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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

YASUYOSHI IKE[1]* AND DON B. CLEWELL[2,3]

Department of Microbiology, School of Medicine, Gunma University, Maebashi, Japan,[1] and Department of Biologic and Materials Sciences, School of Dentistry,[2] and Department of Microbiology-Immunology, School of Medicine,[3] The University of Michigan, Ann Arbor, Michigan 48109

Received 21 April 1992/Accepted 13 October 1992

The hemolysin (Hly/Bac) determinant in strains of *Enterococcus faecalis* was found to be present on plasmids in different incompatibility groups (conferring different sex pheromone responses) as well as on the chromosome. Of 33 Hly/Bac plasmids identified in clinical isolates, the related pheromone for 30 was cAD1; the related pheromone for another two (pYI1 and pYI3) or one (pYI2) was cOB1 or cYI2, respectively. The representative Hly/Bac plasmids pAD1, pYI1, pOB1, and pYI2, which responded to pheromones cAD1, cOB1, cOB1, and cYI2, respectively, were compatible with one another. As additions to the incompatibility group IncHly of pAD1, groups for pOB1, pYI1, and pYI2 were designated IncHlyII, IncHlyIII, and IncHlyIV, respectively. Eleven of the 30 plasmids conferring a response to cAD1 were very similar to pAD1 on the basis of their restriction endonuclease profiles. *Eco*RI fragment D, F, or H containing parts of the Hly/Bac gene(s) of pAD1 hybridized to similar *Eco*RI fragments from each of the other three representatives of incompatibility groups (i.e., pOB1, pYI1, and pYI2) and to homologous DNA representing the chromosome of the plasmid-free Hly/Bac strain YI6-1.

Enterococcus faecalis subsp. *zymogenes* is distinguished from other *E. faecalis* strains by its production of a cytotoxin able to lyse human, rabbit, and horse erythrocytes. Strains producing this beta-hemolysin also produce a bacteriocin, and the hemolysin and bacteriocin are mediated by the same genetic determinant (1, 3, 4, 25).

The Hly/Bac determinants examined to date have been associated with a conjugative plasmid which usually transfers in broth matings at a frequency of 10^{-3} to 10^{-1} within a few hours (4, 13, 24, 29, 34). The Hly/Bac plasmids examined so far bear structural similarities to the conjugative plasmid pAD1 (24, 34). pAD1 is a 59.6-kb plasmid originally identified in *E. faecalis* DS16 (39), and it belongs to the incompatibility class IncHly (13). The pAD1 *hly/bac* determinant was previously shown to contribute to pathogenicity in a mouse model (28). In addition to Hly/Bac synthesis, pAD1 has been previously shown to confer both resistance to UV light (8) and a conjugative mating response to the peptide sex pheromone cAD1 secreted by recipient cells (5, 12, 14, 16).

All of the Hly/Bac plasmids examined so far belong to the same incompatibility group (13), and pAD1 is representative of this group. It has not been reported whether Hly/Bac can be conferred by different replicons with respect to incompatibility functions or pheromone responses, nor has it been found to be expressed from the chromosome. In this report, we show that *hly/bac* can indeed be found on different types of plasmids as well as on the bacterial chromosome.

Pheromone response of Hly/Bac plasmids. *E. faecalis* recipients secrete multiple peptide pheromones, each specific for a donor harboring a related pheromone-responding plasmid. Once a plasmid is acquired by the recipient, secretion of the related pheromone ceases, whereas other pheromones (unrelated) continue to be produced. If a donor strain responds to a culture filtrate of the recipient strain FA2-2 but does not respond (aggregate) to a culture filtrate of FA2-2(pAD1), the related pheromone for that plasmid is probably cAD1, the pAD1-specific pheromone.

