R	gcg gaa tTC ATT GCG CAG CAA ATC ATT GC	pMG1110
B9P6180F	aac gcg tcg ACA GGA ATT GAG ACA TAC GCT	pMG1111
B9P9015R	aac gcg tcg acT TCG TCA AAT CCA TTT CCC CTA	pMG1111
B9P8823F	ggc gga tcc GCA GCA GAA TTA GCA GGA GCG	pMG1112, pMG1113
B9P9599R	gcc gga tcc CAA AAG TCA TAC ATG ACC TCC	pMG1112
B9P10335R	gcc gga tcc CTG TAT AAA TCC ATA CTA CAC	pMG1113

[a] Underlining indicates the following restriction endonuclease recognition sequences: GAATTC; EcoRI, GTCGAC; SalI, GGATCC; BamHI. Lowercase letters indicate incorporated tag sequences.

istic. *E. faecalis* FA2-2 strains carrying pYI12, pYI14, or pYI15 were tested for bacteriocin production against the indicator strains *S. aureus* FDA209P, *E. faecalis* FA2-2 and OG1-10, *Enterococcus faecium* BM4105RF, *E. hirae* ATCC 9790, *Enterococcus durans* ATCC 49135, *Enterococcus raffinosus* JCM8733, *Enterococcus gallinarum* BM4174, *S. agalactiae*, *S. pyogenes*, *Listeria monocytogenes*, and *Listeria denitrificans*. Each of the three bacteriocinogenic strains only showed bacteriocin activity against

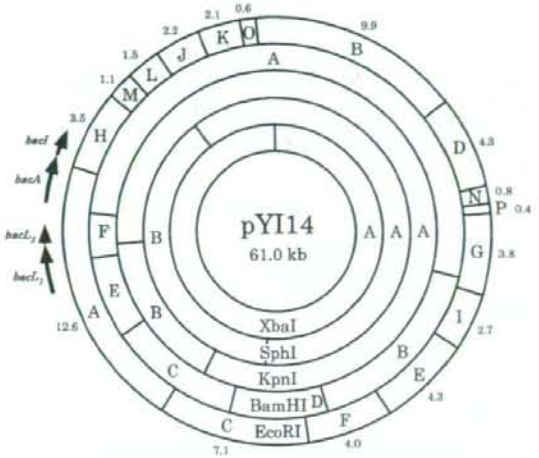

FIG. 1. Physical map of pYI14 showing the locations of bacteriocin 41 determinants $bacL_1$, $bacL_2$, *bacA*, and *bacI*. Each value is the size of the fragment in kilobases.

E. faecalis. Plasmid pYI14, isolated from strain YI714, was used as the representative plasmid encoding the bacteriocin.

The donor *E. faecalis* OG1-10(pYI14) and recipient *E. faecalis* FA2-2 formed a mating aggregate in the mating mixture. When OG1-10(pYI14) cells were exposed to *E. faecalis* FA2-2 culture filtrate (pheromone) for 4 h at 37°C, the OG1-10(pYI14) cells showed aggregation. Agarose gel electrophoresis of the EcoRI restriction fragments of pYI14 DNA was carried out, and the DNA was transferred to a membrane for Southern hybridization. The membrane was hybridized with a DNA probe containing the pheromone response genes of the pheromone-responsive plasmid pPD1 or plasmid pMG326, which contains the putative surface exclusion protein gene and the N-terminal region of the aggregation substance gene of pPD1 (14, 41). Each probe hybridized to specific pYI14 EcoRI fragments (data not shown). These results indicated that plasmid pYI14 was a pheromone-responsive plasmid.

Restriction map of pYI14. To determine the order of the EcoRI fragments, a relational clone set was obtained. The order of EcoRI fragments was determined to be A-H-M-L-J-K-O-B-D-N-P-G-I-E-F-C (Fig. 1). Each clone was digested with BamHI, KpnI, SphI, and XbaI, and the cleavage sites were determined (Fig. 1). Restriction sites within the EcoRI A and H fragments were also confirmed by sequencing (see the supplemental material).

Bacteriocin activity of the cloned DNA fragment. To examine the bacteriocin activity of the relational clones, each clone was introduced into *E. faecalis* OG1-10 and the resulting transformant was examined for bacteriocin activity. *E. faecalis* OG1-10 carrying plasmid pHT1100, which contained the EcoRI A and H fragments (16.1 kb), exhibited the bacteriocin activity (Fig. 2). *E. faecalis* OG1-10 carrying either the EcoRI A (12.6 kb) or HM (4.6 kb) fragments (plasmids pHT1101 and pHT1102, respectively) did not exhibit bacteriocin activity (Fig. 2). *E. faecalis* OG1-10 carrying the EcoRI HM fragments showed resistance to the bacteriocin activity of *E. faecalis* OG1-10(pYI14). These results indicated that the bacteriocin determinant of pYI14 is located on the EcoRI A and H (AH) fragments and the immunity gene (i.e., the gene for resistance to its own bacteriocin) is located on the EcoRI H fragment.

DNA sequence analysis. The EcoRI AH fragments were sequenced, and computer analysis was used to identify open reading frames (ORFs) within the sequence. Fifteen ORFs (ORF1 to ORF15) were located in the region spanning map positions 0 to 12 kbp, as indicated by the numerical scale shown in Fig. 2, where position 0 is the BamHI site located between BamHI fragments E and C and position 12 kbp is the EcoRI site located between the EcoRI H and M fragments. (Fig. 1 and 2 and Table 2; see Fig. S1 in the supplemental material). Figure 2 shows the ORFs that have a deduced ribosome-binding site in the 20-base region upstream of the predicted start codon and the potential promoters for initiation of transcription.

Generation of Tn*5* or mini-Tn*7* insertion mutants. To examine the location of the bacteriocin determinant, mutants with altered bacteriocin expression were generated by Tn*5* or mini-Tn*7* insertion into pHT1100. The precise locations of Tn*5* or mini-Tn*7* insertions in the ORFs were determined by DNA sequence analysis (see Fig. S1 in the supplemental material), and the results are shown in Fig. 2 and Table 3. Tn*5* insertions into ORF7, ORF8, and ORF11 resulted in defective bacteriocin activity in *E. faecalis* OG1-10. Insertion of mini-Tn*7* into the C-terminal region of ORF11 also resulted in defective bacteriocin activity in *E. faecalis* OG1-10. *E. faecalis* OG1S carrying each of the insertion mutants showed resistance to the bacteriocin activity of *E. faecalis* OG1-10(pYI14), indicating that the mutant plasmids retained immunity to the bacteriocin.

Generation of deletion mutants by end filling after cleavage with a restriction enzyme. Mutant pHT1100 plasmids with BamHI fragment deletions within were also generated to examine the location of the bacteriocin determinant as described in Materials and Methods (Fig. 2) (47). Deletion mutant plasmids pMG1103 and pMG1104 possessed deletions of the 4.1-kbp BamHI E fragment between map positions 0 kb and 4.1 kb and the 2.2-kbp BamHI F fragment between map positions 4.1 kb and 6.3 kb, respectively. Plasmid pMG1103, which had a deletion in the amino-terminal region of ORF7 and had lost the six ORFs located upstream of ORF7, did not exhibit bacteriocin activity but retained immunity to the bacteriocin. Plasmid pMG1104, which had deletions within the carboxyl-terminal region of ORF7, ORF8, and ORF9 and the amino-terminal region of ORF10, did not exhibit bacteriocin activity but retained immunity to the bacteriocin. These results implied that the gene for immunity is located downstream of ORF11.

