Identification of Pheromone-Induced Surface Proteins in *Streptococcus faecalis* and Evidence of a Role for Lipoteichoic Acid in Formation of Mating Aggregates

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The conjugative transfer of the *Streptococcus faecalis* plasmid pAD1 is characterized by a 10,000-fold increase in frequency following sex pheromone (cAD1) induction. Before the increase in plasmid transfer, donor cells synthesize a proteinaceous adhesin that facilitates the formation of mating aggregates. Four novel surface proteins appearing after exposure of pAD1-containing cells to sex pheromone have been identified. Thirty minutes after induction, a 130-kilodalton (kDa) protein was detectable by Western blotting. A 74-kDa protein, the major species present, and a pair of bands at 153 and 157 kDa were evident 45 min after induction. Induced cells containing another conjugative *S. faecalis* plasmid, pPD1, gave rise to three high-molecular-weight proteins of the same size (130, 153, and 157 kDa) as those synthesized by pAD1-containing cells. These proteins cross-reacted with antisera raised against induced cells containing pAD1. However, the major protein species produced by pPD1-containing cells had a molecular weight of 78,000 and did not cross-react significantly with the corresponding band of the pAD1 system. Pheromone-induced transfer of the two plasmids, when both were present in the same cell, was independent; induction was limited to the pheromone-specified plasmid. The possibility that lipoteichoic acid might act as a receptor (binding substance) for the induced adhesin protein was also explored. Free lipoteichoic acid (isolated from *S. faecalis*) inhibited clumping of induced cells, apparently by acting as a competitive inhibitor of the cellular binding substance.

Transfer of certain conjugative plasmids in *Streptococcus faecalis* is induced by a system of sex pheromones (8, 9). Potential recipient cells excrete small peptides which elicit a mating response in exposed donor cells. The pheromones are plasmid specific; when a cell acquires a certain plasmid, it shuts off the corresponding pheromone activity, but continues producing pheromones specific for different *S. faecalis* plasmids (7, 9). Exposure of donor cells to a pheromone for 90 min increases the frequency of transfer of resident plasmid DNA by about 4 orders of magnitude over that of the uninduced state (8, 9). This increase in capacity to donate DNA, which is chloramphenicol and rifampin sensitive, can be separated into two phases: surface changes, allowing donors and recipients to aggregate into clumps, and changes which allow the plasmid DNA to be transferred between the two cells (4; for a review, see reference 6).

The substance induced on the donor cell surface has been termed aggregation substance. This component, it is postulated, binds to a cell surface receptor, called binding substance. (Binding substance is presumed to be present on donors, as well as recipients, since self-clumping occurs upon induction by filtrates of recipients; the latter phenomenon is the basis of the pheromone assay.) The binding of the two surface determinants is dependent on the presence of divalent cations and phosphate (25). The formation of aggregates by induced cells containing the bacteriocin plasmid pPD1 was shown to be sensitive to trypsin, pronase, sodium dodecyl sulfate (SDS) (0.05%), and heat, whereas treatment

with lysozyme, lipase, and glutaraldehyde or the presence of a number of different sugars had no effect (25). Immunoelectron microscope studies of induced cells revealed the presence of a uniform coating of fuzzy surface material which was absent from uninduced cells (25). From these data, it was inferred that the aggregation substance was a protein.

The synthesis of novel proteins associated with the induction by pheromones was first noted in the case of the plasmid pPD1 (15, 25). Crossed immunoelectrophoresis revealed that the cell surface contains antigenic material not associated with uninduced cells. This substance is important for aggregation, as Fab fragments of antibody raised against induced cells inhibit the clumping of induced cells (25). A 78-kilodalton (kDa) protein is the predominant species induced in cells containing the plasmid. In another study involving the conjugative *S. faecalis* plasmid pCF10, Tortorello and Dunny found several higher-molecular-weight proteins induced by pheromones (19).

In this report, the synthesis of novel proteins associated with the induction of the hemolysin-bacteriocin plasmid pAD1 (5, 18) is examined. Earlier work, including fluorescent antibody staining, suggests that induced cells containing pAD1 produce a surface substance that cross-reacts immunologically with induced pPD1-containing cells (25). Evidence presented in this paper shows that, while cells carrying pAD1 or pPD1 produced some related proteins upon induction with pheromone, the major induced protein species were not identical. In addition, studies of the conjugative transfer of these two plasmids support the finding that the induction process is plasmid specific. Finally, we provide data bearing on the nature of the binding substance.

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TABLE 1. Bacterial strains and plasmids

Strain or plasmid	Relevant markers	Comments or references
Strains		
FA2-2	<i>rif fus</i>	5
JH2-2	<i>rif fus</i>	12
39-5	<i>str</i>	Original isolate of pPD1-carrying strain (11)
OG1-10	<i>str</i>	5
OG1X	<i>gel str</i>	Gelatinase-negative derivative of OG1-10 (11)
Plasmids		
pAD1	Hemolysin-bacteriocin, responds to cAD1	57-kb ^a conjugative plasmid from <i>S. faecalis</i> DS16 (5, 18)
pPD1	Bacteriocin, responds to cPD1	54-kb conjugative plasmid from <i>S. faecalis</i> 39-5 (17, 25)
pAM714	Hemolysin-bacteriocin, responds to cAD1, <i>erm</i>	pAD1::Tn917 (10)
pAM351	Bacteriocin, responds to cPD1, <i>tet</i>	pPD1::Tn916 (11)
pAM378	Responds to cAM373, <i>tet</i>	pAM373::Tn918 (pAM373 is a 36-kb conjugative plasmid from <i>S. faecalis</i> RC73) (3)

^a kb, kilobase.

