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Genetic Analysis of the pAD1 Hemolysin/Bacteriocin Determinant in *Enterococcus faecalis*: Tn*917* Insertional Mutagenesis and Cloning

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Thirty-seven nonhemolytic/nonbacteriocinogenic mutations in *Enterococcus* (*Streptococcus*) *faecalis* plasmid pAD1 were generated by Tn*917* insertion. All were found to belong to one of two complementation classes. Each class of mutants secreted either hemolysin/bacteriocin (Hly/Bac) component A or L into the culture medium. DNA encoding Hly/Bac was cloned in *Escherichia coli* in which both components of the hemolysin were expressed individually and collectively. The region encoding components A and L was further defined by deletion analysis and physically mapped. A total of approximately 8.4 kilobases of pAD1 DNA were observed to be required for hemolysin expression. Hly/Bac activity of the wild-type and the inactive L substance was observed to be heat stable. Active Hly/Bac resulting from incubating separately secreted components A and L was also found to be heat stable. The results indicate that component A activates component L and that activated component L possesses the Hly/Bac activity. Component A was also observed to be associated with host immunity to the Hly/Bac.

In certain geographical locations as many as 60% of clinical *Enterococcus* (formerly *Streptococcus*) *faecalis* isolates produce a cytolytic toxin which lyses human, rabbit, and horse erythrocytes (20, 23). This hemolysin also possesses bacteriolytic activity and mediates the lysis of a broad range of gram-positive bacteria (2, 4, 6). Epidemiological studies have shown that hemolysin/bacteriocin (Hly/Bac) production correlates with the presence of conjugative plasmids which transfer at a frequency of 10^{-1} to 10^{-3} in broth matings (23). A high percentage of the hemolysin plasmids bear structural similarities to the conjugative plasmid pAD1 (20, 26). pAD1 is a 59.6-kilobase (kb) plasmid originally identified in *E. faecalis* DS16 (30) and belongs to the incompatibility class IncHly (9). The pAD1 *hly/bac* determinant was shown to contribute to pathogenicity in a mouse model (24). In addition to Hly/Bac synthesis, pAD1 has been shown to confer resistance to UV light (7) and a conjugative mating response to the peptide sex pheromone cAD1 secreted by recipient cells (5, 11, 13).

Early studies on the physical and chemical nature of the *E. faecalis* Hly/Bac found the hemolytic activity to be heat sensitive and inhibited by a teichoic acid produced by hemolytic strains (10). Granato and Jackson (16) isolated two types of *Streptococcus zymogenes* (*E. faecalis*) X-14 hemolysin mutants by chemical mutagenesis. Each type of mutant produced a single complementing substance, demonstrating that the hemolysin comprised two components. These components were termed A and L substance, since A appeared to play an activator role and L, when activated, possessed the lytic activity.

In this report, we present results of a genetic study of the *hly/bac* determinant of pAD1. Insertional mutagenesis with the erythromycin resistance transposon Tn*917* (5 kb) (28, 29) was performed to isolate mutations in the *hly/bac* determinant. The location of Tn*917* insertions into pAD1 was determined and correlated with the observed mutant complementation class. Based on the localization of the Hly/Bac-encoding genes, the region was cloned and reconstructed in *Escherichia coli*. Expression and externalization of both Hly/Bac components by this heterologous host allowed refinement of the location of component A- and L-encoding regions on a physical map. Physical and biochemical properties of Hly/Bac components A and L and their interaction are reported.

MATERIALS AND METHODS

Bacteria, media, and reagents. The strains and plasmids used in this study are listed in Table 1. *E. faecalis* strains were grown in antibiotic medium 3 (AB3; Difco Laboratories, Detroit, Mich.) for DNA isolation. Strains of *E. coli* were grown in L broth (27) supplemented with 100 μg of ampicillin (Sigma Chemical Co., St. Louis, Mo.) per ml for the selection of plasmids. For detection of insertional inactivation of the *lacZα* gene contained in the cloning vectors used, 50 μM isopropyl-β-D-thiogalactopyranoside (Sigma) and 0.01% 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (Sigma) were included in the media. Unless otherwise indicated, the medium used for other aspects of this study was brain heart infusion (BHI, Difco). Solid media were prepared by the addition of 1.5% (wt/vol) agar (Difco). Growth of cultures at 37°C was monitored with a Klett-Summerson colorimeter (Klett Manufacturing Co., Long Island City, N.Y.) with a no. 54 filter. Todd-Hewitt agar (Difco) plates containing 4% horse blood (Colorado Serum Co., Denver, Colo.) were used for detecting hemolysis on solid media. Rabbit blood (Colorado Serum Co.) was used in liquid hemolysis assays. Plasmids pUC18 and pUC19 (32) (Bethesda Research Laboratories, Inc., Gaithersburg, Md.) and pT7T318U and pT7T319U (Pharmacia Fine Chemicals, Piscataway, N.J.) were obtained commercially.

Generation of pAD1::Tn*917* derivatives. Tn*917* was inserted into pAD1 by erythromycin-induced transposition as

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TABLE 1. Bacterial strains and plasmids

Strain or plasmid	Genotype or phenotype	Plasmid content	Comments
Enterococcus faecalis			
FA2-2	*rif fus*	None	Derivative of JH2 (8)
JH2SS	*str spc*	None	Derivative of JH2 (29)
OG1-10	*str*	None	Derivative of OG1 (13, 15)
OG1X	*str*	None	Protease-negative mutant of OG1-10 (22)
DS16	*tet*	pAD1 (Hly/Bac) pAD2 [Sm Km Em (Tn*917*)]	Clinical isolate (30)
Escherichia coli			
JM109	*recA1 endA1 gyrA96 thi hsdR17 supE44 relA1* λ^- Δ(*lac-proAB*) (F' *traD36 proAB lacI*^{q}ZΔ*M15*)		J. Messing (32)
DH5α	*recA1 endA1 gyrA96 thi-1* hsdR17 *supE44 relA1* φ80 *lacZΔM15* (r_K^- m_K^+)		Bethesda Research Laboratories
Plasmid			
pAD1	*hly/bac*		59.6-kb conjugative plasmid from DS16 (8, 14, 21, 30)
pPD1	*bac*		54-kb conjugative plasmid from strain 39-5 (31)
pAM714	*hly/bac erm*		pAD1::Tn*917*, wild-type Hly/Bac and transfer (21)
pUC18/19	*bla lacZα*		J. Messing (32)
pT7T318U/19U	*bla lacZα*		Similar to pUC18/19, also contains promoters for bacteriophage T3 and T7 RNA polymerases, and phage f1 origin of replication (Pharmacia)

described previously (14, 21, 29). When matings between donor strain DS16 and recipient FA2-2 were involved, nonhemolytic mutants were selected on blood agar containing rifampin (25 μg/ml), fusidic acid (25 μg/ml), and erythromycin (12.5 μg/ml). Nonhemolytic transconjugants susceptible to streptomycin (500 μg/ml) and kanamycin (500 μg/ml) were analyzed further.

