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# Broad Geographical Distribution of a Cytotoxin Gene Mediating Beta-Hemolysis and Bacteriocin Activity Among *Streptococcus faecalis* Strains

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Conjugative hemolysin-bacteriocin plasmids were isolated from *Streptococcus faecalis* var. *zymogenes* strains of diverse geographical origins. Cloned DNA fragments containing the hemolysin-bacteriocin gene(s) from one of these plasmids (pAD1) hybridized to two *Eco*RI fragments of identical size from each of the five plasmids examined. Results of hybridization experiments in which total plasmid DNA was used suggested that the plasmids shared extensive homology. Two of the plasmids, pAD1 from strain DS16 (Ann Arbor, Mich.) and pAMγ1 from strain DS5 (Miami, Fla.), were 100% homologous and had identical *Eco*RI restriction patterns (eight fragments each). There was no detectable homology between the plasmid-mediated hemolysin determinants of *S. faecalis* and DNA from other beta-hemolytic streptococci (Lancefield groups A, B, F, or H).

Bacterial hemolysin production, recognized by the ability of a producer strain to cause zones of clearing on blood agar plates, is common among gram-positive and gram-negative species (4). A number of exotoxins produced by pathogenic *Clostridium*, *Staphylococcus*, and *Streptococcus* spp. also exhibit hemolysin activity (27). Although hemolysis is not generally considered to be essential for pathogenesis, the hemolysin molecule may lyse other cells, such as leukocytes, resulting in lowered host resistance (27). Recently, a cloned hemolysin determinant of *Escherichia coli* was shown to enhance virulence in an animal model (26). Bacteriocin production has also been described in a wide range of gram-positive and gram-negative bacteria. These small polypeptide antibiotics are thought to provide the producer strain with a selective advantage over other related strains by killing them (4). Bacteriocin and hemolysin production is often mediated by bacterial plasmids (4).

The production of a cytotoxin, active in the lysis of human, rabbit, and horse erythrocytes, distinguishes the *zymogenes* variety of *Streptococcus faecalis* (Lancefield group D) from other *S. faecalis* strains. This beta-hemolysin appears to be identical to a distinct bacteriocin, type I of Brock and Davie (2), that is active against a wide variety of gram-positive bacteria, including most *Streptococcus* spp. (3). Thus, *S. faecalis* var. *zymogenes* is unique among bacteriocin- and hemolysin-producing bacterial species in that both activities may be mediated by the same genetic determinant. In all cases studied thus far, the hemolysin-bacteriocin (Hly-Bcn) activity of *S. faecalis* strains has been shown to reside on a conjugative plasmid (1, 7).

In this communication, we address the molecular relatedness of Hly-Bcn determinants associated with plasmids from clinical *S. faecalis* isolates of diverse geographical origin. Additional homologies among the plasmids were also examined. No homology was detected between the Hly-Bcn determinants of *S. faecalis* (group D) and DNA from other beta-hemolytic streptococcal species.

## MATERIALS AND METHODS

The beta-hemolytic clinical isolates of *S. faecalis* var. *zymogenes* used in this study are described in Table 1. The properties of other *S. faecalis* strains, used as sources of purified plasmid or total cell DNA, are listed in Table 2. Culture conditions were as previously described (20).

The cloning of Hly-Bcn-specific DNA from plasmid pAD1 in *E. coli* and the separation and isolation of cloned fragments from vector DNA will be described elsewhere (manuscript in preparation).

Plasmid isolation, purification of total cell DNA, restriction endonuclease digestion and agarose gel electrophoresis, DNA blotting and hybridization, and the preparation of $^{32}$P-labeled DNA probes were all carried out as previously described (20).