Strains and plasmids used in this study are listed in Table 1. More than 50% of hemolytic clinical isolates of *E. faecalis* were previously found to carry transferable hemolysin determinants (24, 29). Pheromone responses (aggregation) of the plasmids were examined as previously described (14, 16). Thirty-three conjugative hemolysin plasmids isolated from those clinical strains were introduced into *E. faecalis* FA2-2 or OG1-10, where they were found to exhibit an aggregation response to culture filtrates of FA2-2; however, only three strains responded to culture filtrates of FA2-2(pAD1). Thus, the related pheromone for 30 (about 90%) of the hemolysin plasmids appears to be cAD1. The strains containing pYI1, pYI3, or pYI2, which responded to culture filtrates of FA2-2(pAD1), were examined with regard to their responses to culture filtrates of one another. The Hly/Bac plasmid pOB1, which responds to the pheromone cOB1, was included in the experiment (5, 35). FA2-2 or OG1-10 strains carrying pYI1, pYI3, or pOB1 did not respond to culture filtrates of FA2-2 strains carrying any one of these plasmids, but they did respond to culture filtrates of FA2-2(pYI2). This implies that pYI1, pYI3, and pOB1 respond to the same pheromone and that cOB1 is the representative peptide. OG1-10(pYI2) responded to culture filtrates of FA2-2 carrying pYI1, pYI3, or pOB1, indicating that the related pheromone was different from cAD1 and cOB1; the pheromone was therefore designated cYI2. These results indicate that there are at least three different Hly/Bac plasmid groups with respect to their pheromone responses.

* Corresponding author.

TABLE 1. Bacterial strains and plasmids

Strain or plasmid	Genotype or phenotype	Plasmid content	Description, source, or reference(s)
Strains			
E. faecalis			
FA2-2	*rif fus*	None	Derivative of JH2 (11)
JH2SS	*str spc*	None	Derivative of JH2 (38)
OG1X	*str*	None	Gelatinase-negative mutant of OG1-10 (26)
DS16C1	*tet*	pAD2[Sm Km Em(Tn*917*)]	DS16 cured of pAD1 (19)
YI6	*tet hly/bac*	pYI6(Sm Em, 10 kb)	This study
YI6-1	*tet hly/bac*	None	YI6 cured of pYI6
UV202	*rif fus*	None	UV-sensitive mutant of JH2-2 (40)
Plasmids			
pAD1	*hly/bac*		59.6-kb conjugative plasmid from DS16 (11, 17, 23, 39)
pAM714	*hly/bac erm*		pAD1::Tn*917*, wild-type Hly/Bac and transfer (23)
pAM210	*hly/bac tet*		pAD1::Tn*916*, hyper hemolysin and transfer (18)
pOB1	*hly/bac*		64.7-kb conjugative plasmid (5, 35)
pYI1	*hly/bac*		57.5-kb conjugative plasmid (this study)
pYI2	*hly/bac*		56-kb conjugative plasmid (this study)
pGL101	Ap^{r}[a]		Derivative of pBR322
pAM120LT	Ap^{r}		pGL101 carrying *Eco*RI fragment F of pAD1 (22)
pAM170LT	Ap^{r}		pGL101 carrying *Eco*RI fragment D of pAD1 (22)
pAM160LT	Ap^{r}		pGL101 carrying *Eco*RI fragment H of pAD1 (8)

[a] Ap^{r}, ampicillin resistant.

Structure of Hly/Bac plasmids. Eleven plasmids that responded to cAD1 (Table 2), as well as pOB1, pYI1, and pYI2, were chosen for structural analyses. Figure 1a shows *Eco*RI restriction profiles of each of the plasmids responding to cAD1. The plasmids exhibited *Eco*RI profiles similar to that of pAD1. Nine of the plasmids were essentially indistinguishable from pAD1. The plasmids also have *Sal*I restriction profiles similar to that of pAD1 (three fragments each, except for pMG709) (Fig. 1b). Figure 2a shows *Eco*RI restriction profiles of pOB1, pYI1, and pYI2. Plasmid pYI1 (lane 3) exhibited an *Eco*RI profile similar to that of pAD1 (lane 5). pOB1 (lane 1) and pYI2 (lane 2) had *Eco*RI digestion patterns that were very different from that of pAD1. pOB1 and pYI2 had only two *Eco*RI fragments (i.e., F and H) and three *Eco*RI fragments (i.e., C, F, and H), respectively, identical to those of pAD1.