Isolation of plasmid DNA and pAD1::Tn*917* insertion mapping. Because FA2-2 is relatively refractory to lysozyme treatment, plasmid DNA was introduced into strain OG1-10 or OG1X by conjugation. Plasmids were then isolated by the Sarkosyl lysate procedure, followed by CsCl-ethidium bromide equilibrium centrifugation as previously described (8). The location of Tn*917* insertions on the *Eco*RI map of pAD1 was determined by *Eco*RI and *Sal*I restriction enzyme analysis as described previously (8, 14, 15, 21, 28). Plasmids were isolated from *E. coli* strains by the alkaline lysis procedure, followed by dye-buoyant density gradient purification as previously described (27). Competent cells of *E. coli* JM109 and DH5α were prepared and transformed as described previously (19).

Cloning of *E. faecalis hly/bac* determinant. All restriction enzymes were obtained from Bethesda Research Laboratories and used according to their specification. Transposon analysis revealed that the *E. faecalis hly/bac* determinant encompassed pAD1 *Eco*RI fragments F, H, I, and portions of C and D as illustrated in Fig. 1. Since the *hly/bac* determinant centered around pAD1 *Eco*RI fragment F, this fragment was initially cloned into *Eco*RI-linearized pUC18, resulting in construction pKSG-F18. Candidate restriction enzymes useful for cloning the entire *hly/bac* determinant were identified by hybridization of ^{32}P-labeled, denatured pKSG-F18 to pAD1 target DNA after digestion with *Hin*dIII, *Sal*I, *Cla*I, *Bcl*I, *Hin*cII, *Ava*I, *Ava*II, *Hpa*I, or *Pst*I as described previously (27). Cleavage of pAD1 DNA with *Bcl*I generated a 7.3-kb fragment which hybridized to the cloned *Eco*RI F fragment of pAD1 and was selected for further analysis.

*Bcl*I restriction fragments of pAD1 were cloned into *Bam*HI-linearized pUC18 DNA. No detectably hemolytic clones were obtained after *E. coli* transformation. Therefore, 800 transformants in which the vector *lacZα* gene had been inactivated were picked and screened by colony hybridization as described previously (27) with the radiolabeled pKSG-18F probe. Two clones demonstrating positive hybridization signals were found to contain only the 7.3-kb *Bcl*I fragment of pAD1. These clones, pBCL3 and pBCL4, possessed the pAD1 insert in the same orientation, and pBCL3 was selected for further study.

A physical map of the cloned 7.3-kb *Bcl*I fragment of pAD1 was prepared by digesting pBCL3 with restriction enzymes singly and in combination as described previously (3). As shown in Fig. 1, 940 base pairs of the pAD1 *Eco*RI D fragment were contained within the 7.3-kb *Bcl*I insert, suggesting that additional regions of *Eco*RI fragment D as identified by transposon mutagenesis were required for expression. The insert contained within pBCL3 was subcloned as an *Sst*I-*Xma*I fragment into the multifunction vectors pT7T318U and pT7T319U, resulting in constructions pRAS18 and pRAS19 (Fig. 1). Additional pAD1 sequences were then spliced onto the 940 base pairs of pAD1 *Eco*RI fragment D contained within the cloned 7.3-kb *Bcl*I insert as follows. A 1.7-kb *Ava*I-*Sal*I pAD1 DNA fragment contained within pAD1 *Eco*RI fragment D was gel purified (1) and ligated into similarly cleaved pRAS18 (after destruction of the vector *Sma*I-*Xma*I-*Ava*I recognition site by *Xma*I digestion followed by S1 nuclease trimming [1]). The resulting constructions, pRAS18-3 and pRAS28-4 (Fig. 1), expressed the hemolytic phenotype. Southern blot comparisons of pAD1 and pRAS18-3 and pRAS28-4 digested singly and in combination with *Eco*RI, *Eco*RV, *Sal*I, *Ava*I, *Ava*II, *Hin*cII, and *Hin*dIII revealed that no detectable rearrangements occurred during the process of cloning the functional *hly/bac* determinant.

Deletion and complementation analysis of cloned *hly/bac* determinant. Direct subcloning of restriction fragments was