TABLE 1. Clinical isolates of *S. faecalis* var. *zymogenes* used in this study

| Strain | Geographical origin | Plasmid (size [kilobases]) | Plasmid-associated phenotypes[a] | References |
|---|---|---|---|---|
| DS5 | Miami, Fla. | pAMα1 (9.1) | $Tet^r$ | 7, 9 |
| | | pAMβ1 (26.5) | $MLS^r$ | 7, 10 |
| | | pAMγ1 (60.0) | $Hly^+$ $Bcn^+$ $UV^r$ PR | 7, 10, 12 |
| | | pAMγ2 (52.8) | $Bcn^+$ PR | 7 |
| | | pAMγ3 (45.8) | PR | 7 |
| DS16 | Ann Arbor, Mich. | pAD1 (60.0) | $Hly^+$ $Bcn^+$ $UV^r$ PR | 7, 25 |
| | | pAD2 (22.7) | $MLS^r$ $Str^r$ $Kan^r$ | 7, 25 |
| JH1 | London, England | pJH1 (80.7) | $MLS^r$ $Str^r$ $Kan^r$ $Neo^r$ $Tet^r$ | 17, 20 |
| | | pJH2 (58.0) | $Hly^+$ $Bcn^+$ PR | 7, 16, 20 |
| 5952 | Ann Arbor, Mich. | pOB1 (64.7) | $Hly^+$ $Bcn^+$ PR | 7, 22 |
| | | pOB2 (42.3) | $Bcn^+$ (streptocin 101) | 7, 22 |
| 39-5 | Philadelphia, Pa. | pPD1 (56.4) | $Bcn^+$ $UV^r$ PR | 7, 29 |
| | | pPD2 (14.6) | Cryptic | 7, 29 |
| | | pPD3 (7.8) | Cryptic | 7, 29 |
| | | pPD4 (5.3) | Cryptic | 7, 29 |
| | | pPD5 (59.7) | $Hly^+$ $Bcn^+$ | 7, 29 |
| | | pPD6 (54.3) | Cryptic | 29 |

[a] Abbreviations: Tet, tetracycline; Str, streptomycin; Kan, kanamycin; Neo, neomycin; MLS, macrolide-lincosamide-streptogramin $B_\alpha$ antibiotics; Hly, hemolysin; Bcn, bacteriocin; PR, pheromone responder.

## RESULTS

The regulation or expression of plasmid-mediated Hly-Bcn activity in *S. faecalis* strains may be altered by the insertion of antibiotic resistance transposons into specific regions of the plasmids (14, 15, 20). Transpositional mutagenesis by the streptococcal transposons Tn*916* (14, 22) and Tn*917* (24) was used to locate the Hly-Bcn gene(s) on a restriction endonuclease map of plasmid pAD1 (7; Fig. 1). *Eco*RI fragment F of pAD1 and a *Bam*HI-*Sal*I fragment incorporating part of *Eco*RI fragments F and D and all of fragment H have been cloned into *E. coli*, with plasmid pACYC184 (6) used as the vector (manuscript in preparation). The cloned fragments were separated from vector DNA and used to prepare $^{32}P$-labeled probes for the hybridization experiments described below.