pAD1, pMG711, pYI1, pYI2, and pOB1 were chosen for homology studies with the cloned pAD1 *Eco*RI fragment D, F, or H, each of which contained a part of the Hly/Bac determinant of pAD1. The fragments F and H each contained only a segment of the Hly/Bac gene(s), whereas about 1.5 kb of the 4.1-kb D fragment contained part of the Hly/Bac determinant (25). Each of the three probes hybridized to specific *Eco*RI fragments from each of the Hly/Bac plasmids (Fig. 2). In all cases, fragments homologous to the *Eco*RI fragments F and H also appeared to be identical in

TABLE 2. Phenotypes of representative strains used in structural analyses of hemolysin plasmids that responded to cAD1[a]

Hly plasmid examined[b]	Transfer frequency of Hly plasmid from FA2-2 to JH2SS	Phenotype of wild-type strains in which Hly plasmid was identified[c]	Source[d]
pMG701	3×10^{-2}	Hly (7, 11.6%)	J
pMG702	2×10^{-2}	Hly Tc Sm (2, 3.3%)	J
pMG703	10^{-1}	Hly Tc Cm Em Km Gm (11, 18.3%)	G
pMG704	10^{-2}	Hly Tc Cm Em Km Gm	I
pMG705	4×10^{-2}	Hly Tc Cm Em Km Gm	G
pMG706	10^{-2}	Hly Tc Km Gm (1, 1.7%)	I
pMG707	2×10^{-2}	Hly Tc Cm Sm (1, 1.7%)	I
pMG708	10^{-1}	Hly Tc Cm (2, 3.3%)	I
pMG709	5×10^{-2}	Hly Tc Cm	G
pMG710	10^{-1}	Hly Tc (12, 20%)	I
pMG711	2×10^{-1}	Hly	G

[a] Sixty hemolytic strains were isolated from patients in three geographically dispersed hospitals in Japan and were reported previously (24, 29). Of the 60 strains, 27 were from Gunma University Hospital (Maebashi City), 31 were from Isesaki City Hospital (Isesaki City), and 2 were from Jikei University Hospital (Tokyo). Representatives of these strains examined in detail are listed in this table. Antibiotic concentrations used in selective plates were as follows (in micrograms per milliliter): erythromycin (Em), 25; streptomycin (Sm), 500; spectinomycin, 500; kanamycin (Km), 500; gentamicin (Gm), 200; tetracycline (Tc), 6; rifampin, 25; and fusidic acid, 25. Hemolysin (Hly) detection was on Todd-Hewitt agar (Difco Laboratories, Detroit, Mich.) containing 4% horse blood (Toyo Serum Co., Tokyo, Japan). For the transfer of hemolytic properties, broth matings were carried out as previously described (24, 29).

[b] The hemolysin plasmids are not associated with drug resistance.

[c] All strains came from separate patients. All hemolytic properties transferred to FA2-2 at a frequency of about 10^{-3} per donor. Transconjugants acquiring only hemolytic properties were found in all of the matings, indicating that the hemolytic determinant is not linked to the drug resistance determinant (24). Numbers and percentages of strains representing this phenotype from among 60 Hly/Bac strains are shown in parentheses.

[d] J, Jikei University Hospital; G, Gunma University Hospital; I, Isesaki City Hospital.