Generation of four-nucleotide insertion (deletion) mutants. Mutants with changes in ORF7 and ORF10 were generated to obtain mutants with in-frame changes in the determinant by blunt ending the recessed 3′ terminus of the BamHI site or the prominent 3′ terminus of the KpnI cleavage site within pHT1100 DNA that had been partially digested with these enzymes prior to ligation (Fig. 2) (45). Blunt ending the BamHI and KpnI sites resulted in the insertion of four nucleotides (5′-GATC-3′) with the Klenow enzyme in the case of the BamHI site and the deletion of four nucleotides (5′-GTAC-3′) with the T4 DNA polymerase DNA-blunting kit

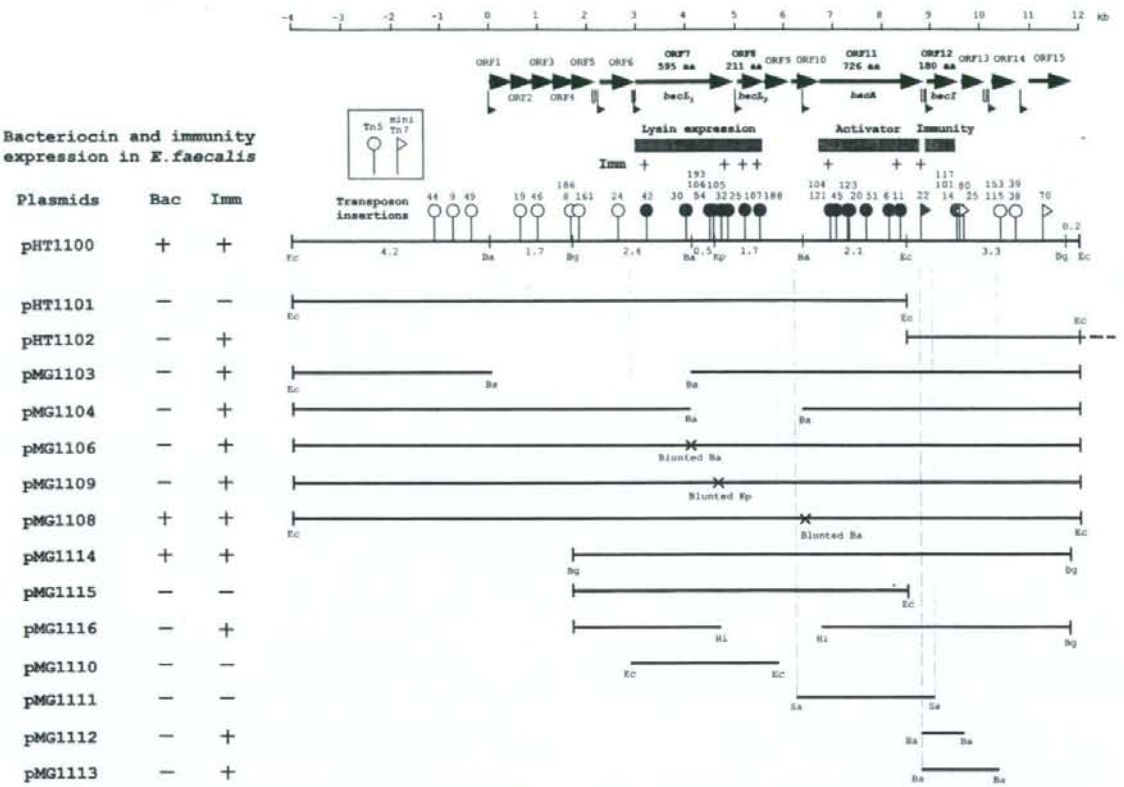

FIG. 2. Physical maps of the 16.1-kb region containing EcoRI fragments A (12.6 kb) and H (3.5 kb) in pYI14 (which is carried on pHT1100), transposon insertions, and subclones. The zero position of the numerical scale (top horizontal line) indicates the BamHI endonuclease recognition site located between the BamHI C and E fragments, and it runs in a clockwise direction on the physical map of Fig. 1. Thick horizontal arrows indicate the predicted ORFs and the direction of ORF transcription. The flags and hairpins below the ORFs indicate the potential promoter regions and inverted repeat sequences. The horizontal lines under the map represent the cloned pYI14 DNA fragments in the derivative plasmids listed on the left. Small vertical bars at ends of the lines represent the endonuclease recognition sites for cloning. The dotted vertical lines represent the ends of the amplified PCR fragment of pYI14 DNA used to clone the bacteriocin determinant. The endonuclease recognition sites incorporated for the cloning of the PCR products are indicated. Abbreviations of the endonuclease recognition sites: Eco, EcoRI; Ba, BamHI; Kp, KpnI; Bg, BglII; Hi, HindIII; Sa, SalI. Bac +, normal bacteriocin expression; Bac −, no bacteriocin expression; Imm +, resistance to bacteriocin 41; Imm −, sensitive to bacteriocin 41. The vertical lines with circular or triangular heads on the pHT1100 map show the points of transposon insertion. The circular heads indicate Tn*5*, and the triangular heads indicate mini-Tn*7* and its orientations. The heads represent the levels of bacteriocin 41 expression in *E. faecalis* strains as follows: open heads, normal bacteriocin expression; black heads, no bacteriocin expression; gray heads, weak bacteriocin expression (Fig. 3A). The values on the insertions indicate the numbers of insertions and correspond to those shown in Table 3 (see also Fig. S1 in the supplemental material). The cross marks on the clones indicate the mutated endonuclease recognition sites (a four-base insertion or deletion). aa, amino acids.

(Takara) in the case of the KpnI site. The pMG1106 and pMG1109 mutants that resulted from the blunt ending of the BamHI site and KpnI sites in ORF7 did not exhibit bacteriocin activity but retained the immunity activity, indicating that ORF7 is essential for bacteriocin expression. The pMG1108 mutant, which resulted from the end filling of the BamHI site in ORF10, expressed both bacteriocin and immunity activity, suggesting that ORF10 is not essential for bacteriocin expression.

Subcloning of the bacteriocin determinant and generation of the derivative mutants. The 10.0-kb BglII fragment that is located between 1.7 kb and 11.7 kb on the map was cloned into shuttle vector pLZ12-Km (19) (Fig. 2), and the cloned plasmid was designated pMG1114. pMG1114 expressed both bacteriocin activity and immunity, indicating that the bacteriocin determinant was located within the 10.0-kb BglII fragment. Deletion mutants pMG1115 and pMG1116 were generated from pMG1114. pMG1115 had a deletion of the 3.3-kbp EcoRI/BglII fragment between 8.4 kb and 11.7 kb on the map, which contains the C-terminal region of ORF11. pMG1115 did not express either the bacteriocin or immunity, indicating that ORF11 is necessary for bacteriocin activity. pMG1116 had a deletion of two HindIII fragments totaling 1.4 kb that were located between 5.2 kb and 6.6 kb on the map and contains the C-terminal region of ORF7 and all of ORF8, ORF9, and ORF10. pMG1116 did not express the bacteriocin but ex-