Eight plasmids were chosen for homology studies with the cloned Hly-Bcn-specific DNA from pAD1. They were all large (46 to 65 kilobases) conjugative plasmids. Five of the plasmids mediated Hly-Bcn activity (pAD1, pAMγ1, pJH2, pPD5, and pOB1), two were $Hly^-$ but coded for the production of bacteriocin (pAMγ2 and pPD1), and one was negative with respect to Hly and Bcn activity (pAMγ3). After isolation from the appropriate *S. faecalis* strains (see legend to Fig. 2 and Table 2), the plasmids were digested with restriction endonuclease *Eco*RI and the fragments separated by agarose gel electrophoresis (Fig. 2B). DNA in the gel was blotted onto nitrocellulose paper which was then incubated with $^{32}P$-labeled *Eco*RI fragment F or the *Bam*HI-*Sal*I fragment from pAD1. Both probes hybridized to *Eco*RI fragments from each of the Hly-Bcn plasmids (Fig. 2, lanes A, D, E, F, and I), but not to DNA from the $Hly^-$ $Bcn^+$ plasmids (lanes B, G, and H) or to pAMγ3 (lane C). *Eco*RI fragment F of pAD1 hybridized to a fragment of the same size produced by *Eco*RI digestion of each of the Hly-Bcn plasmids (Fig. 2C). On the basis of the restriction map shown in Fig. 1, it was expected that the pAD1 *Bam*HI-*Sal*I fragment would hybridize to *Eco*RI fragments D, F, and H of pAD1, and this was indeed the case (Fig. 2A, lane D). The *Eco*RI digest of pAMγ1 (Fig. 2A, lane A) produced the same hybridization pattern as pAD1. Each of the remaining Hly-Bcn plasmids, pOB1 (Fig. 2A, lane E), pPD5::Tn*916* (lane F), and pJH2 (lane I), also contained three *Eco*RI fragments that hybridized to the *Bam*HI-*Sal*I fragment from pAD1. In all cases, two of these homologous fragments appeared to be identical in size to *Eco*RI fragments F and H of pAD1. None of the homology exhibited by pPD5::Tn*916* was due to the presence of the transposon, since the $Hly^-$ $Bcn^+$ plasmid, pPD1::Tn*916* (Fig. 2A, lane G), had no detectable homology with either probe. These results suggested that the Hly-Bcn determinants on all five of the plasmids examined were very closely related and probably had a common origin.

A second series of experiments was conduct-

TABLE 2. Streptococcal strains used as sources of plasmid or total cell DNA

| Strain | Species | Plasmids present | Derivation or comment[a] | Reference |
|---|---|---|---|---|
| JH2-2 | *S. faecalis* | None | Rifampin and fusidic acid resistant | 17 |
| DT11 | *S. faecalis* | pAMα1 | $Tet^r$ transconjugant of DS5 × JH2-2 | 12 |
| YA101 | *S. faecalis* | pAMα1, pAMγ1 | $Tet^r$ $Hly^+$ $Bcn^+$ transconjugant of DS5 × JH2-2 | 11 |
| YA102 | *S. faecalis* | pAMα1, pAMγ2 | $Tet^r$ $Hly^+$ $Bcn^+$ transconjugant of DS5 × JH2-2 | 11 |
| JH2-2 (pAMα-1, pAMγ3) | *S. faecalis* | pAMα1, pAMγ2 | $Tet^r$ $Hly^-$ $Bcn^-$ transconjugant of DS5 × JH2-2 | This study |
| DL77 | *S. faecalis* | pJH2 | $Hly^+$ $Bcn^+$ transconjugant of JH1 × JH2-2 | 20 |
| DS16C2 | *S. faecalis* | pAD1 | Isolate of DS16 cured of pAD2 | This study |
| OG1-RF1 | *S. faecalis* | None | Spontaneous *S. faecalis* mutant resistant to rifampin and fusidic acid | 13 |
| OG1-RF1 (pOB1) | *S. faecalis* | pOB1 | $Hly^+$ $Bcn^+$ transconjugant of 5952 × OG1RF1 | This study |
| OG1-10 | *S. faecalis* | None | $Str^r$ mutant of OG1 | 13 |
| DJ2 | *S. faecalis* | None | $Tet^r$ transconjugant of DS16 × JH2-2; contains TN916 in chromosome | 13 |
| OG1-10 (pPD1) | *S. faecalis* | pPD1 | $Hly^-$ $Bcn^+$ transconjugant of 39-5 × OG1-10 | This study |
| OG1-10 (pPD1::Tn*916*) | *S. faecalis* | pPD1::Tn*916* | $Hly^-$ $Bcn^+$ $Tet^r$ transconjugant of (39-5 × DJ2) × (OG1-10) | This study |
| OG1-10 (pPD5::Tn*916*) | *S. faecalis* | pPD5::Tn*916* | $Hly^+$ $Bcn^+$ $Tet^r$ transconjugant of (39-5 × DJ2) × (OG1-10) | This study |
| AC1 | *S. pyogenes* | pAC1 | Clinical isolate harboring a 26.5-kilobase MLS resistance plasmid | 8 |
| MV759 | *S. agalactiae* | None | Beta-hemolytic, $Str^r$ group B *Streptococcus* | From M. V. Burdett, Duke University |
| DL812 | *S. anginosus* | pAMβ1 | $MLS^r$ transformant of group F *Streptococcus*, DL8 | 18 |
| DL1 | *S. sanguis* | None | Challis strain of *S. sanguis*, beta-hemolytic | 19 |