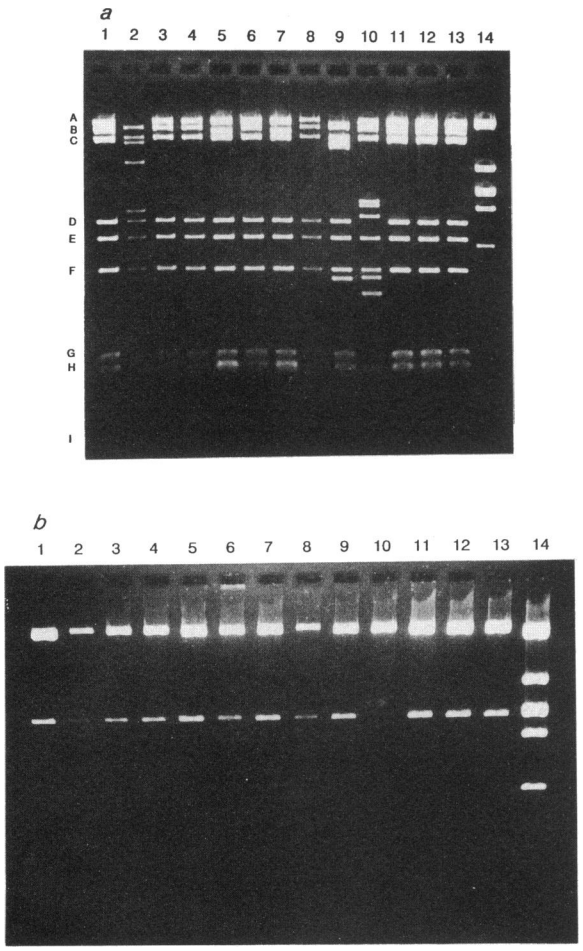

FIG. 1. (a) Agarose gel electrophoresis of *Eco*RI restriction fragments of Hly/Bac plasmids that responded to cAD1. Plasmid DNA was isolated by a small-scale alkaline lysis method described previously (17, 37). Lanes: 1 and 13, pAD1 of *Eco*RI fragments A through I with corresponding molecular sizes of 19.3, 15.4, 12.0, 4.1, 3.4, 2.7, 1.5, 1.2, and 0.2 kb (10, 17, 24); 14, λ DNA; 2 through 12, pMG701 through pMG711. (b) Agarose gel electrophoresis of *Sal*I restriction fragments of Hly/Bac plasmids that responded to cAD1. Lanes: 14, *Eco*RI fragments of λ DNA; 1 and 13, pAD1 fragments; 2 through 12, pMG701 through pMG711.

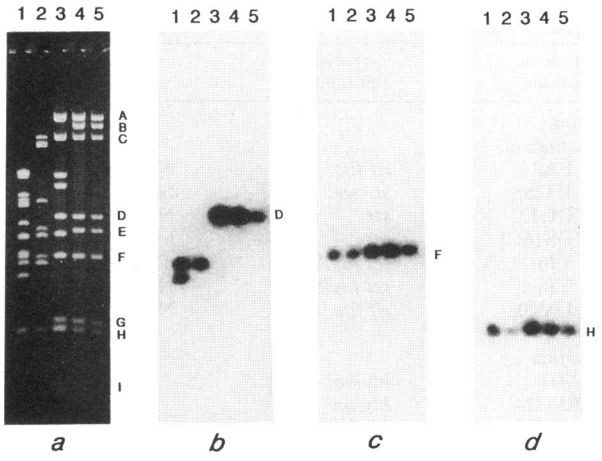

FIG. 2. Hybridization of cloned pAD1 *Eco*RI fragment D (pAM170LT), F (pAM120LT), or H (pAM160LT) to *Eco*RI fragments from Hly/Bac plasmids of pOB1, pYI2, pYI1, pMG711, or pAD1. (a) Agarose gel electrophoresis of *Eco*RI fragments from pOB1 (lane 1), pYI2 (lane 2), pYI1 (lane 3), pMG711 (lane 4), or pAD1 (lane 5). The preparation of ^{32}P-labeled DNA probes made use of a Nick Translation Kit 2 (Nippon Gene, Toyama, Japan). Duplicate gels were Southern blotted onto Biodyne A Nylon membrane (Pall Co., Glen Cove, N.Y.) and hybridized (37) to ^{32}P-labeled cloned pAD1 *Eco*RI fragment D (b), *Eco*RI fragment F (c), and *Eco*RI fragment H (d).

size to their respective fragments. In the case of pMG711 and pYT1, fragments homologous to *Eco*RI fragment D were identical in size to *Eco*RI fragment D of pAD1, but in the case of pYI2 and pOB1, they were smaller than *Eco*RI fragment D of pAD1. These results indicate that determinants encoded in *Eco*RI fragment D, F, or H of pAD1 were conserved in the Hly/Bac plasmids of clinical isolates.