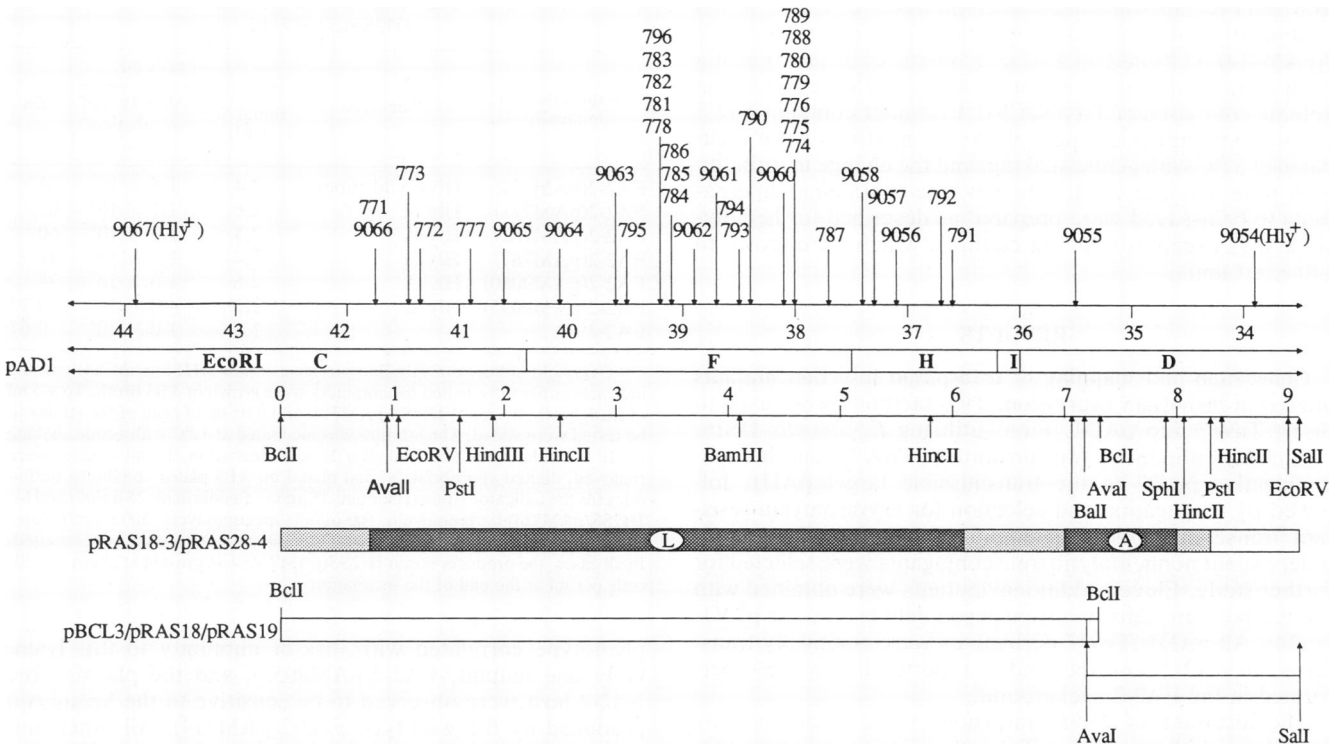

FIG. 1. Map of Hly/Bac-encoding region of pAD1. Top: Arrows indicate the positions of Tn*917* insertions relative to the Hly/Bac-encoding region of the pAD1 physical map (14). Numbers of insertions correspond to the mutant pAD1::Tn*917* plasmid designations, having the prefix pAM. Bottom: The location of restriction sites throughout the cloned determinant. Plasmid pRAS18-3 contains the entire cloned region inserted into vector pT7T318U with map position 0 adjacent to the vector *lacP* and confers a hemolytic phenotype to the *E. coli* host (see text for derivation). Plasmid pRAS28-4 is identical to pRAS18-3, except that the insert in pT7T319U is inverted relative to the vector *lacP*. Hemolytic deletion derivatives of pRAS18-3 and pRAS28-4 were constructed by deleting map positions 8.3 to 9.2. Separate nonhemolytic derivatives possessed deletions spanning map positions 0 to 1.6 and 7.9 to 9.2. A frameshift introduced into the *Bam*HI recognition site by filling the cohesive termini with the large fragment of DNA polymerase I and deoxynucleotide triphosphates (27) also eliminated the hemolytic phenotype.

used to construct deletion derivatives of pRAS18-3 and pRAS28-4. Complementation analysis was performed by cross streaking pRAS18-3 and pRAS28-4 deletion derivatives with each other and with *E. faecalis* mutants FA2-2(pAM9055) and FA2-2(pAM307) (8) producing either Hly/Bac component A or L alone.

Soft agar assay for bacteriocin production and immunity. As previously described (12), 0.05 ml of an overnight culture of the indicator strain grown in AB3 was added to 5 ml of molten soft agar (0.75%) and poured onto an AB3 agar plate. After solidification, each tester strain was streaked into the soft agar. Zones of inhibition around the streaks were monitored after overnight incubation at 37°C.

Hemolysin assays. *E. faecalis* strains were grown in BHI at 37°C with slow shaking. At an optical density of 110 Klett units (Klett-Summerson colorimeter; no. 54 filter), chloramphenicol was added to a final concentration of 100 μg/ml and the bacteria were removed by centrifugation at 43,000 × *g* for 30 min. Hemolysin activities were measured in the resulting supernatants.

Rabbit erythrocytes stored 18 to 30 days after collection were found to be reproducibly sensitive to the *E. faecalis* hemolysin in hemolysin assays. Fresh erythrocytes were fairly refractory to the hemolysin, whereas erythrocytes stored longer than 30 days were observed to autolyse spontaneously. Before use, erythrocytes were washed four times with Tris-NaCl buffer (10 mM Tris hydrochloride [pH 7.4], 150 mM NaCl) and used at a concentration of 2% (vol/vol) in the hemolysin assay mixture.

Hemolysin assays were performed in a 30-ml volume of Tris-NaCl buffer containing 50 mM $MgCl_2$, 2% (vol/vol) washed erythrocytes, and hemolysin-containing supernatant (10 to 30% [vol/vol]). Hemolysis occurred at 37°C with gentle shaking. Samples were removed at various times, and intact erythrocytes were pelleted (5,800 × *g*, 5 min). Hemoglobin released into the supernatant was measured in Klett units after subtraction of the optical density observed for parallel control assays of nonhemolytic FA2-2 culture supernatants.

Complementing activities in hemolysis assays of two types of nonhemolytic mutants. Hemolytic assays were performed essentially as described above except that crude wild-type hemolysin was replaced by mixtures of culture supernatants derived from nonhemolytic mutants. Values obtained were normalized to that obtained when the reaction contained equal (10 ml) amounts of each complementing culture supernatant.

Liquid bacteriolysis assay. The bacteriocin indicator culture was grown to 90 Klett units in BHI at 37°C, at which time 12.5 μg of chloramphenicol per ml was added. The cells were harvested and washed with Tris-NaCl buffer and resuspended in 0.1 of the original volume of Tris-NaCl containing additionally 20 mM $CaCl_2$. After 30 min at ambient temperature, the cells were again pelleted and resuspended in the same buffer. Washed indicator cells (about 1 ml) were added

to a level of 110 Klett units to 10-ml assay mixtures which contained 3 ml of culture supernatant derived from FA2-2(pAM714), plasmid-free FA2-2, or mixtures of supernatants from cultures of nonhemolytic mutants, with the remaining volume composed of Tris-NaCl-$CaCl_2$ buffer containing 12.5 μg of chloramphenicol per ml. Assay mixtures were incubated at 37°C with gentle shaking, and the change in turbidity with time was recorded. The Hly/Bac-containing supernatants to be assayed were prepared as described for hemolysin assays except for mutant cultures, which were grown to 140 Klett units.

RESULTS

Generation and mapping of transposon insertion mutants altered in hemolysin expression. Two methods were used to insert Tn*917* into pAD1. First, utilizing *E. faecalis* DS16, erythromycin-induced transposition of Tn*917* from the nonconjugative pAD2 to the transmissible target pAD1, followed by conjugation and selection for erythromycin-resistant transconjugants, was employed (21, 29). Twenty-six independent nonhemolytic transconjugants were selected for further study. Eleven additional mutants were obtained with the temperature-sensitive transposon delivery vector pTV1-ts (14). All pAD1::Tn*917* derivatives were conjugally transferred to FA2-2, and the studies reported below were performed in the FA2-2 background.

The locations of Tn*917* insertions were determined by *Eco*RI and *Sal*I digestions of the plasmids containing mutations as described previously (8, 14, 21). The sites of insertion are shown in Fig. 1.