TABLE 2. ORFs encoded on the BamHI/EcoRI 11,952-bp-spanning region

ORF	Gene	Map location (bp)	Gene/protein size (base pairs/amino acids)	Homology	% Identity/similarity (amino acids)	Function
1		136–588	453/150	*pcfS* (*E. faecalis* pCF10)	98/100	Ssb[a]
2		602–754	153/50	EFB0044 (*E. faecalis* V583 pTEF2)	100/100	
3		766–1344	579/192	*pcfT* (*E. faecalis* pCF10)	89/90	Thermonuclease
4		1350–1670	321/106	*pcfU* (*E. faecalis* pCF10)	93/97	
5		1827–2009	183/60	Efae03001107 (*E. faecium*)	50/67	
6		2204–2920	717/238	Lipoprotein (*E. faecalis* V583)	31/45	
7	*bacL*$_1$	3058–4845	1,788/595	Lysozyme (*B. subtilis* bacteriophage B103) Lysin (*S. agalactiae* prophage lambda Sa1) Muramidase (*L. plantarum* WCFS1)	37/52 (1–151) 46/63 (160–309) 24/41 (318–577)	Lysin (bacteriocin 41)
8	*bacL*$_2$	5031–5666	636/211			Lysin expression
9		5689–6120	432/143	ORF50 (*S. pneumoniae* bacteriophage MM1)	31/51	Holin
10		6123–6650	528/175	EF0637 (*E. faecalis* V583)	27/45	
11	*bacA*	6693–8873	2,181/726	*ybfG* (*B. subtilis*) *ykuG* (*B. subtilis*)	41/56 40/55	Lysin activator
12	*bacI*	8981–9523	543/180			Immunity
13		9590–10165	576/191			
14		10308–10640	333/110	EFB0057 (*E. faecalis* V583 pTEF2)	98/100	
15		11080–11781	702/233			

[a] Ssb, single-stranded binding protein.

pressed immunity. Analysis of the insertion mutants and deletion mutants showed that ORF7, ORF8, ORF11, and ORF12 are necessary for bacteriocin expression.

Extracellular complementation of nonbacteriocinogenic mutants. Extracellular complementation experiments to express bacteriocin activity were performed with ORF7 or ORF8 and ORF11 mutant strains on soft agar plates containing the indicator strain. OG1-10(pMG1106) and OG1-10(pMG1109), which were ORF7 mutants prepared by blunt ending, were streaked in proximity to streaks of either OG1-10 carrying the pHT1100 derivatives of the Tn*5* insertion mutants in ORF11 or OG1-10(pHT1101) with a deletion in ORF11. This experiment showed that there was complementation of the bacteriocin activity at the streak junction. When OG1-10(pHT1101) was streaked in proximity to streaks of OG1-10 carrying the pHT1100 derivatives of the Tn*5* insertion mutants in ORF7 or ORF8 and the end-filled mutants of ORF7, complementation of the bacteriocin activity was observed at the streak junction. These results indicated that the mutants fell into one of two complementation groups. Representative results are shown in Fig. 3 and Table 4. The OG1-10(pHT1100), OG1-10(pHT1101), and OG1-10(pMG1106) strains were inoculated in proximity in soft agar containing the indicator strain (Fig. 3B). Bacteriolysis was observed around the wild-type strain and also between OG1-10(pHT1101) and the wild-type strain or OG1-10(pMG1106), respectively. Bacteriolysis was also observed surrounding OG1-10(pHT1101). Figure 3C shows the complementation activity that resulted from cross-streaking of OG1-10(pMG1106) and OG1-10(pHT1101) on soft agar containing the indicator strain. Bacteriolysis was observed at the junction of the two strains. Based on these observations, the two complementation substances were tentatively designated L (lysin) and A (activator). OG1-10(pHT1101) and the ORF11 mutants were presumed to be defective in bacteriocin component A synthesis and tentatively assigned an $L^+ A^-$ phenotype. The ORF11 gene was designated *bacA*. OG1-10(pMG1106) and the ORF7 and ORF8 mutants were presumed to be defective in bacteriocin component L synthesis and tentatively assigned an $L^- A^+$ phenotype. The ORF7 and ORF8 genes were designated *bacL*$_1$ and *bacL*$_2$, respectively.

Cloning of component L, component A, and the immunity genes. The PCR product of each ORF was cloned to analyze its function in bacteriocin expression (Fig. 2). Cloned pMG1110, pMG1111, pMG1112, and pMG1113 contained ORF7/8 (*bacL*$_1$ and -*L*$_2$), ORF11 (*bacA*), ORF12, and ORF12/13, respectively (Fig. 2). Each of the individually cloned fragments did not express bacteriocin activity. pMG1112 and pMG1113, which contained ORF12 and ORF12/13, expressed immunity to the bacteriocin activity, indicating that ORF12 was the immunity gene, and it was designated *bacI*.

Extracellular complementation between cloned L and A components. Cross streaks of strains carrying the two cloned fragments were made on bacteriocin assay plates. When OG1-10(pMG1110), which contained ORF7 (*bacL*$_1$) and ORF8 (*bacL*$_2$), was streaked across a preexisting streak of OG1S (pMG1111), which contained ORF11 (*bacA*), a large area of bacteriolysis was observed around the two crossed strains (Table 4). Growth of the two strains was markedly inhibited. These data indicated that the product of each strain complemented to produce an active bacteriocin, but the two strains have no immunity to the bacteriocin; therefore, growth of the strains was inhibited by the bacteriocin.

DNA sequence analysis of ORFs located in the region containing the bacteriocin 41 determinant. A homology search of the 15 ORFs contained in the 12-kbp region was performed by BLAST against the protein databases, and the results are shown in Table 2 (1). ORF7 (*bacL*$_1$), ORF8 (*bacL*$_2$), ORF11 (*bacA*), and ORF12 (*bacI*) were essential for the expression of bacteriocin 41. *bacL*$_1$ encoded a 595-amino-acid protein. Computer analysis suggested that the deduced *bacL*$_1$-encoded protein had a signal peptide sequence and that a potential signal peptidase processing site corresponding to the L-K-A sequence was located at positions 19 to 21 (Fig. 4A). Comparison