[a] Abbreviations: Tet, tetracycline; Bcn, bacteriocin; Str, streptomycin; MLS, macrolide-lincosamine-streptogramin $B_\alpha$ antibiotics.

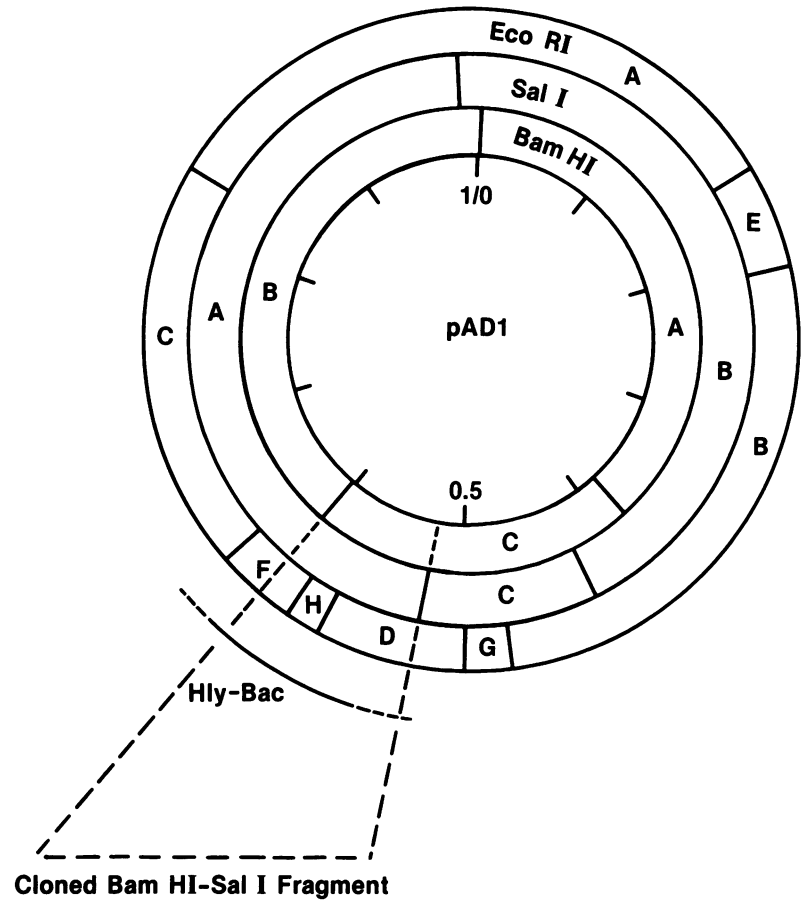


FIG. 1. Physical map of the *S. faecalis* Hly-Bcn plasmid, pAD1, showing the approximate position of the Hly-Bcn determinant(s) (redrawn, with modifications, from Clewell [7]). Also shown are the partial and complete *Eco*RI fragments included in the cloned *Bam*HI-*Sal*I fragment described in the text.