Incompatibility of Hly/Bac plasmids. To examine the incompatibility properties of the Hly/Bac plasmids, each of the Hly/Bac plasmids was tagged with transposon Tn*917*(Em) or Tn*916*(Tc) to distinguish incoming and resident plasmids in new transconjugants. Matings between donors and recipients carrying distinguishable Hly/Bac plasmids were performed (Table 3). The frequency of plasmid transfer to a recipient cell already carrying an isogenic plasmid was lower than that when the recipient was plasmid free. Transfer frequencies to recipient cells carrying heterogenic plasmids were as high as those when the recipients were plasmid free. For mating experiments with cells carrying pYI1 and pOB1, which respond to the same pheromone, the transfer frequency of each was somewhat lower than that when the recipient was plasmid free. Drug resistance in transconjugants derived from the mating experiments with cells carrying heterogenic plasmids was examined. After 30 transconjugants derived from each mating experiment were purified, they all remained erythromycin and tetracycline resistant. When the transconjugants were tested as donors in subsequent matings, the two markers were found to be unlinked (data not shown). These results indicate that pAD1, pOB1, pYI1, and pYI2 are able to coexist and belong to different incompatibility groups. Mating experiments using the plasmid pMG711 (one of the plasmids responding to cAD1) were also performed, and the transfer of pMG711::Tn*917* from FA2-2 to JH2SS carrying each of the plasmids indicated in Table 3 was similar to that with pAM714 (data not shown).

In the mating experiments with cells carrying isogenic plasmids, aggregation-mating functions were not observed; this was expected, since the related pheromone was shut down (6, 10, 26). To test the transferability of the plasmid to the recipient cells and the incompatibility of the isogenic plasmids, the donor cells were exposed (45 min) to an FA2-2 culture filtrate (pheromone) to induce aggregation-mating functions before a short (20-min) mating period. The short matings were between the induced donor cells and the uninduced recipient cells. Transfer frequencies of the induced plasmids to the uninduced recipient cells carrying isogenic plasmids were about 10^{-5} to 10^{-6} per donor in each mating experiment (Table 4), and the frequencies were about 2 orders of magnitude lower than those when the recipients were plasmid free (7). Fifty transconjugants derived from each mating experiment were examined for their drug resistance. All transconjugants were erythromycin resistant (conferred by the incoming plasmid), but they lost tetracycline resistance (encoded by the resident plasmid), reflecting the

TABLE 3. Transfer frequencies of Hly/Bac plasmids from donor strains to recipients carrying Hly/Bac plasmids[a]

Plasmid from donor cells of JH2SS	Transfer frequency to recipient cells of FA2-2 carrying the following plasmid:				
	pAM210(Tc)	pOB1::Tn*916*(Tc)	pYI1::Tn*916*(Tc)	pYI2::Tn*916*(Tc)	None
pAM714(Em)	$<10^{-7}$	2.8×10^{-2}	2×10^{-3}	2.8×10^{-2}	2.8×10^{-2}
pOB1::Tn*917*(Em)	2.8×10^{-2}	$<10^{-7}$	2×10^{-5}	2.8×10^{-2}	8×10^{-3}
pYI1::Tn*917*(Em)	8×10^{-3}	2.6×10^{-5}	9×10^{-7}	2.8×10^{-2}	9×10^{-3}
pYI2::Tn*917*(Em)	2×10^{-2}	4×10^{-2}	3×10^{-2}	2×10^{-7}	1×10^{-2}

[a] Hly/Bac plasmids tagged with an erythromycin resistance marker were constructed by transferring Hly/Bac plasmids into strain DS16C1 carrying the nonconjugative pAD2 and selecting for transposition of Tn*917* from pAD2 as described by Tomich et al. (38). Hly/Bac plasmids tagged with a tetracycline resistance determinant were constructed by first transferring the Hly/Bac plasmids into strain OG1X::Tn*916* and then using the plasmid-containing OG1X::Tn*916* derivatives as donors in filter matings with recipient strain FA2-2, selecting for transconjugants that were tetracycline resistant and hyperhemolytic as described previously (9, 18, 19, 27). Overnight cultures of donor and recipient strains were used. Broth matings were carried out as described previously (15, 32). The mixtures were incubated at 37°C with gentle agitation for 4 h and then vortexed. Portions of the mixtures were then plated on Todd-Hewitt agar plates containing erythromycin, rifampin, and fusidic acid for selection of transconjugants.