TABLE 3. Transposon insertion mutants of pHT1100 and bacteriocin expression

Insertion no. in Fig. 2	Plasmid(s)[a]	Transposon	Map position (kb)[b]	Insertion location	Bac[c]	Imm[d]
	pYI14				++	+
	pHT1100				++	+
44	pMG1105-44	Tn*5*	−1.2	Upstream of ORF1	++	+
9	pMG1105-9	Tn*5*	−0.8	Upstream of ORF1	++	+
49	pMG1105-49	Tn*5*	−0.4	Upstream of ORF1	++	+
19	pMG1105-19	Tn*5*	0.7	ORF2	++	+
46	pMG1105-46	Tn*5*	1.0	ORF3	++	+
8, 186	pMG1105-8, -186	Tn*5*	1.7	Between ORF4 and ORF5	++	+
161	pMG1105-161	Tn*5*	1.9	ORF5	++	+
24	pMG1105-24	Tn*5*	2.6	ORF6	++	+
42	pMG1105-42	Tn*5*	3.2	ORF7 (*bacL*$_1$)	−	+
30	pMG1105-30	Tn*5*	4.0	ORF7 (*bacL*$_1$)	−	+
54, 105, 193	pMG1105-54, -105, -193	Tn*5*	4.5	ORF7 (*bacL*$_1$)	−	+
105	pMG1105-105	Tn*5*	4.6	ORF7 (*bacL*$_1$)	−	+
32	pMG1105-32	Tn*5*	4.7	ORF7 (*bacL*$_1$)	−	+
25	pMG1105-25	Tn*5*	4.8	ORF7 (*bacL*$_1$)	−	+
107	pMG1105-107	Tn*5*	5.2	ORF8 (*bacL*$_2$)	−	+
188	pMG1105-188	Tn*5*	5.5	ORF8 (*bacL*$_2$)	−	+
104, 121	pMG1105-104, -121	Tn*5*	6.9	ORF11 (*bacA*)	−	+
45	pMG1105-45	Tn*5*	7.1	ORF11 (*bacA*)	−	+
123	pMG1105-123	Tn*5*	7.3	ORF11 (*bacA*)	−	+
20	pMG1105-20	Tn*5*	7.3	ORF11 (*bacA*)	−	+
51	pMG1105-51	Tn*5*	7.7	ORF11 (*bacA*)	−	+
6	pMG1105-6	Tn*5*	8.1	ORF11 (*bacA*)	−	+
11	pMG1105-11	Tn*5*	8.4	ORF11 (*bacA*)	−	+
22	pMG1107-22	Mini-Tn*7*	8.7	ORF11 (*bacA*)	−	+
14, 101, 117	pMG1105-14, -101, -117	Tn*5*	9.5	ORF12 (*bacI*)	+	±
80	pMG1107-80	Mini-Tn*7*	9.5	Between ORF12 and ORF13	++	+
25	pMG1107-25	Mini-Tn*7*	9.6	ORF13	++	+
115, 153	pMG1105-115, -153	Tn*5*	10.4	ORF14	++	+
38, 39, 163	pMG1105-38, -39, -163	Tn*5*	10.7	Between ORF14 and ORF15	++	+
87	pMG1107-87	Mini-Tn*7*	11	Between ORF14 and ORF15	++	+
74	pMG1107-74	Mini-Tn*7*	11	Between ORF14 and ORF15	++	+
76	pMG1107-76	Mini-Tn*7*	11.1	Between ORF14 and ORF15	++	+
69	pMG1107-69	Mini-Tn*7*	11.1	ORF15	++	+
59	pMG1107-59	Mini-Tn*7*	11.2	ORF15	++	+
70	pMG1107-70	Mini-Tn*7*	11.3	ORF15	++	+
40	pMG1107-40	Mini-Tn*7*	11.4	ORF15	++	+
80	pMG1107-80	Mini-Tn*7*	11.4	ORF15	++	+
83	pMG1107-83	Mini-Tn*7*	11.5	ORF15	++	+

[a] The host strain of the derivative was *E. faecalis* OG1S (OG1-10).
[b] The map position is the distance from the junction between EcoRI fragments A and H. Minus values indicate the opposite direction.
[c] Bac, bacteriocin expression. Symbols: ++, normal bacteriocin expression; +, weak bacteriocin expression (Fig. 3A); −, no bacteriocin expression.
[d] Imm, immunity expression. Symbols: +, positive expression; −, no expression; ±, weak expression.

of the primary structure of the deduced amino acid sequence of the BacL$_1$ protein showed significant homology with the cell wall lytic enzymes found in gram-positive bacteria (Fig. 4A) (32). Of the 595 amino acid residues of the BacL$_1$ protein, the N-terminal 151 amino acid residues showed a high level of homology with the lysozyme encoded on *Bacillus subtilis* bacteriophage B103 (accession number Q37896) (37). The 150-amino-acid sequence from residue 160 to residue 309, which is located in the center of the *bacL*$_1$-encoded protein, showed a high level of homology with the N-terminal amino acid residues of the lysin encoded on the *S. agalactiae* prophage lambda Sa1 (accession number NP 687631) (43), and the C-terminal 260 amino acid residues showed a high level of homology with the C-terminal amino acid residues of the muramidase of *Lactobacillus plantarum* WCFS1 (accession number CAD64901) (30). The *bacL*$_1$-encoded protein harbored a three-repeat structure of an almost identical amino acid sequence (Fig. 4B). The three-repeat structure located at the C terminus of the *bacL*$_1$-encoded protein corresponded to the homologous C-terminal region of the *L. plantarum* WCS1 muramidase, which is thought to be a choline-binding region (28, 51). The repeat structure was composed of three copies of an almost identical 74-amino-acid sequence. The first copy was located between amino acid residues 333 and 406, the second copy was located between amino acid residues 424 and 497, and the third copy was located between amino acid residues 520 and 593. *bacL*$_2$ encoded a 211-amino-acid protein and did not show any significant homology with other reported proteins. There was no obvious leader peptide with hydrophobic residues at the N-terminal peptide of the deduced *bacL*$_2$-encoded protein. *bacA* encoded a 726-amino-acid protein and showed a significant degree of homology with *ybfG* and *ykuG* of *B. subtilis*, but the function of these proteins is unknown (Fig. 5) (accession numbers CAB12014 for YbfG and CAA10870 for YkuG, respectively). The *bacA* protein had a putative signal peptide sequence, and a potential signal peptidase processing site corresponding to

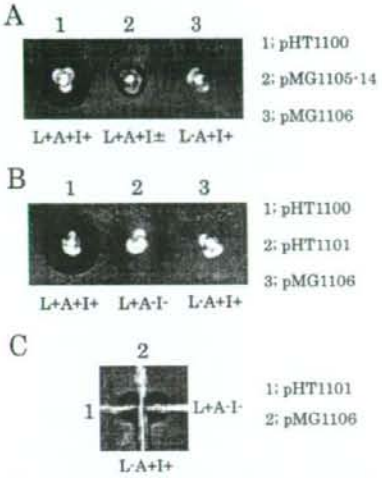

FIG. 3. Bacteriocin expression assay by the soft-agar method with *E. faecalis* OG1-10 carrying the representative pYI14 bacteriocin derivatives (A) and complementation assays (B and C). The indicator strain was *E. faecalis* OG1-10. The strains used are shown in Fig. 2 and Table 3. (A) 1, OG1-10(pHT1100) wild type; 2, OG1-10(pMG1105-14, a transposant of pHT1100::Tn*5*) (Tn*5* inserted in the C-terminal region of ORF12); 3, OG1-10(pMG1106) in-frame $bacL_1$ mutant. (B) 1, OG1-10(pHT1100); 2, OG1-10(pHT1101) *bacA* and *bacI* deletion mutant; 3, OG1-10(pMG1106). (C) 1; OG1-10(pHT1101), 2; OG1-10(pMG1106). L, $bacL_1$ and $bacL_2$ expression; A, *bacA* expression; I, immunity expression; +, positive expression; −, no expression; ±, weak expression.

the V-S-G sequence was located at positions 19 to 21 (Fig. 5). The *bacA* protein contained a 60-amino-acid sequence corresponding to the putative peptidoglycan-binding domain, which was located between amino acids 81 and 140 in the *bacA*-encoded protein, suggesting that the BacA protein could be directed to the bacterial cell surface.

DISCUSSION

Bacteriocin 41 of strain YI714 was encoded on *E. faecalis* pheromone-responsive plasmid pYI14 (61 kbp) and was only active against *E. faecalis*. The EcoRI AH fragments of pYI14, which conferred the bacteriocin activity, were cloned and used for genetic analysis of the bacteriocin determinant. Transposon insertion and deletion mutant analysis of the EcoRI AH fragments and further subcloning of the bacteriocin determinant showed that a 6.6-kb fragment of pYI14 was the minimum-size fragment required for bacteriocin expression. The 6.6-kb region contained six ORFs, which were designated $bacL_1$, $bacL_2$, ORF9, ORF10, *bacA*, and *bacI*. All of the ORFs were oriented in the same direction and in that order. The insertion mutants were classified into one of two complementation classes for component L and component A. Each class showed extracellular complementation to produce the active bacteriocin. A series of PCR products containing the L-encoding region for component L, the A-encoding region for component A, and the immunity-encoding region for resistance to the bacteriocin were subcloned into *E. faecalis* OG1-10. The subclones for the L-encoding, A-encoding, and immunity-encoding regions contained $bacL_1$ and $bacL_2$, *bacA*, and *bacI*, respectively. The subclone containing $bacL_1$ and $bacL_2$ produced an L component capable of extracellular complementation with the A component for expression of bacteriocin activity, indicating that $bacL_1$ and $bacL_2$ were required for component L. These results indicated that of the ORFs within the 6.6-kb region, $bacL_1$, $bacL_2$, and *bacA* are essential for the production of the active bacteriocin, and *bacI* is the immunity gene for resistance to the bacteriocin that is produced.