ed to determine whether the homologies observed among the five Hly-Bcn plasmids extended beyond the Hly-Bcn gene(s). Five identical blots, containing the electrophoretically separated *Eco*RI fragments from each of the Hly-Bcn plasmids (Fig. 3B) were prepared. Each blot was incubated in a hybridization reaction with one of the five $^{32}$P-labeled Hly-Bcn plasmids. The results obtained with the total pAD1 DNA probe are shown in Fig. 3A. Eight fragments of identical size, from pAMγ1 (Fig. 3A, lane A) and pAD1 (lane B), hybridized to the pAD1 probe. Two additional fragments in the *Eco*RI digest of pAMγ1 that did not hybridize to pAD1 were shown in a separate experiment (data not shown) to have been cleavage products of the tetracycline resistance plasmid, pAMα1, present in the same strain from which pAMγ1 had been isolated (YA101; Table 2). In the reciprocal experiment, in which pAMγ1 was used as a probe, all of the *Eco*RI fragments of pAD1 hybridized to pAMγ1. Thus, pAD1, originally from strain DS16, and pAMγ1, originally from strain DS5, appeared to be identical by both restriction analysis and DNA-DNA hybridization.

Plasmids pJH2 (Fig. 3A, lane C), pPD5::Tn*916* (lane D), and pOB1 (lane E) had *Eco*RI digestion patterns that were quite different from those of pAD1 and pAMγ1. However, the pAD1 probe hybridized to all of the pJH2 fragments and to 5 of 9 and 8 of 15 *Eco*RI fragments from pPD5::TN*916* and pOB1, respectively (Fig. 3A). When these three plasmids were used as probes, pJH2 and pOB1 hybridized to all of the *Eco*RI fragments from pAD1 and pAMγ1, whereas pPD5::Tn*916* hybridized to all but the corresponding G fragments (data not shown). These results suggested that all five of the Hly-Bcn plasmids examined share considerable DNA sequence homology and were probably derived from a common ancestral origin.

Experiments were conducted to determine whether the plasmid-mediated Hly-Bcn determi-

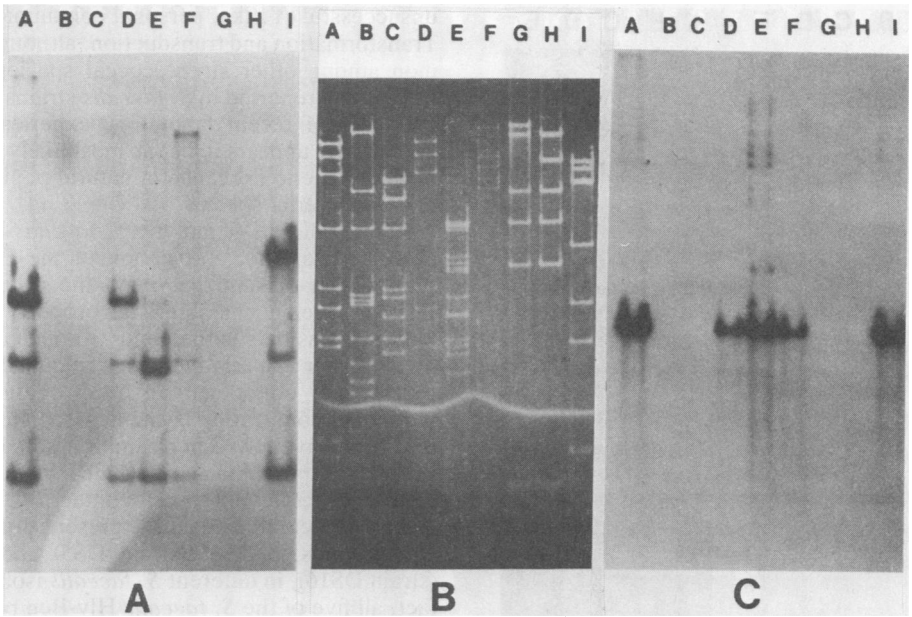


FIG. 2. Identification of Hly-Bcn determinants on five different Hly-Bcn plasmids. (B) Agarose gel electrophoresis of *Eco*RI-digested pAMγ1 (lane A), pAMγ2 (lane B), pAMγ3 (lane C), pAD1 (lane D), pOB1 (lane E), pPD5::Tn*916* (lane F), pPD1::Tn*916* (lane G), pPD1 (lane H), and pJH2 (lane I). Duplicate gels were blotted onto nitrocellulose paper and hybridized to $^{32}$P-labeled, cloned pAD1 *Eco*RI fragment F (autoradiogram, C) or to the cloned pAD1 *Bam*HI-*Sal*I fragment (A).