Cloning and expression of *E. faecalis hly/bac* determinant in *E. coli*. The approximate location of the *E. faecalis hly/bac* determinant on pAD1 as determined above was observed to include *Eco*RI fragments F, H, and I and portions of *Eco*RI fragments C and D. Because of a lack of restriction enzyme recognition sites flanking this entire portion of pAD1, the region was first cloned as a single *Bcl*I fragment onto which an *Ava*I-*Sal*I fragment of pAD1 was spliced (Fig. 1). A detailed physical map of the cloned *E. faecalis hly/bac* region of pAD1 is shown in Fig. 1.

Interestingly, the cloned *hly/bac* determinant contained within plasmids pRAS18-3 and pRAS28-4 (in opposite orientations) was observed to be optimally expressed on BHI plates containing erythrocytes. Little hemolysis was observable on L agar plates lacking glucose, even though comparable growth was observed. A minimum of 0.2% glucose was found to be required in L blood agar plates to result in detectable hemolysis. Optimum hemolysis occurring on L blood agar plates containing 0.4% glucose was nonetheless significantly less than that observed on BHI blood agar plates (which contain 0.2% glucose [data not shown]). Since glucose affected hemolysis expression in both orientations, vector influence on this phenomenon appears to be minimal.

Bacteriocin activity and immunity. Thirty-seven nonhemolytic mutants and wild-type FA2-2(pAM714) were tested for the production of bacteriocin activity with FA2-2, OG1-10, and *E. faecium* (ATCC 9790) indicator strains as described in Materials and Methods. None of the mutant strains produced zones of inhibition on the indicator strain lawns, demonstrating that the hemolytic and bacteriocinogenic phenotypes coincide.

E. faecalis strains harboring pAD1 are resistant (immune) to bacteriocin activity, allowing the producing strain to survive the effects of the lysin. The mutant strains were therefore tested to determine whether loss of the Hly/Bac phenotype correlated with loss of immunity to this lysin. Only one mutant, FA2-2(pAM9055), and the plasmid-free FA2-2 host were observed to be sensitive to the hemolysin produced by the wild-type FA2-2(pAM714). All other mutants were found to have retained resistance to this bacteriocin. All strains tested were inhibited by FA2-2(pPD1), which encodes production of an unrelated bacteriocin.

TABLE 2. Immunity to Hly/Bac produced by *E. faecalis* JH2SS(pAM714)[a]

Strain tested for immunity	Phenotype	No. of cells/ml		b/a
		Initial (a) (10^7)	After 18 h of incubation (b)	
FA2-2(pAM714)	Hly (wild type)	2.4	1.5×10^9	63
FA2-2(pAM771)	Hly A	2.6	1.7×10^9	65
FA2-2(pAM782)	Hly A	2.5	1.5×10^9	60
FA2-2(pAM787)	Hly A	3.4	1.4×10^9	41
FA2-2(pAM9060)	Hly A	2.6	1.0×10^9	38
FA2-2(pAM9055)	Hly L	2.3	4.0×10^6	0.17
FA2-2		1.5	1.0×10^7	0.67

[a] Overnight cultures of Hly/Bac producer strain JH2SS(pAM714) and the indicator strain to be tested for immunity were grown in AB3 broth. To 4.5 ml of fresh broth, 0.5 ml of JH2SS(pAM714) and 0.05 ml of each of the strains to be tested were added. The mixture was incubated at 37°C with gentle shaking for 18 h. At the initial time and after 18 h of incubation, 0.1-ml samples were removed, diluted appropriately, and plated on AB3 plates containing antibiotics for the indicator strain (rifampin, 25 μg/ml; fusidic acid, 25 μg/ml) and for JH2SS(pAM714) (streptomycin, 500 μg/ml; spectinomycin, 500 μg/ml). After overnight incubation of the plates at 37°C, the colonies grown were counted. In all cases, the producer strain JH2SS(pAM714) was present at about 1×10^9 cells per ml at the end of the incubation.

The immunity of mutant strains to the bacteriocin activity resulting from mixtures of complementing cultures was also determined. As summarized in Table 2, the results of liquid bacteriocin assays paralleled those obtained with agar overlays. FA2-2 and FA2-2(pAM9055) cell counts decreased after overnight incubation, whereas the other four mutant strains and the wild-type FA2-2(pAM714) control increased, representing a difference of about 2 orders of magnitude. Together with the results of the agar overlay bacteriocin tests, these results indicate that Tn*917* insertions between map positions 36.7 and 41.8 kb of pAD1 do not affect host bacteriocin immunity, whereas insertions at 35.4 kb (as for pAM9055) abrogate immunity.

Extracellular complementation between hemolysin components produced by nonhemolytic mutants. Streaks of the only

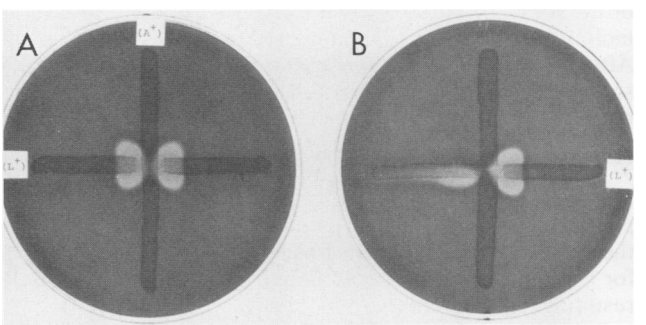

FIG. 2. Complementation on blood agar by the two classes of nonhemolytic pAD1::Tn*917* mutants obtained. (A) Butterfly wing hemolysis. The L-producing strain FA2-2(pAM9055) was streaked first, crossed by A-producing mutant FA2-2(pAM771). (B) Asymmetrical hemolysis. The A-producing strain FA2-2(pAM771) was streaked first, crossed by a streak of L-producing FA2-2(pAM9055) from right to left. Hemolysis at 24 h is shown.

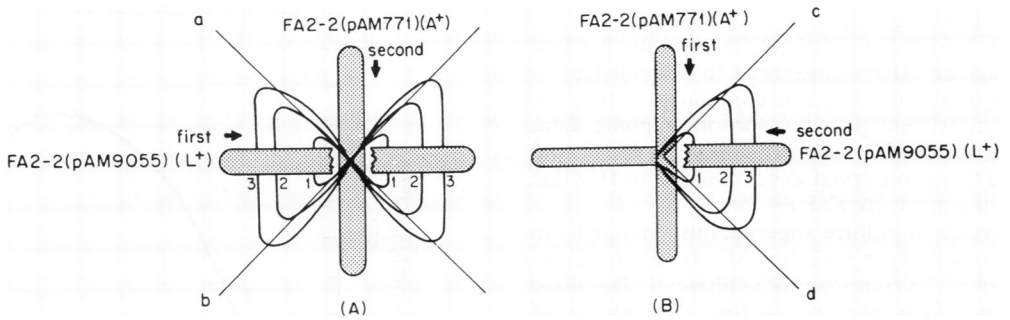

FIG. 3. Schematic representation of the advance of hemolysis along the FA2-2(pAM9055) streak with time. The plates shown in Fig. 2 were incubated for 3 days at 37°C. The hemolysis zone was traced after days 1, 2, and 3. In addition to advancing along the component L-producing FA2-2(pAM9055) streak, hemolysis also advanced along lines a and b in plate A and c and d in plate B.

mutant observed above to be susceptible to bacteriocin, FA2-2(pAM9055), made in proximity to streaks of all other obtained mutants resulted in a complementation of activities at the streak junctions on both blood agar and bacteriocin assay plates. No other complementing combinations were found. Therefore, all mutants fall into one of two complementation groups.