Tn*5* insertions into $bacL_1$ or $bacL_2$ of the bacteriocin determinant did not result in a detectable polar effect on the expression of the downstream *bacA* or *bacI* gene, and insertion into *bacA* also did not result in a polar effect on the expression of *bacI*. Both component determinants and *bacI* were expressed when each of the determinants was cloned into vector plasmid pAM401 in either orientation within an *E. faecalis* OG1-10 background. These results suggested that a significant amount of transcription of the $bacL_1$ and $bacL_2$, *bacA*, and *bacI* genes can occur from different promoters.

In the complementation experiment between the $L^+ A^-$ and $L^- A^+$ strains, bacteriocin activity was observed around the $L^+ A^-$ strain. When the wild-type $L^+ A^+$ and mutant $L^+ A^-$ strains were inoculated in proximity to the bacteriocin assay, bacteriolysis was observed around the $L^+ A^-$ strain. The complementation experiment between the wild-type $L^+ A^+$ and mutant $L^- A^+$ strains did not show any bacteriocin activ-

TABLE 4. Extracellular *trans*-complementation analysis of bacteriocin 41 activity[a]

Plasmid(s)	Genotype	Phenotype[b]	pMG1105-42, pMG1105-25	pMG1106, pMG1109	pMG1105-107, pMG1105-188	pMG1105-104, pMG1105-11	pHT1101	pMG1110	pMG1111
pMG1105-42, pMG1105-25	$bacL_1\ L_2^+\ A^+\ I^+$	$L^-A^+I^+$	NT						
pMG1106, pMG1109	$bacL_1\ L_2^+\ A^+\ I^+$	$L^-A^+I^+$	C^-	NT					
pMG1105-107, MG1105-188	$bacL_1^+\ L_2\ A^+\ I^+$	$L^-A^+I^+$	C^-	C^-	NT				
pMG1105-104, pMG1105-11	$bacL_1^+\ L_2^+\ A\ I^+$	$L^+A^-I^+$	C^+	C^+	C^+	NT			
pHT1101	$bacL_1^+\ L_2^+\ A\ I$	$L^+A^-I^-$	C^+	C^+	C^+	C^-	NT		
pMG1110	$bacL_1^+\ L_2^+\ A\ I$	$L^+A^-I^-$	C^+	C^+	C^+	C^-	C^-	NT	
pMG1111	$bacL_1\ L_2\ A^+\ I$	$L^-A^+I^-$	C^-	C^-	C^-	C^+	C^+	C^+	NT

[a] C^+; bacteriocin activity was detected at the intersection of the two strains by the soft-agar assay, C^-; no bacteriocin activity, NT; not tested.
[b] L, expression of lytic protein(s); A, activator expression; I, immunity expression.

FIG. 4. Comparison of the amino acid sequence of the predicted $BacL_1$ protein (ORF7) of bacteriocin 41 with the amino acid sequence of the cell wall lytic enzymes of gram-positive bacteria (A) and the repeat sequences found in the $BacL_1$ protein (B). Lysozyme, *B. subtilis* bacteriophage B103 (accession number Q37896); lysin, *S. agalactiae* prophage lambda Sa1 (accession number NP 687631); muramidase, *L. plantarum* WCFS1 (accession number CAD64901).

ity. These results suggested that the activator of component A modified component L, that the activated component L possessed the bacteriocin activity, and also that an excess of component A existed in the extracellular medium.

The β-hemolysin/bacteriocin (cytolysin) determinant encoded on pAD1 consists of the eight genes *cylR2*, *cylR1*, $cylL_L$, $cylL_S$, *cylM*, *cylB*, *cylA*, and *cylI* (2, 8, 9, 17, 18, 39). $CylL_L$ and $CylL_S$ are the cytolysin structural subunits. The $CylL_L$ and $CylL_S$ proteins are modified posttranslation by $CylL_M$ (2), and the modified $CylL_L$ and $CylL_S$ proteins are secreted via $CylL_B$, which is the ATP-binding exporter (16). The extracellular cytolysin precursors $CylL_L$ and $CylL_S$ are converted to the active cytolysin by CylA (2, 22). In an early study of the β-hemolysin/bacteriocin (cytolysin) determinant (22), two functional domains within the operon were identified and it was found that one region encodes the toxin precursor L component, which is now known to be encoded by$CylL_1$, $CylL_2$, CylM, and CylB, and the other region encodes an activator A component, which is now known to be encoded by CylA and CylI (2, 8, 9, 17, 18, 39). In the complementation experiment between the A component-producing strain or the wild-type strain and the L component-producing strain on blood agar plates, the β-hemolysis zone occurred around or along the L component-producing strain (22), indicating that the A component activates the L component extracellularly and that the activated L component possesses the β-hemolysin/bacteriocin activity and an excess of extracellular A component is present in the culture medium of the wild-type strain (24). These observations are similar to the extracellular complementation observed between the L component-producing strain and the A component-producing strain for bacteriocin 41.

The deduced amino acid sequence encoded by $bacL_1$ showed a high degree of homology with the cell wall lytic enzymes and murein hydrolases of lysozyme, lysine, and the muramidase of gram-positive bacteria (32). These enzymes cleave glycan strains either between the *N*-acetylmuramic acid and *N*-acetylglucosamine or at the alternative acetylglucosamine-muramic acid glycoside linkage (34). Sequence alignments of the murein hydrolases of the gram-positive bacteria show that most of these enzymes display a domain structure. In general, these enzymes harbor an N-terminal signal peptide, followed by a second domain containing the enzymatic activity. In addition, these proteins harbor repeat structures or cell wall-targeting structures that flank either the N- or C-terminal side of the enzymatic domain (40). The repeated domains direct the murein hydrolase to its receptor on the cell surface of gram-positive bacteria (51). Murein hydrolase is usually synthesized as a preproenzyme, and after cleavage of the N-terminal signal peptide, the soluble proenzyme is secreted into the extracellular environment. The repeated domains or cell wall-targeting domains direct the proenzyme to its receptor on the bacterial cell surface. Proteolytic cleavage or activation of the proenzyme generates the mature enzyme (32).