nants of *S. faecalis* strains were related to the beta-hemolysin genes of streptococci belonging to other Lancefield groups. Total cell DNA was isolated from *Streptococcus pyogenes* AC1 (group A), *Streptococcus agalactiae* MV759 (group B), *S. faecalis* JH2-2 (group D), *Streptococcus anginosus* DL812 (group F), *Streptococcus sanguis* DL1 (group H), and *S. faecalis* DS16-C2 (group D). One microgram of each DNA preparation was digested with *Eco*RI and the resulting fragments were separated by agarose gel electrophoresis (Fig. 4B). Strain AC1 (Fig. 4B, lane A), MV759 (lane B), and DL812 (lane D) produce chromosomally determined beta-hemolysins that are active in the lysis of sheep, horse, human, and rabbit erythrocytes. The hemolysin of strain DL1 (Fig. 4B, lane E), which does not contain any detectable plasmids, and the pAD1-mediated trait of strain DS16-C2 (lane F) are active on horse, human, and rabbit, but not sheep, erythrocytes. Strain DL1 did not, however, express any bacteriocin activity against *S. faecalis* JH2-2, an indicator strain for the plasmid-associated type 1 bacteriocin. Strain JH2-2 (Fig. 4B, lane C) is a nonhemolytic, plasmidless *S. faecalis* control. To determine the limits of detectability of the probe, 60 (Fig. 4B, lane G), 30 (lane H), and 15 (lane I) ng of *Eco*RI-digested pAMγ1 DNA, representing approximately 2, 1, and 0.5 copies of plasmid DNA per genome equivalent of streptococcal chromosomal DNA, respectively, were included on the same gel. The separated DNA fragments were transferred to a nitrocellulose blot which was incubated with $^{32}$P-labeled pAD1 DNA in a hybridization reaction (Fig. 4A). The assay was sensitive enough to detect all of the *Eco*RI fragments from as little as 15 ng (Fig. A, lane I) of pAMγ1, as well as pAD1 DNA (approximately one plasmid copy per genome equivalent; Fig. 4A, lane F) present in the total DNA preparation from strain DS16-C2. There was absolutely no detectable homology between pAD1 and DNA from strains MV759 (lane B), JH2-2 (lane C), or DL1 (lane E). The very faint band seen in Fig. 4A, lanes A (strain AC1) and D (strain DL812) of the autoradiograph was not observed in similar experiments in which cloned *Eco*RI fragment F or the *Bam*HI-*Sal*I fragment from pAD1 was used as a probe. It may be significant that strains AC1 and DL812 each harbor a 26-kilobase macrolide-lincosamide-streptogramin $B_\alpha$ resistance plasmid (8, 18). These two plasmids are virtually identical (28), and one of them has been shown to contain two *Eco*RI cleavage sites (21). The larger *Eco*RI fragment from this plasmid is 21 kilobases in size, which is the calculated size of the light band observed in Fig. 4A, lanes A and D. Whether the two macrolide-lincosamide-streptogramin $B_\alpha$ resistance plasmids share any sequence homology with plasmid pAD1 has not been determined.