FA2-2(pAM9055), the only representative obtained of one complementation class, and FA2-2(pAM771), a mutant phenotypically representative of the other class, were selected for the following experiments. Cross streaks of the two mutant strains were made on two blood agar plates. The pattern of hemolysis observed at the junctions of the streak depended markedly on the order in which the two mutants were streaked (Fig. 2 and 3). When FA2-2(pAM771) was streaked across a preexisting streak of FA2-2(pAM9055), a butterfly wing pattern of hemolysis was observed at the junction. The hemolyzed area was observed to expand outward along 45° axes with time (Fig. 3A). This observation suggests that an optimal proportion of the complementing activities occurred along this track and that an excess of the activity produced by FA2-2(pAM771) was inhibitory. In contrast, streaks made in the opposite order, that is, FA2-2(pAM771) crossed over by FA2-2(pAM9055), resulted in a butterfly wing of hemolysis only on the side in which the FA2-2(pAM9055) streak entered the FA2-2(pAM771) streak (Fig. 3B). In both cases, growth of FA2-2(pAM9055) was markedly inhibited at the junctions of the cross streak (Fig. 2 and 3). The departing arm of the horizontal streak made with FA2-2(pAM9055) shown in Fig. 2B and 3B was subcultured and found to contain predominantly FA2-2(pAM771), confirming that complementing activities produced by mixing of the strains at the junction was lethal to FA2-2(pAM9055) and accounting for the absence of the hemolysis pattern on the departing side of this cross.

Because of the similarity between the inhibition of hemolysis and bacteriolysis observed under streaks of FA2-2(pAM771) and the inhibition of hemolysis previously observed by Granato and Jackson (16) under conditions of hemolysin component A excess, FA2-2(pAM771) was presumed to be defective in hemolysin component L synthesis and was tentatively designated A^+L^-. Similarly, FA2-2(pAM9055), the only representative of the second complementation class, was tentatively designated A^-L^+.

Localization of component A and L genes on cloned *hly/bac* determinant by deletion and complementation analysis. Expression of the cloned *hly/bac* determinant by *E. coli* facilitated efforts to further localize the respective component A- and L (17, 18)-encoding genes. Both Hly/Bac components A and L were independently externalized by *E. coli*, despite the outer membrane barrier, and were fully functional in the extracellular environment (Fig. 4). Complementation on blood agar plates between deletion derivatives of pRAS18-3 and pRAS28-4 secreting only one *E. faecalis* Hly/Bac component and transposon mutants of *E. faecalis* known to secrete Hly/Bac component A or L allowed the

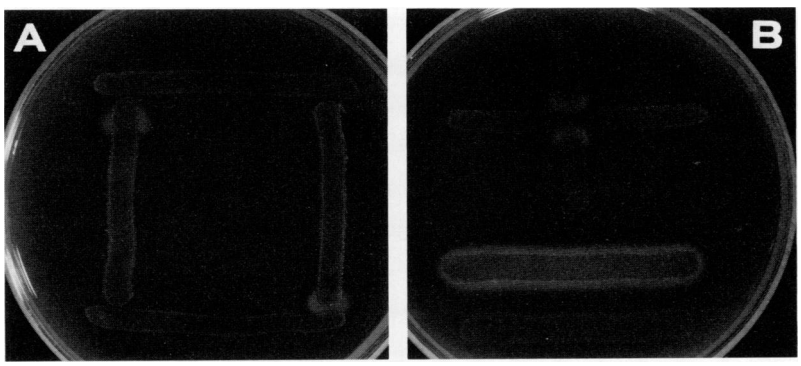

FIG. 4. Complementation by *E. coli* clones secreting either component A or component L. (A) Complementation between the component A-producing FA2-2(pAM771) streaked across the top, component L-producing *E. faecalis* FA2-2(pAM9055) streaked across the bottom, and *E. coli* clones DH5α(pBCL3) (producing component L, left [Fig. 1]) and DH5α(pRAS18-3E) (a deletion derivative of pRAS18-3 harboring only the component A-encoding *Eco*RI-*Sal*I fragment spanning map positions 6.6 to 9.2; right). (B) Complementation between DH5α(pBCL3) (top, vertical) and DH5α(pRAS18-3E) (top, horizontal). The fully hemolytic clone DH5α(pRAS18-3) is streaked in the lower center, and DH5α(pT7T318U) (vector control) is streaked below.

location of A and L gene boundaries to be determined on the physical map. A 1.5-kb region of pAD1 centrally located within *Eco*RI fragment D was observed to be sufficient to confer the ability to produce Hly/Bac component A (Fig. 1). In contrast, but consistent with transposon mapping data, nearly 7 kb including pAD1 *Eco*RI fragments I, H, F, and portions of D and C was observed to be required for Hly/Bac synthesis of component L by *E. coli*.

Hemolytic activities of culture supernatants. Hemoglobin release in assays of wild-type FA2-2(pAM714) culture supernatants was observed to comprise three phases: a lag phase of about 30 min which occurred before the release of detectable hemoglobin, a linear phase which occurred 90 to 120 min into the assay, and a plateau which occurred after the linear phase. The results of hemolysis assays, read after 2 h, were normalized to the value obtained in the assay of wild-type FA2-2(pAM714), which was set at 100. Hemolytic activity was observed to be proportional to the volumes of hemolysin-containing culture supernatant and rabbit erythrocytes in control experiments.