Although the mechanism of activation or the precise mode of action of the $bacL_1$-encoded protein is not known, analysis

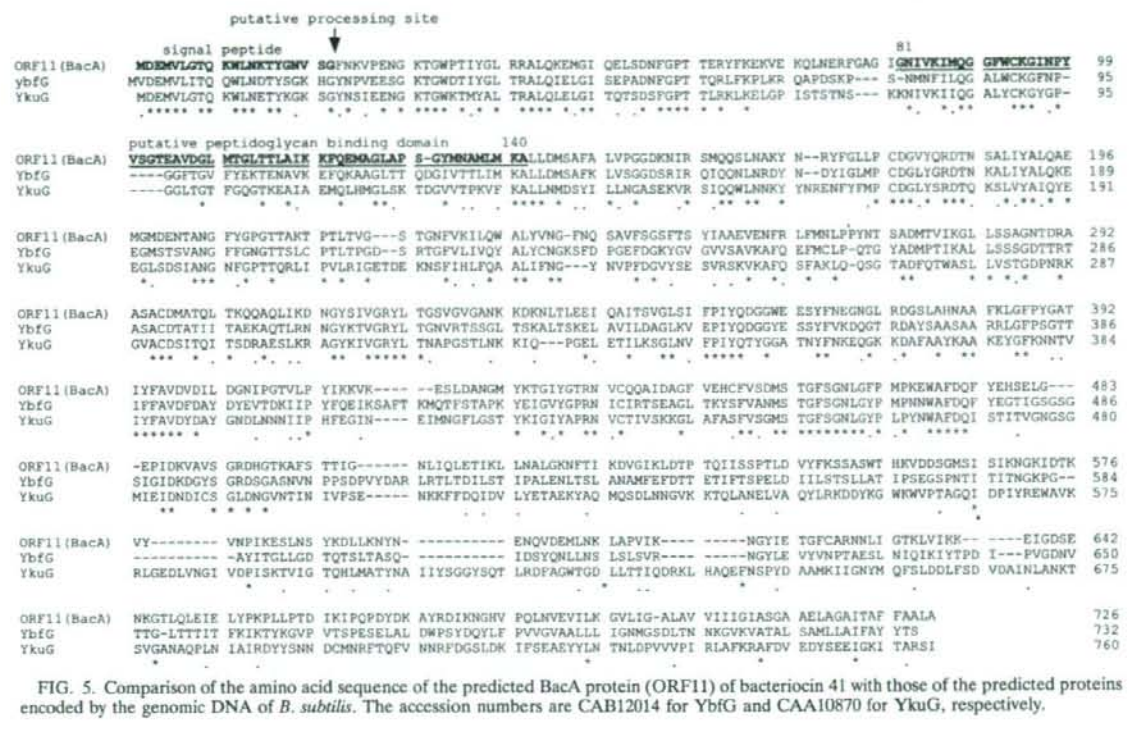

FIG. 5. Comparison of the amino acid sequence of the predicted BacA protein (ORF11) of bacteriocin 41 with those of the predicted proteins encoded by the genomic DNA of *B. subtilis*. The accession numbers are CAB12014 for YbfG and CAA10870 for YkuG, respectively.

of the deduced amino acid sequence of the $bacL_1$-encoded protein suggests that the protein exhibits a domain structure. The domain structure is composed of an N-terminal signal peptide followed by a second domain containing the enzymatic activity and a third domain with the three amino acid sequence repeat structures. The $bacL_1$-encoded protein might be synthesized as a preproenzyme, and after signal peptide cleavage, the soluble proprotein encoded by $bacL_1$ would be secreted into the extracellular environment. The repeat domains might function to direct the proprotein encoded by $bacL_1$ to its receptor on the bacterial cell surface, and the proprotein encoded by $bacL_1$ might be activated by the *bacA* protein, resulting in the generation of the mature $BacL_1$ protein. As described above, $bacL_2$ was also essential for the expression of the L component. The deduced $bacL_2$-encoded protein was a 211-amino-acid protein with no leader peptide. The sequence data implied that the $bacL_2$-encoded protein might modify the $bacL_1$-encoded protein inside the bacterial cell. Bacteriocin 41 only showed bacteriocin activity against *E. faecalis*, which suggested that the $bacL_1$-encoded protein of bacteriocin 41 was highly specific for the glycan strand of the *E. faecalis* cell wall.

Recently, another group reported the discovery of a novel cell wall-degrading bacteriocin, which has been named enterolysin A (EnlA), in an *E. faecalis* strain isolated from fish (36). The bacteriocin gene *enlA* encodes a 343-amino-acid preprotein with a *sec*-dependent signal peptide of 27 amino acids. The mature EnlA protein consists of 316 amino acids and is homologous to the catalytic domains of a variety of cell wall-degrading proteins. It might be that bacteriocin 41 belongs to the same group of enterococcal cell wall-degrading bacteriocins as EnlA, although the details of the mechanism of expression of EnlA, including the immunity factor, have not been clearly elucidated (15). However, our results imply that the mechanism of bacteriocin 41 expression is more complex than the EnlA expression system and that they are divergent systems.

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REFERENCES

1. **Altschul, S. F., W. Gish, W. Miller, E. W. Myers, and D. J. Lipman.** 1990. Basic local alignment search tool. J. Mol. Biol. **215**:403–410.
2. **Booth, M. C., C. P. Bogie, H.-G. Sahl, R. J. Siezen, K. L. Hatter, and M. S. Gilmore.** 1996. Structural analysis and proteolytic activation of *Enterococcus faecalis* cytolysin, a novel lantibiotic. Mol. Microbiol. **21**:1175–1184.
3. **Brock, T. D., B. Peacher, and D. Pierson.** 1963. Survey of the bacteriocins of enterococci. J. Bacteriol. **86**:702–707.
4. **Chow, J. W., L. A. Thal, M. B. Perri, J. A. Vazquez, S. M. Donabedian, D. B. Clewell, and M. J. Zervos.** 1993. Plasmid-associated hemolysin and aggregation substance production contribute to virulence in experimental enterococcal endocarditis. Antimicrob. Agents Chemother. **37**:2474–2477.
5. **Clewell, D. B.** 1981. Plasmids, drug resistance, and gene transfer in the genus *Streptococcus*. Microbiol. Rev. **45**:409–436.