TABLE 4. Pheromone-mediated plasmid transfer between *E. faecalis* strains carrying Hly/Bac plasmid[a]

Donor and induction	Recipient	No. of transconjugants/donor cell[b]
JH2SS[pAM714(Em)]		
−	FA2-2	$<1 \times 10^{-7}$
+	FA2-2	7×10^{-3}
−	FA2-2[pAM210(Tc)]	$<1 \times 10^{-7}$
+	FA2-2[pAM210(Tc)]	3×10^{-5}
JH2SS[pOB1::Tn*917*(Em)]		
−	FA2-2	$<1 \times 10^{-7}$
+	FA2-2	3×10^{-4}
−	FA2-2[pOB1::Tn*916*(Tc)]	$<1 \times 10^{-7}$
+	FA2-2[pOB1::Tn*916*(Tc)]	3×10^{-6}
JH2SS[pYI1::Tn*917*(Em)]		
−	FA2-2	$<1 \times 10^{-7}$
+	FA2-2	1×10^{-2}
−	FA2-2[pYI1::Tn*916*(Tc)]	$<1 \times 10^{-7}$
+	FA2-2[pYI1::Tn*916*(Tc)]	7×10^{-5}
JH2SS[pYI2::Tn*917*(Em)]		
−	FA2-2	$<5 \times 10^{-7}$
+	FA2-2	3×10^{-4}
−	FA2-2[pYI2::Tn*916*(Tc)]	$<3 \times 10^{-7}$
+	FA2-2[pYI2::Tn*916*(Tc)]	4×10^{-6}
JH2SS[pOB1::Tn*917*(Em)]		
−	FA2-2[pYI1::Tn*916*(Tc)]	$<1 \times 10^{-7}$
+	FA2-2[pYI1::Tn*916*(Tc)]	2×10^{-4}
JH2SS[pYT1::Tn*917*(Em)]		
−	FA2-2[pOB1::Tn*916*(Tc)]	$<1 \times 10^{-7}$
+	FA2-2[pOB1::Tn*916*(Tc)]	2×10^{-2}

[a] Pheromone induction and the mating experiments were performed as previously described (6). A freshly prepared single colony of each strain was inoculated in N2GT broth, and the strain was grown overnight at 37°C. Using fresh overnight cultures grown in N2GT broth, we diluted each strain separately 1:20 in fresh broth and grew it to late log phase (about 5×10^8 cells per ml). The culture to be induced (+) was then diluted 1:10 into a 1:1 mixture of fresh broth and culture filtrate of the plasmid-free strain FA2-2. In parallel, the other, uninduced culture (−) was diluted 1:10 into fresh broth. Both cultures were then incubated (37°C) for 45 min (with shaking), after which time equal volumes of each were mixed together to give a 1:1 ratio of donor to recipient cells. The mixture was incubated (with shaking) for 20 min at 37°C and then blended in a Vortex mixer and plated on selective medium. Todd-Hewitt broth selective agar plates containing rifampin (25 μg/ml), fusidic acid (25 μg/ml), and erythromycin (25 μg/ml) were used for the selection of incoming erythromycin resistance plasmids into FA2-2 recipients.

[b] Seven independent experiments were performed and showed similar results. Values show the representative results of the experiments.

incompatibility of the plasmids. The lower transfer frequencies are assumed to be due to a preference for the resident plasmid (7).

For mating experiments with cells carrying pYI1 and pOB1, which respond to the same pheromone and were compatible, the transfer frequencies were intermediate relative to the frequencies of compatible or incompatible mating experiments with the controls (Table 3). The transfer frequencies of the short matings between the pheromone-induced donor cells and the uninduced recipient cells were as high as those when the recipients were plasmid free (Table 4). The intermediate transfer frequencies may be due to the related pheromones being mostly, but not completely, shut down; the incoming plasmids were not excluded by the compatible resident plasmid.

UV resistance and pheromone inhibitor. pAD1 bears a determinant for resistance to UV light (8, 40). This trait is most easily observed when the plasmid is located in a UV-sensitive host such as *E. faecalis* UV202 (8). pYI1, pYI2, pOB1, and each of the 11 plasmids that responded to cAD1 were transferred to strain UV202. UV202 strains containing any 1 of the 11 plasmids responding to cAD1 or pYI1 exhibited the UV resistance phenotype, whereas this was not the case with strains harboring pYI2 or pOB1.