Complementation and inhibition in mixed culture supernatants of FA2-2(pAM771) and FA2-2(pAM9055). The data of Fig. 2 and 3 suggested that a maximum ratio of component A to component L exists, above which hemolysis is inhibited, and below which hemolysis occurs. To determine the stoichiometry of component A-component L interaction in more quantitative experiments, mixtures of culture fluids derived from these mutants were combined in various proportions. In experiments in which the amount of supernatant derived from the component A-producing strain [FA2-2(pAM771)] varied from 1 to 10 ml and the supernatant derived from the component L-producing mutant [FA2-2(pAM9055)] was held constant at 10 ml, the level of hemolytic activity was observed to increase, reach a hyperbolic maximum, and then decrease (Fig. 5). When the converse experiment was performed, that is, the amount of L supernatant was varied from 1 to 10 ml and the A supernatant was held at 10 ml in the hemolysis reaction, hemolysis was inhibited until an L:A ratio of 5:10 was reached followed by an exponential increase in hemolysis to the point where the ratio was 1.0. These results supported the qualitative observations made in complementation tests performed on blood agar in that a relative excess of the A component is inhibitory. Furthermore, these results were consistent with previous observations on the kinetics and stoichiometry of component A and L interaction (16), confirming our tentative ascriptions of A and L phenotype to the two mutant classes.

The addition of culture fluid derived from the A-expressing mutant FA2-2(pAM771) to hemolytically active supernatant derived from the wild type FA2-2(pAM714) resulted in a significant decrease in hemolytic activity over controls (Fig. 6). In contrast, the addition of component L-containing supernatant resulted in a six- to sevenfold increase in the level of hemolytic activity. The similarities in the shapes of the curves obtained by quantitating hemolysis with time in the above experiments suggest that the inhibition results from a reduced level of active lysin. Furthermore, these results suggest that in the proportions of A and L produced by the wild-type FA2-2(pAM714) in liquid culture, excess A activity is available and capable of activating additional component L.

Thermostability of components A and L and the active lysin. Previous reports on the inhibition of cytolytic activity derived from *S. zymogenes* X-14 identified lipoteichoic acid produced by hemolytic strains as being able to inhibit the hemolytic reaction (10). Lipoteichoic acid inhibition was

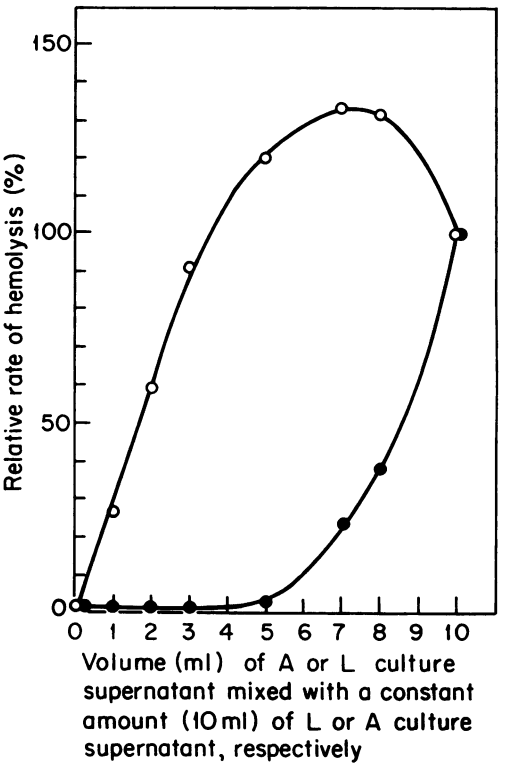

FIG. 5. Hemolysis resulting from mixtures of culture supernatants of the L-producing strain FA2-2(pAM9055) and the A-producing strain FA2-2(pAM771). The assay was performed as described in Materials and Methods. The values represent the relative rate of hemolysis, where the value corresponding to a mixture containing both A and L in equal amounts (10 ml each) was taken as 100. Hemolysis was assessed 90 min after lysis began to occur. Symbols: ○, mixture containing various amounts of A culture supernatant and a constant amount (10 ml) of L supernatant; ●, mixture containing various amounts of L supernatant and constant amount (10 ml) of A culture supernatant.

observed to be insensitive to pretreatment of the lipoteichoic acid preparation with heat (10). It was therefore of interest to determine whether the inhibition of hemolytic activity by component A was related to the above observations on the effect of lipoteichoic acid. The inhibitory activity of culture fluid containing component A was found to be sensitive to heat treatment (65°C, 15 min) (Fig. 6). Heat exposure of culture fluid containing A resulted in some inhibition, but no more than that of an unheated culture supernatant of FA2-2 (plasmid-free).

Heat treatment of component A also eliminated its ability to complement component L (Fig. 7). Treatment for as little as 5 min was observed to eliminate component A activity. In contrast, component L which had been heat treated (98°C, 15 min) was activated by native component A to an extent identical to non-heat-treated component L (Fig. 7). Extended heat treatment of component L (30 min, 98°C) also had no effect on the ability of component L to be activated by native component A (data not shown). It can therefore be concluded that component A inhibition of hemolytic activity is unrelated to previously observed heat-resistant LTA inhibition, and the ability of component L to interact with native component A is heat resistant.

The ability of the actively lytic moiety to withstand heat treatment was also investigated. Hemolytic activity was

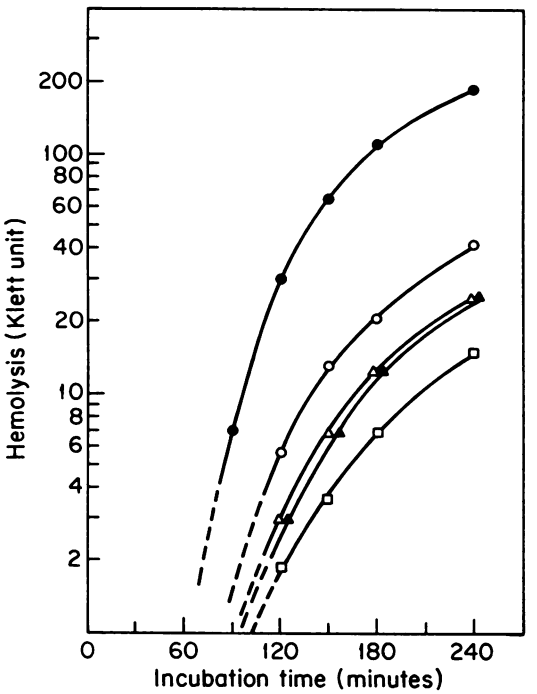

FIG. 6. Inhibition of wild-type hemolysin by A-containing supernatant. Strains used were FA2-2(pAM714) (wild-type hemolysin), FA2-2(pAM771) (A-producing mutant), FA2-2(pAM9055) (L-producing mutant), and FA2-2 (plasmid-free control). The culture supernatant of each strain was prepared as described in Materials and Methods. Each mixture contained 10% (vol/vol) FA2-2(pAM714) supernatant plus 20% (vol/vol) of one of the other supernatants. Symbols: ○, FA2-2(pAM714) culture supernatant; △, addition of FA2-2 culture supernatant; □, addition of A-containing supernatant; ●, addition of L-containing supernatant; ▲, addition of heated A-containing supernatant.