6. **Clewell, D. B., and G. M. Dunny.** 2002. Conjugation and genetic exchange in enterococci, p. 265–300. *In* M. S. Gilmore, D. B. Clewell, P. Courvalin, G. M. Dunne, B. E. Murray, and L. B. Rice. (ed.), The enterococci: pathogenesis, molecular biology and antibiotic resistance. ASM Press, Washington, DC.
7. **Clewell, D. B., P. K. Tomich, M. C. Gawron-Burke, A. E. Franke, Y. Yagi, and F. Y. An.** 1982. Mapping of *Streptococcus faecalis* plasmids pAD1 and pAD2 and studies relating to transposition of Tn*917*. J. Bacteriol. **152:**1220–1230.
8. **Coburn, P. S., L. E. Hancock, M. C. Booth, and M. S. Gilmore.** 1999. A novel means of self-protection, unrelated to toxin activation, confers immunity to the bactericidal effects of the *Enterococcus faecalis* cytolysin. Infect. Immun. **67:**3339–3347.
9. **Coburn, P. S., C. M. Pillar, B. D. Jett, W. Haas, and M. S. Gilmore.** 2004. *Enterococcus faecalis* senses target cells and in response expresses cytolysin. Science **306:**2270–2272.
10. **Coque, T. M., J. E. Patterson, J. M. Steckelberg, and B. E. Murray.** 1995. Incidence of hemolysin, gelatinase, and aggregation substance among enterococci isolated from patients with endocarditis and other infections and from feces of hospitalized and community-based persons. J. Infect. Dis. **171:**1223–1229.
11. **Dunny, G. M., B. L. Brown, and D. B. Clewell.** 1978. Induced cell aggregation and mating in *Streptococcus faecalis*: evidence for a bacterial sex pheromone. Proc. Natl. Acad. Sci. USA **75:**3479–3483.
12. **Dunny, G. M., R. A. Craig, R. L. Carron, and D. B. Clewell.** 1979. Plasmid transfer in *Streptococcus faecalis*: production of multiple sex pheromones by recipients. Plasmid **2:**454–465.
13. **Fujimoto, S., H. Hashimoto, and Y. Ike.** 1991. Low cost device for electrotransformation and its application to the highly efficient transformation of *Escherichia coli* and *Enterococcus faecalis*. Plasmid **26:**131–135.
14. **Fujimoto, S., H. Tomita, E. Wakamatsu, K. Tanimoto, and Y. Ike.** 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.
15. **Franz, C. M. A. P., M. J. van Belkum, W. H. Holzapfel, H. Abriouel, and A. Galvez.** 2007. Diversity of enterococcal bacteriocins and their grouping in a new classification scheme. FEMS Microbiol. Rev. **31:**293–310.
16. **Gilmore, M. S., R. A. Segarra, and M. C. Booth.** 1990. An HlyB-type function is required for expression of the *Enterococcus faecalis* hemolysin/bacteriocin. Infect. Immun. **58:**3914–3923.
17. **Gilmore, M. S., R. A. Segarra, M. C. Booth, C. P. Bogie, L. R. Hall, and D. B. Clewell.** 1994. Genetic structure of the *Enterococcus faecalis* plasmid pAD1-encoded cytolytic toxin system and its relationship to lantibiotic determinants. J. Bacteriol. **176:**7335–7344.
18. **Haas, W., B. D. Shepard, and M. S. Gilmore.** 2002. Two-component regulator of *Enterococcus faecalis* cytolysin responds to quorum-sensing autoinduction. Nature **415:**84–87.
19. **Hanski, E., P. A. Horvitz, and M. G. Caparon.** 1992. Expression of protein F, the fibronectin-binding protein of *Streptococcus pyogenes* JRS4, in heterologous streptococcal and enterococcal strains promotes their adherence to respiratory epithelial cells. Infect. Immun. **60:**5119–5125.
20. **Ike, Y., and D. B. Clewell.** 1984. Genetic analysis of the pAD1 pheromone response in *Streptococcus faecalis*, using transposon Tn*917* as an insertional mutagen. J. Bacteriol. **158:**777–783.
21. **Ike, Y., and D. B. Clewell.** 1992. Evidence that the hemolysin/bacteriocin phenotype of *Enterococcus faecalis* subsp. *zymogenes* can be determined by plasmids in different incompatibility groups as well as by the chromosome. J. Bacteriol. **174:**8172–8177.
22. **Ike, Y., D. B. Clewell, R. A. Segarra, and M. S. Gilmore.** 1990. Genetic analysis of the pAD1 hemolysin/bacteriocin determinant in *Enterococcus faecalis*: Tn*917* insertional mutagenesis and cloning. J. Bacteriol. **172:**155–163.
23. **Ike, Y., R. A. Craig, B. A. White, Y. Yagi, and D. B. Clewell.** 1983. Modification of *Streptococcus faecalis* sex pheromones after acquisition of plasmid DNA. Proc. Natl. Acad. Sci. USA **80:**5369–5373.
24. **Ike, Y., S. E. Flannagan, and D. B. Clewell.** 1992. Hyperhemolytic phenomena associated with insertions of Tn*916* into the hemolysin determinant of *Enterococcus faecalis* plasmid pAD1. J. Bacteriol. **174:**1801–1809.
25. **Ike, Y., H. Hashimoto, and D. B. Clewell.** 1984. Hemolysin of *Streptococcus faecalis* subspecies *zymogenes* contributes to virulence in mice. Infect. Immun. **45:**528–530.
26. **Ike, Y., H. Hashimoto, and D. B. Clewell.** 1987. High incidence of hemolysin production by *Enterococcus* (*Streptococcus*) *faecalis* strains associated with human parenteral infection. J. Clin. Microbiol. **25:**1524–1528.
27. **Jack, R. W., J. R. Tagg, and B. Ray.** 1995. Bacteriocins of gram-positive bacteria. Microbiol. Rev. **59:**171–200.
28. **Jedrzejas, M. K.** 2001. Pneumococcal virulence factors: structure and function. Microbiol. Mol. Biol. Rev. **65:**187–207.
29. **Jett, B. D., H. G. Jensen, R. E. Nordquist, and M. S. Gilmore.** 1992. Contribution of the pAD1-encoded cytolysin to the severity of experimental *Enterococcus faecalis* endophthalmitis. Infect. Immun. **60:**2445–2452.
30. **Kleerebezem, M., J. Boekhorst, R. van Kranenburg, D. Molenaar, O. P. Kuipers, R. Leer, R. Tarchini, S. A. Peters, H. M. Sandbrink, M. W. E. J. Fiers, W. Stiekema, R. M. Klein Lankhorst, P. A. Bron, S. M. Hoffer, M. N. Nierop Groot, R. Kerkhoven, M. de Vries, B. Ursing, W. M. de Vos, and R. J. Siezen.** 2003. Complete genome sequence of *Lactobacillus plantarum* WCFS1. Proc. Natl. Acad. Sci. USA **100:**1990–1995.
31. **LeBlanc, D. J., L. N. Lee, D. B. Clewell, and D. Behnke.** 1983. Broad geographical distribution of a cytotoxin gene mediating beta-hemolysis and bacteriocin activity among *Streptococcus faecalis* strains. Infect. Immun. **40:** 1015–1022.
32. **López, R., and E. García.** 2004. Recent trends on the molecular biology of pneumococcal capsules, lytic enzymes, and bacteriophage. FEMS Microbiol. Rev. **28:**553–580.
33. **Martínez-Bueno, M., M. Maqueda, A. Galvez, B. Samyn, J. V. Beeumen, J. Coyette, and E. Valdivia.** 1994. Determination of the gene sequence and the molecular structure of the enterococcus peptide antibiotic AS-48. J. Bacteriol. **176:**6334–6339.
34. **Navarre, W. W., and O. Schneewind.** 1999. Surface proteins of gram-positive bacteria and mechanisms of their targeting to the cell wall envelope. Microbiol. Mol. Biol. Rev. **63:**174–229.
35. **Nes, I. F., D. B. Diep, and H. Holo.** 2007. Bacteriocin diversity in *Streptococcus* and *Enterococcus*. J. Bacteriol. **189:**1189–1198.
36. **Nilsen, T., I. F. Nes, and H. Holo.** 2003. Enterolysin A, a cell wall-degrading bacteriocin from *Enterococcus faecalis* LMG 2333. Appl. Environ. Microbiol. **69:**2975–2984.
37. **Pecenková, T., V. Benes, J. Paces, C. Vlcek, and V. Paces.** 1997. Bacteriophage B103: complete DNA sequence of its genome and relationship to other *Bacillus* phages. Gene **199:**157–163.
38. **Sambrook, J., and D. W. Russell.** 2001. Molecular cloning: a laboratory manual, 3rd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
39. **Segarra, R. A., M. C. Booth, D. A. Morales, M. M. Huycke, and M. S. Gilmore.** 1991. Molecular characterization of the *Enterococcus faecalis* cytolysin activator. Infect. Immun. **59:**1239–1246.
40. **Sheehan, M. M., J. L. García, R. López, and P. García.** 1997. The lytic enzyme of the pneumococcal phage Dp-1: a chimeric lysin of intergeneric origin. Mol. Microbiol. **25:**717–725.
41. **Shiojima, M., H. Tomita, K. Tanimoto, S. Fujimoto, and Y. Ike.** 1997. High-level plasmid-mediated gentamicin resistance and pheromone response of plasmids present in clinical isolates of *Enterococcus faecalis*. Antimicrob. Agents Chemother. **41:**702–705.
42. **Tanimoto, K., and T. Iino.** 1985. Additional genes essential for replication of the mini-F plasmid from origin I. Mol. Gen. Genet. **198:**358–359.
43. **Tettelin, H., V. Masignani, M. J. Cieslewicz, J. A. Eisen, S. Peterson, M. R. Wessels, I. T. Paulsen, K. E. Nelson, I. Margarit, T. D. Read, L. C. Madoff, A. M. Wolf, M. J. Beanan, L. M. Brinkac, S. C. Daugherty, R. T. DeBoy, A. S. Durkin, J. F. Kolonay, R. Madupu, M. R. Lewis, D. Radune, N. B. Fedorova, D. Scanlan, H. Khouri, S. Mulligan, H. A. Carty, R. T. Cline, S. E. Van Aken, J. Gill, M. Scarselli, M. Mora, E. T. Iacobini, C. Brettoni, G. Galli, M. Mariani, F. Vegni, D. Maione, D. Rinaudo, R. Rappuoli, J. L. Telford, D. L. Kasper, G. Grandi, and C. M. Fraser.** 2002. Complete genome sequence and comparative genomic analysis of an emerging human pathogen, serotype V *Streptococcus agalactiae*. Proc. Natl. Acad. Sci. USA **99:**12391–12396.
44. **Tomich, P. K., F. Y. An, and D. B. Clewell.** 1980. Properties of erythromycin-inducible transposon Tn*917* in *Streptococcus faecalis*. J. Bacteriol. **141:**1366–1374.
45. **Tomita, H., and D. B. Clewell.** 2000. A pAD1-encoded small RNA molecule, mD, negatively regulates *Enterococcus faecalis* pheromone response by enhancing transcription termination. J. Bacteriol. **182:**1062–1073.
46. **Tomita, H., S. Fujimoto, K. Tanimoto, and Y. Ike.** 1996. Cloning and genetic organization of the bacteriocin 31 determinant encoded on the *Enterococcus faecalis* pheromone-responsive conjugative plasmid pYI17. J. Bacteriol. **178:** 3585–3593.
47. **Tomita, H., S. Fujimoto, K. Tanimoto, and Y. Ike.** 1997. Cloning and genetic and sequence analysis of the bacteriocin 21 determinant encoded on the *Enterococcus faecalis* pheromone-responsive conjugative plasmid pPD1. J. Bacteriol. **179:**7843–7855.
48. **Tomita, H., and Y. Ike.** 2005. Genetic analysis of transfer-related regions of the vancomycin resistance *Enterococcus* conjugative pHTβ: identification of *oriT* and a putative relaxase gene. J. Bacteriol. **187:**7727–7737.
49. **Tomita, H., C. Pierson, S. K. Lim, D. B. Clewell, and Y. Ike.** 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.
50. **Wirth, R., F. Y. An, and D. B. Clewell.** 1986. Highly efficient protoplast transformation system for *Streptococcus faecalis* and a new *Escherichia coli-S. faecalis* shuttle vector. J. Bacteriol. **165:**831–836.
51. **Wren, B. W.** 1991. A family of clostridial and streptococcal ligand-binding proteins with conserved C-terminal repeat sequences. Mol. Microbiol. **5:**797–803.