Cells harboring pAD1 specifically shut down production of endogenous cAD1, and they also secrete a plasmid-encoded peptide, iAD1, which acts as a competitive inhibitor of cAD1 (6, 10, 26). Inhibitor activity was therefore examined as described previously (10, 26) in culture filtrates of FA2-2 carrying pYI1, pYI2, or pOB1. A culture filtrate of FA2-2(pYI2) secreted an inhibitor (8 U) to its own pheromone activity; however, inhibitor activity was not detected in culture filtrates of FA2-2(pYI1) or FA2-2(pOB1).

Hly/Bac determinant present on the chromosome. About half (26 strains) of the Hly/Bac strains of *E. faecalis* clinical isolates did not conjugatively transfer the hemolytic trait in broth matings (24, 29). The hemolysin determinant in these strains may exist on a nontransferable plasmid or on the chromosome. Seventeen of the 26 strains exhibited induced clumping upon exposure to the FA2-2 culture filtrate. It is possible that the clumping responses are due to coresident plasmids. A nonclumping strain, YI6, was selected for further study. In addition to having the Hly/Bac phenotype, *E. faecalis* YI6 was resistant to erythromycin, streptomycin, and tetracycline and harbored a 10-kb plasmid. An erythromycin- and streptomycin-sensitive derivative (YI6-1) was isolated from YI6 by ethidium-bromide treatment and penicillin screening. The 10-kb plasmid DNA was not detected in YI6-1 by agarose gel electrophoresis, indicating that it had

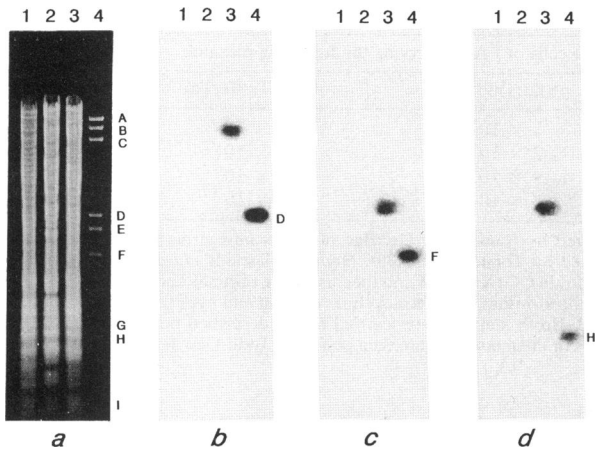

FIG. 3. Hybridization of cloned pAD1 *Eco*RI fragment D (pAM170LT), F (pAM120LT), or H (pAM160LT) to *Eco*RI-digested chromosomal DNAs of *E. faecalis* YI6-1, OG1X, or FA2-2. Chromosomal DNAs were isolated by the Sarkosyl lysate procedure and then subjected to CsCl-ethidium bromide equilibrium centrifugation as previously described (11). (a) Agarose gel electrophoresis of *Eco*RI-digested FA2-2 (lane 1), OG1X (lane 2), YI6-1 (lane 3), or pAD1 (lane 4). Duplicate gels were Southern blotted onto Biodyne A Nylon membrane (Pall Co.) and hybridized (37) to ^{32}P-labeled cloned pAD1 *Eco*RI fragment D (b), F (c), or H (d).

been lost by the curing conditions. Erythromycin- and streptomycin-resistant transformants of YI6-1 were isolated by electroporation with plasmid DNA from YI6 (20). These results indicate that erythromycin and streptomycin resistance was encoded by the 10-kb plasmid of YI6.

We radioactively labeled DNA in experiments in an effort to rule out the presence of other plasmids in YI6-1 (30). YI6-1 and YI6 were labeled with [^{3}H]thymidine (5 μCi/ml) and [^{14}C]thymidine (3 μCi/ml), respectively, in 5 ml of broth culture. Equal volumes of each culture were mixed, and whole-cell lysate was prepared by the Sarkosyl lysate method and analyzed by cesium chloride-ethidium bromide equilibrium centrifugation. After the DNAs were fractionated, the radioactivity of covalently closed circular DNA was detected in YI6 but not in YI6-1 (data not shown). These results indicate that YI6-1 was free of covalently closed circular DNA molecules and that the Hly/Bac and tetracycline determinants of YI6-1 were located on the chromosome.