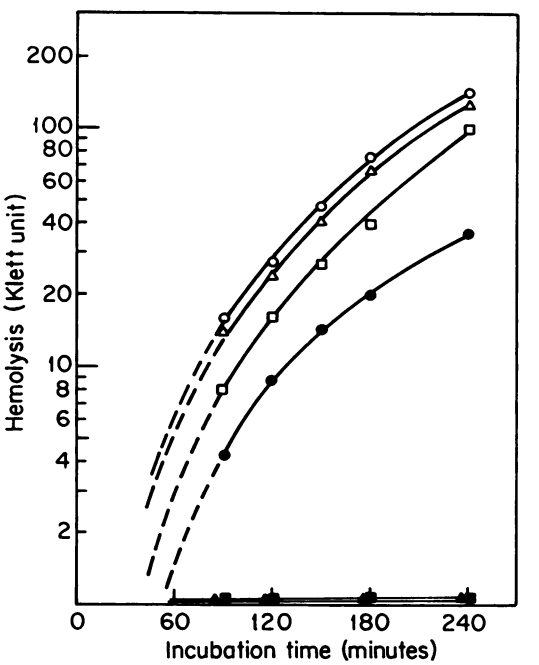

FIG. 7. Heat sensitivity of the A component and heat resistance of the L component. Assay mixtures contained 30% (vol/vol) L-containing supernatant [FA2-2(pAM9055)] plus 21% (vol/vol) A-containing supernatant [FA2-2(pAM771)]. Heat treatments were at 98°C for 15 min. Symbols: ○, native A plus native L; △, native A plus heat-treated L culture supernatant; ▲, native L plus heat-treated A; ■, heated immediately upon mixing native A and L; ●, A and L mixed together for 3 h and then heated; □, A and L mixed together for 3 h with no heat treatment.

easily detected in supernatants derived from wild-type FA2-2(pAM714) which had been heat treated at either 65 or 98°C for 15 min and was approximately 60% of that observed for controls which were not heat treated (data not shown). The reduced level of hemolytic activity observed suggests that some component A-mediated activation of component L occurs during the hemolysis assay and that this additional activation is arrested by heat treatment. The effect of similar heat treatment on the ability of separately secreted components A and L to interact was investigated (Fig. 7). These results show that, like component L, the active lytic moiety once formed is heat stable.

Because of the above-described effects of heat on the activities of components A and L in hemolysis assays, it was of interest to determine whether similar effects would be detected in bacteriolytic assays. Mixtures of component A and L containing culture fluids were bacteriolytic (Fig. 8). Furthermore, the bacteriolytic activity was destroyed by heat treatment of component A supernatants but not those containing component L. These results demonstrate that bacteriolytic and hemolytic activities are manifestations of the same lytic moiety.

DISCUSSION

A total of 37 nonhemolytic mutations in pAD1 were generated by Tn*917* insertion. All were classified into one of two types: mutants which produced only Hly/Bac component A or L. No mutants were observed which were deficient in both components. Each class secreted the respective Hly/Bac component into the culture medium.

The region of pAD1 shown by transposon mapping to be related to Hly/Bac synthesis was cloned in *E. coli*. The hemolysin was expressed by this heterologous host, as were the separately subcloned component A- and L-encoding regions. Transposon mutagenesis revealed that a minimum of 6.5 kb of pAD1 is required for Hly/Bac synthesis, whereas deletion analysis showed no more than 8.4 kb to be required. The component A-encoding region is confined to 1.6 kb of pAD1 (map positions 34.3 to 35.9). In contrast, the component L-encoding region spans 7.2 kb (map positions 36.6 to 42.8). This observation suggests that component L (previously reported to be 11,000 daltons when purified from *S. zymogenes* X-14 [18]) synthesis is a complex process which may involve auxiliary functions, possibly related to processing, externalization, and regulation. The observations that (i) Tn*917* insertions into component A- and component L-encoding regions do not result in a detectable polar effect on expression of the other component and (ii) both component determinants are expressed in both orientations when cloned in *E. coli* suggest that transcription of component A and L genes occurs at a significant frequency from separate promoters.

The wild-type activated hemolysin produced by FA2-2(pAM714) as well as unactivated L component are heat resistant. The activator component A is heat labile. As was previously observed for the interaction between *S. zymogenes* X-14 components A and L (16), a discrete optimum in component A concentration exists which results in maximal

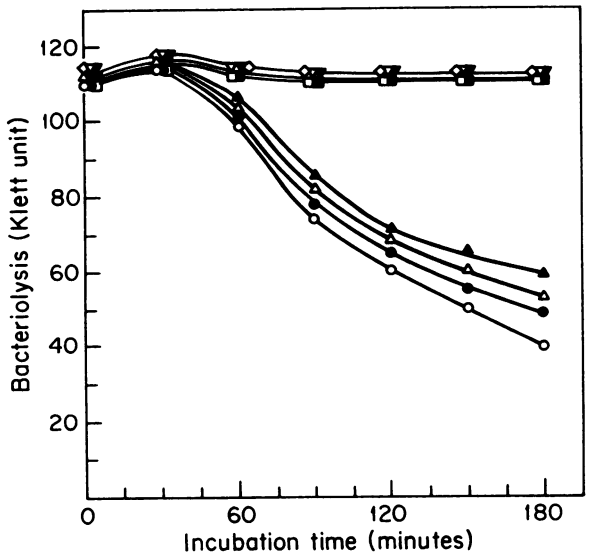

FIG. 8. Bacteriolytic activity of culture supernatants. The bacteriocin assays were performed as described in Materials and Methods. *E. faecalis* OG1-10 was used as the indicator strain in this experiment. When mixtures of culture supernatants were involved, the 10-ml assay mixture contained 2.1 ml of the A-containing supernatant [FA2-2(pAM771)] and 3 ml of the L-containing supernatant [FA2-2(pAM9055)]. For individual assays of supernatants, 3 ml was used in the 10-ml assay. Heating was at 98°C for 15 min. Symbols: ○, FA2-2(pAM714) culture supernatant; ●, heat-treated FA2-2(pAM714) culture supernatant; △, mixture of A-containing supernatant and L-containing supernatant; ▲, mixture of A-containing supernatant with heat-treated L-containing supernatant; □, L-containing supernatant; ■, A-containing supernatant; ▽, mixture of heat-treated A-containing supernatant with native L-containing supernatant; ▼, heat treatment immediately after mixing L- and A-containing supernatants; ◇, FA2-2 culture supernatant.

activation of component L. These results suggest that the activator modifies component L, which, once altered, possesses lytic activity. The observations that (i) excess component A is inhibitory to hemolytic activity and (ii) transposon inactivation of component A results in loss of immunity to the bacteriolytic effect suggest that component A not only activates the lysin but also mediates immunity to the bacteriolytic effect. Since activation of component L is observed in the extracellular milieu, the types of potential component A modifications of component L are limited. Speculatively, component A could effect both activation of and resistance to the Hly/Bac through a covalent modification such as proteolytic cleavage.