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Identification and Characterization of Two Novel Methyltransferase Genes That Determine the Serotype 12-Specific Structure of Glycopeptidolipids of *Mycobacterium intracellulare*▽

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The *Mycobacterium avium* complex is distributed ubiquitously in the environment. It is an important cause of pulmonary and extrapulmonary diseases in humans and animals. The species in this complex produce polar glycopeptidolipids (GPLs); of particular interest is their serotype-specific antigenicity. Several reports have described that GPL structure may play an important role in bacterial physiology and pathogenesis and in the host immune response. Recently, we determined the complete structure of the GPL derived from *Mycobacterium intracellulare* serotype 7 and characterized the serotype 7 GPL-specific gene cluster. The structure of serotype 7 GPL closely resembles that of serotype 12 GPL, except for O methylation. In the present study, we isolated and characterized the serotype 12-specific gene cluster involved in glycosylation of the GPL. Ten open reading frames (ORFs) and one pseudogene were observed in the cluster. The genetic organization of the serotype 12-specific gene cluster resembles that of the serotype 7-specific gene cluster, but two novel ORFs (*orfA* and *orfB*) encoding putative methyltransferases are present in the cluster. Functional analyses revealed that *orfA* and *orfB* encode methyltransferases that synthesize *O*-methyl groups at the C-4 position in the rhamnose residue next to the terminal hexose and at the C-3 position in the terminal hexose, respectively. Our results show that these two methyltransferase genes determine the structural difference of serotype 12-specific GPL from serotype 7-specific GPL.

The *Mycobacterium avium* complex (MAC) consists of two species, *M. avium* and *Mycobacterium intracellulare*, which are opportunistic pathogens of humans and animals. Human exposure to the MAC is common because organisms of this complex are ubiquitous in the environment: they have been isolated from water, soil, plants, house dust, and other sources. In fact, the MAC is the most common cause of disease attributable to nontuberculous mycobacteria in humans (9). The majority of MAC infections are acquired environmentally, and person-to-person transmission is considered to be rare. The treatment of MAC infection is difficult because the organisms are often resistant to standard antituberculosis drugs.

Many antigenic or immunoregulatory glycolipids with structural diversity are expressed on the mycobacterial cell wall. These molecules are considered to be involved in bacterial virulence through host immune responses (5, 14, 22, 23). It is necessary to elucidate the molecular structure, biochemical characteristics, and biological functions of the lipid components to better understand the mechanisms of pathogenesis and drug resistance of the MAC. The most prominent feature of the MAC is the presence of antigenic glycolipids, the glycopeptidolipids (GPLs), which are present on the cell surface (1). The standard method for differentiation of MAC strains is serologic typing based on the oligosaccharide (OSE) residue of the GPL. GPLs contain a tetrapeptide-amino alcohol core, D-phenylalanine-D-*allo*-threonine-D-alanine-L-alaninol (D-Phe-D-*allo*-Thr-D-Ala-L-alaninol), with an amido-linked 3-hydroxy or 3-methoxy C_{26}-to-C_{34} fatty acid at the N terminus of D-Phe (4). The D-*allo*-Thr and terminal L-alaninol are further linked with 6-deoxy-talose (6-d-Tal) and 3,4-di-*O*-methyl-rhamnose (3,4-di-*O*-Me-Rha), respectively. This core GPL is present in all species of the MAC and shows a common antigenicity (1). In the serotype-specific GPLs, a haptenic OSE is linked with the 6-d-Tal residue. To date, 31 distinct serotype-specific polar GPLs have been identified biochemically; the complete structures of GPLs are partly defined for serotype 1 to 4, 7, 8, 9, 12, 14, 17, 19 to 21, 25, and 26 GPLs (7, 10). On the other hand, it has been reported that serotype-specific GPLs participate in pathogenesis and immunomodulation in the host (2, 13). Modification of the GPL structure might play an important role not only in antigenicity but also in host immune responses and bacterial physiology (18). Recently, chemical synthesis of various haptenic OSEs was demonstrated, and the genes encoding glycosylation pathway enzymes for the biosynthesis of GPLs were identified and characterized (8, 12, 19, 21). However, genes responsible for serotype-specific glycosylation have yet to be analyzed for most of the serotypes.

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