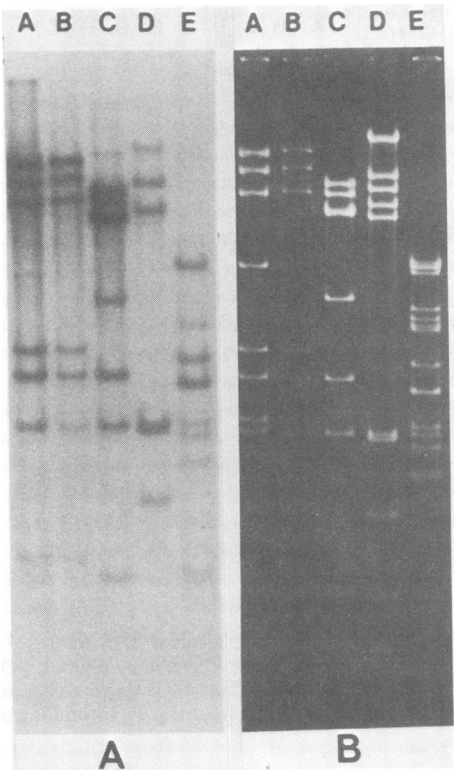


FIG. 3. Molecular relationships among Hly-Bcn plasmids. (C) Agarose gel electrophoresis of *Eco*RI-digested Hly-Bcn plasmids pAMγ1 (lane A), pAD1 (lane B), pJH2 (lane C), pPD5::Tn*916* (lane D), and pOB1 (lane E). Blotted DNA from the gel was hybridized to $^{32}$P-labeled pAD1 DNA (autoradiogram, A).

## DISCUSSION

We examined molecular relationships among Hly-Bcn plasmids harbored by clinical *S. faecalis* isolates of diverse geographical origins. We conclude from the data presented that the Hly-Bcn genetic determinants of all five strains examined are very closely related, if not identical, suggesting that the Hly-Bcn trait has been disseminated among *S. faecalis* strains in natural environments.

The dissemination of genetic traits among bacterial populations can occur by a number of mechanisms. Individual determinants may be transferred between replicons within the same cell by transposition or by classical recombination. Chromosomal DNA and, more commonly, smaller replicons such as plasmids and phages may be exchanged between bacterial cells by transformation, transduction, or conjugation. Preliminary attempts to detect the translocation of the Hly-Bcn determinant of the *S. faecalis* strains examined in this study to other plasmids, or to the host chromosomes, have thus far been unsuccessful (Y. Ike, personal communication). Transformation and transduction, although common among other streptococcal species, have never been reported in *S. faecalis* strains (7). On the basis of recent laboratory experience (7), conjugation appears to be the most likely mechanism of genetic exchange in natural populations of this bacterial species.

Among all beta-hemolytic *S. faecalis* isolates examined to date, association of the Hly-Bcn trait with a plasmid has been the rule (1, 7). Furthermore, a characteristic property of the Hly-Bcn plasmids is the ability to mediate their own transfer at a relatively high frequency ($10^{-3}$ to $10^{-1}$ per donor cell) in broth culture. The particular conjugation system associated with four of the five Hly-Bcn plasmids studied makes use of sex pheromones produced by recipient cells to generate cell-to-cell contact (7). Such a mechanism could explain the presence of identical plasmids, pAMγ1 (strain DS5) and pAD1 (strain DS16), in different *S. faecalis* isolates. In fact, all five of the *S. faecalis* Hly-Bcn plasmids examined were found to share considerable DNA homology. The dual Hly-Bcn activity appears to be unique among *S. faecalis* strains and has not been reported in other beta-hemolytic streptococcal species. It may be significant that the pheromone-enhanced conjugation system and the plasmids involved also appear to be confined to *S. faecalis* strains (7).

The role, if any, of hemolysis in streptococcal infections has not been established, but the bacteriocin activity of the *S. faecalis* hemolysin is lethal to a wide variety of gram-positive bacteria (3) and may provide the host strain with a selective advantage. If a beta-hemolytic *S. faecalis* strain also had other selectively advantageous properties, such as multiple antibiotic resistance, one might expect wide dissemination of that strain. Two of the isolates included in this study, DS16 (Ann Arbor) and JH1 (London), share enough such atypical properties to suggest a recent common ancestral origin. First, their Hly-Bcn plasmids, pAD1 (strain DS16) and pJH2 (strain JH1), share at least 90% DNA homology with each other. Second, each strain contains a chromosome-borne tetracycline resistance transposon (20, 24) with at least some homology between them (20). Finally, each strain harbors a multiple-antibiotic resistance plasmid, pAD2 (strain DS16) and pJH1 (strain JH1), mediating resistance to streptomycin, kanamycin, neomycin, and erythromycin (17, 25). The erythromycin resistance determinant of pAD2 is on a transposon, Tn*917* (24), and that of pJH1 has been shown to transfer independently of the other resistance genes to plasmid pJH2 (M. Banai and D. J. LeBlanc, unpublished observations). Plasmid pJH1 also carries a tetracy-