The hemolysin of YI6-1 was active on erythrocytes of horses, humans, and rabbits, as with the activity conferred by pAD1. YI6-1 and FA2-2(pAD1) also exhibited immunity to each other's bacteriocin activity.

In addition, when *Eco*RI fragments D, F, and H of pAD1 were used as probes to detect their related determinants in the YI6-1 chromosome, all hybridized to specific *Eco*RI fragments (Fig. 3). The F and H fragments appeared to hybridize to the same *Eco*RI fragment, which had a size of about 4.4 kb, whereas the fragment revealed with the D probe was much larger (13.3 kb).

Concluding comments. The hemolytic clinical isolates in the present study originated from patients in Japan. The fact that the majority (90%) of those that exhibited a pheromone response contained plasmids closely resembling pAD1, which originated in the United States and was previously found to be closely related to certain Hly/Bac plasmids such as pJH2 (31) and pIP964 (2), which were identified in Europe, illustrates the global level of dissemination of this highly conjugative system. These elements encoded a response to the sex pheromone cAD1, since they did not exhibit a clumping response when exposed to filtrates of FA2-2 strains harboring pAD1. They are also presumed to be in the same incompatibility group, which has been designated IncHly, as pAD1 (39), pJH2 (31), pAMγ1 (15), and pIP964 (2), by Colmar and Horaud (13). Three other plasmids, pYI1, pYI2, and pYI3, were found to respond to different sex pheromones. pYI1 and pYI3 were found to respond to cOB1, which was previously found to be the pheromone to which the Hly/Bac plasmid pOB1 responds (12, 35). pYI2 responded to filtrates of FA2-2 harboring either pAD1 or pOB1; thus, it recognized still another pheromone, which is here designated cYI2. Different incompatibility groups were also identified and designated Inc HlyII (pOB1), IncHlyIII (pYI1), and IncHlyIV (pYI2). Members of the different incompatibility groups had significantly different restriction endonuclease profiles.

Of the 60 clinical isolates examined, 9 were found to not exhibit a clumping response when exposed to a culture filtrate of *E. faecalis* FA2-2. At least one of these strains appeared to have the *hly/bac* determinant on the chromosome. It is conceivable that other strains among this group could have chromosome-borne *hly/bac* determinants. Alternatively, some could have the determinant on a nonconjugative plasmid or a conjugative plasmid that does not exhibit a pheromone response. There are many plasmids in enterococci that do not confer a pheromone response but do transfer reasonably well on solid media (4). Plasmids from the four incompatibility groups were shown to have significantly homologous *hly/bac* determinants, and the chromosome-borne determinant was also closely related. The fact that each of the determinants conferred immunity to the related bacteriocin activity further attests to the close relationship.

There are probably numerous ways by which the *hly/bac* determinant could locate itself on different replicons. Recombination between various plasmids or between a given plasmid and the chromosome could ultimately give rise to a variety of different locations for the determinant. There is currently no evidence that the *hly/bac* determinant is located on a transposon, although the possibility that in some instances this could be the case has not been ruled out. For the pAD1-related plasmids, the replicon appears to be a highly evolved system whose component parts function efficiently in concert to enable its extensive dissemination. The bacteriocin may play an important role, in that recipients which do not take up the plasmid and acquire immunity are killed. As previously noted, there is evidence that the hemolysin contributes to pathogenicity in an animal model (28). Also, a report by Galli et al. (21) relating to pAD1 has shown that the aggregation substance induced by sex pheromones contains two copies of a motif (RGD) that binds to the integrin family of eukaryotic proteins. Very recently, expression of the aggregation substance was reported to enhance the adherence of bacteria to renal tubular epithelial cells (33). The expression of this protein as well as the entire conjugation system is also subject to phase variation (36); thus, it can sometimes be expressed in the absence of a pheromone. It is therefore likely that pAD1-like plasmids have at least two determinants that contribute to pathogenicity.

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