The Hly/Bac activity encoded by pAD1 appears to differ in several respects from that characterized by Granato and Jackson (16). The two-component hemolysin produced by *S. zymogenes* X-14 was observed to be heat labile (16). Further, the L activity produced by nitrosoguanidine-induced component A-deficient mutants was observed to retain partial hemolytic (16) and bacteriolytic (25) activities (perhaps attributable to a leaky mutation in A or some level of residual A activity). The heat resistance of the pAD1-encoded Hly/Bac and the inactivity of pAD1-encoded component L in the absence of component A toward bacterial cells or erythrocytes demonstrate that although the two component hemolysins may bear some similarities, they appear to be distinct species.

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LITERATURE CITED

1. **Ausubel, F. M., R. Brent, R. E. Kingston, D. D. Moore, J. G. Seidman, J. A. Smith, and K. Struhl.** 1987. Current protocols in molecular biology, p. 2.6.4–2.6.5. John Wiley & Sons, Inc., New York.
2. **Basinger, S. F., and R. W. Jackson.** 1968. Bacteriocin (hemolysin) of *Streptococcus zymogenes*. J. Bacteriol. **96**:1895–1902.
3. **Behnke, D., and M. S. Gilmore.** 1981. Location of antibiotic resistance determinants, copy control, and replication functions on the double-selective streptococcal cloning vector pGB301. Mol. Gen. Genet. **184**:115–120.
4. **Brock, T. D., and J. M. Davie.** 1963. Probable identity of group D hemolysin with bacteriocin. J. Bacteriol. **86**:708–712.
5. **Clewell, D. B.** 1985. Sex pheromones, plasmids, and conjugation in *Streptococcus faecalis*, p. 13–28. *In* H. O. Halvorson and A. Monroy (ed.), The origin and evolution of sex. Alan R. Liss, Inc., New York.
6. **Clewell, D. B.** 1981. Plasmids, drug resistance, and gene transfer in the genus *Streptococcus*. Microbiol. Rev. **45**:409–436.
7. **Clewell, D. B., E. Ehrenfeld, R. Kessler, Y. Ike, A. Franke, M. Madison, J. Shaw, R. Wirth, F. An, M. Mori, C. Kitada, M. Fujino, and A. Suzuki.** 1986. Sex pheromone systems in *Streptococcus faecalis*, p. 131–142. Branbury report 24. Antibiotic resistance genes: ecology, transfer, and expression. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
8. **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.
9. **Colmar, I., and T. Horaud.** 1987. *Enterococcus faecalis* hemolysin-bacteriocin plasmids belong to the same incompatibility group. Appl. Environ. Microbiol. **53**:567–570.
10. **Davie, J. M., and T. D. Brock.** 1966. Effect of teichoic acid on resistance to the membrane lytic agent of *Streptococcus zymogenes*. J. Bacteriol. **92**:1623–1631.
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., and D. B. Clewell.** 1975. Transmissible toxin (hemolysin) plasmid in *Streptococcus faecalis* and its mobilization of a noninfectious drug resistance plasmid. J. Bacteriol. **124**:784–790.
13. **Dunny, G., 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.
14. **Ehrenfeld, E. E., and D. B. Clewell.** 1987. Transfer function of *Streptococcus faecalis* plasmid pAD1: organization of plasmid DNA encoding response to sex pheromone. J. Bacteriol. **169**: 3473–3481.
15. **Ehrenfeld, E. E., R. E. Kessler, and D. B. Clewell.** 1986. Identification of pheromone-induced surface proteins in *Streptococcus faecalis* and evidence of a role for lipoteichoic acid in formation of mating aggregates. J. Bacteriol. **168**:6–12.
16. **Granato, P. A., and R. W. Jackson.** 1969. Bicomponent nature of lysin from *Streptococcus zymogenes*. J. Bacteriol. **100**: 865–868.
17. **Granato, P. A., and R. W. Jackson.** 1971. Characterization of the A component of *Streptococcus zymogenes* lysin. J. Bacteriol. **107**:551–556.
18. **Granato, P. A., and R. W. Jackson.** 1971. Purification and characterization of the L component of *Streptococcus zymogenes* lysin. J. Bacteriol. **108**:804–808.

19. **Hanahan, D.** 1985. Techniques for transformation of *E. coli*, p. 109–136. *In* D. M. Glover (ed.), DNA cloning, vol. 1. A practical approach. IRL Press, Ltd., Oxford, England.
20. **Ike, Y., and D. B. Clewell.** 1987. High incidence of hemolysin production by *Streptococcus faecalis* strains associated with human parenteral infections: structure of hemolysin plasmids, p. 159–164. *In* J. Ferretti and R. Curtiss (ed.), Streptococcal genetics. American Society for Microbiology, Washington, D.C.
21. **Ike, Y., and D. B. Clewell.** 1984. Genetic analysis of pAD1 pheromone response in *Streptococcus faecalis* using transposon Tn*917* as an insertional mutagen. J. Bacteriol. **158:**777–783.
22. **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.
23. **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.
24. **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.
25. **Jackson, R. W.** 1971. Bacteriolysis and inhibition of gram-positive bacteria by components of *Streptococcus zymogenes* lysin. J. Bacteriol. **105:**156–159.
26. **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.
27. **Maniatis, T., E. F. Fritsch, and J. Sambrook.** 1982. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
28. **Shaw, J. H., and D. B. Clewell.** 1985. Complete nucleotide sequence of macrolide-lincosamide-streptogramin B-resistance transposon Tn*917* in *Streptococcus faecalis*. J. Bacteriol. **164:** 782–796.
29. **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.
30. **Tomich, P. K., F. Y. An, S. P. Damle, and D. B. Clewell.** 1979. Plasmid-related transmissibility and multiple drug resistance in *Streptococcus faecalis* subsp. *zymogenes* strain DS16. Antimicrob. Agents Chemother. **15:**828–830.
31. **Yagi, Y., R. Kessler, J. Shaw, D. Lopatin, F. An, and D. Clewell.** 1983. Plasmid content of *Streptococcus faecalis* strain 39-5 and identification of a pheromone (cPD1) induced surface antigen. J. Gen. Microbiol. **129:**1207–1215.
32. **Yanisch-Perron, C., J. Vieira, and J. Messing.** 1985. Improved M13 phage cloning vectors and host strains: nucleotide sequences of the M13mp18 and pUC19 vectors. Gene **33:**103–119.