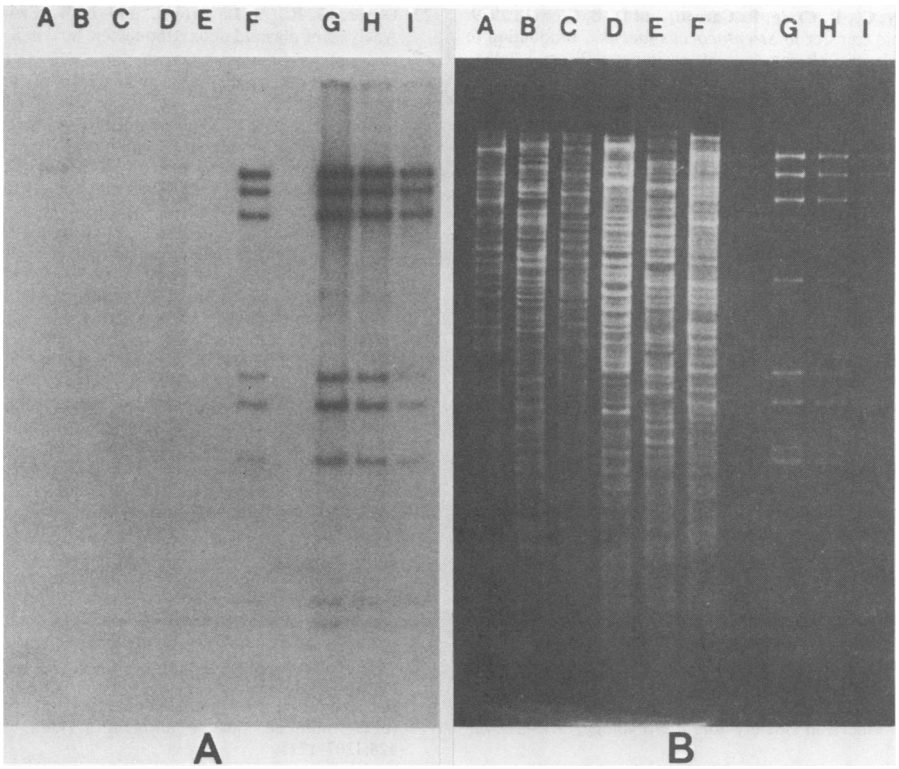


FIG. 4. Hybridization of pAD1 to DNA from beta-hemolytic streptococci. (B) Agarose gel electrophoresis of *Eco*RI-digested total cell DNA from strains AC1 (lane A), MV759 (lane B), JH2-2 (lane C), DL812 (lane D), DL1 (lane E), DS16-C2 (lane F) (1 μg of each), and purified plasmid pAMγ1 at 60 ng (lane G), 30 ng (lane H), and 15 ng (lane I). DNA in the gel was transferred to nitrocellulose paper and mixed with $^{32}$P-labeled pAD1 DNA in a hybridization reaction (autoradiogram, A).

cline resistance determinant that has no homology with the chromosomal trait but belongs to the same homology group, *tetL*, as the tetracycline resistance plasmid, pAMα1, of strain DS5 (5). Furthermore, the erythromycin resistance determinants of pAMβ1 (strain DS5) and Tn*917* (strain DS16) have been placed in the same homology group, class A, by Ounissi and Courvalin (23). As already mentioned, the Hly-Bcn plasmids, pAMγ1 (strain DS5) and pAD1 (strain DS16), appear to be identical. Thus, three of the strains used in this study, DS5, DS16, and JH1, may have a common origin.

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