MATERIALS AND METHODS

Bacteria, media and reagents. The bacterial strains and plasmids used in this study are listed in Table 1. All strains were grown in N2GT (Oxoid Ltd., London, England; nutrient broth no. 2 supplemented with Tris buffer [pH 7.5] and glucose [0.2%]) at 37°C and were monitored with a Klett-Summerson colorimeter (no. 54 filter, Klett Mfg. Co., Long Island City, N.Y.). Antibiotics were used at the following concentrations: tetracycline, 8 µg/ml; erythromycin, 10 µg/ml; streptomycin, 1 mg/ml; rifampin, 25 µg/ml; and fusidic acid, 25 µg/ml. The sources of reagents used in this study have been previously described (5, 9, 10, 25).

Pheromone preparations. Pheromone preparations were made as previously described (9) by centrifugation and filtration of late-log-phase cell cultures. The culture filtrates were autoclaved for 5 min to destroy proteases. cPD1 was prepared from JH2-2(pAM714) and was devoid of cAD1. Conversely, cAD1 was prepared from JH2-2(pAM351) and was devoid of cPD1. cPD1 + cAD1 was prepared from the plasmid-free JH2-2.

Extraction of surface proteins. Extraction of surface proteins was done by a modification of the method described by Kessler and Yagi (15). Broth cultures (30 to 100 ml) of *S. faecalis* cells were grown to 30 Klett units and were exposed to equal volumes of pheromone preparations for 90 min. The induced cells were exposed to 50 mM EDTA, washed as previously described (15), and suspended in 1% of the original volume in either 0.2% SDS (Bio-Rad Laboratories, Richmond, Calif.), Zwittergent 3-12 (Calbiochem-Behring, La Jolla, Calif.), or Triton-X (Sigma Chemical Co., St.

Louis, Mo.) in physiological-buffered saline (0.14 M NaCl, 1.5 mM KH₂PO₄, 15 mM Na₂HPO₄ · 7H₂O, 2.7 mM KCl, pH 7.4). The mixture was vortexed for 1 h at room temperature in a mixer (no. 5432; Eppendorf Geratebau, Netheler + Hinz GmbH, Hamburg, Federal Republic of Germany). The cells were pelleted, and the supernatant was dialyzed overnight at 4°C against 4 liters of 50 mM Tris, pH 7.5, and then stored at -20°C. Protein samples applied to gels were normalized to cell culture volumes.

Preparation of antisera. Glutaraldehyde-fixed pheromone-induced FA2-2(pAM714) and 39-5 cells were used to immunize New Zealand White rabbits as previously described (15). Sera from two rabbits were pooled for each cell type and were stored at -20°C.

SDS-PAGE and Western blotting. The SDS-polyacrylamide gel electrophoresis (PAGE) system used was that of Laemmli (16). Electrophoresis was done with 10% polyacrylamide in the resolving gel and with 5% polyacrylamide in the stacking portion of the gel. Gels were fixed, stained, destained, and dried before being photographed. The gels were fixed and destained in a 7% acetic acid-25% isopropanol solution. Staining was done with Coomassie brilliant blue R-250 (Bio-Rad; 0.2% in the acetic acid-isopropanol solution used above). Protein standards used were from a low-molecular-weight kit (Pharmacia Fine Chemicals, Piscataway, N.J.) and a high-molecular-weight kit (Bio-Rad).

For Western blotting, proteins were separated by SDS-PAGE and then electrophoretically transferred to nitrocellulose filters (20) in a buffer consisting of 25 mM Tris-192 mM glycine (pH 8.3; 20% [vol/vol] with methanol), using a Transphor apparatus (Hoefer Scientific Instruments, San Francisco, Calif.). Enzyme-linked immunosorbent assay of the filters was done by using the directions and reagents from an Immun-blot GAR-HRP (Bio-Rad) assay kit. The filters were soaked overnight in the initial blocking solution (3.0% gelatin in Tris-buffered saline [20 mM Tris {pH 7.5} plus 0.5 M NaCl]). Primary antisera concentrations ranged from 1:100 to 1:500 in Tris-buffered saline-1% gelatin. The filters were washed twice for 10 min each in Tris-buffered saline with 0.05% Tween 20 (Sigma). Horseradish-peroxidase-linked goat anti-rabbit immunoglobulin and the color development kit from the GAR-HRP assay system were used according to the directions of the manufacturer.

Conjugation of plasmids from induced strains. An overnight culture of OG1-10(pAM714, pAM351) was diluted 1:10 into a 1:1 mixture of N2GT plus one of the four pheromone preparations (containing activity specific for pAD1, pPD1, both pAD1 and pPD1, or neither plasmid [uninoculated broth]). The donor cells were induced for 1 h before addition to the recipient cells (FA2-2). The mating mixture contained a 1:10 dilution of the induced donor cells and a 1:10 dilution of the overnight culture of recipient cells in 20 ml of N2GT. After a 15-min incubation at 37°C, the mating mixture was pelleted and suspended in 1/16 of the original volume. Then the cells were serially diluted (0.5 into 4.5 ml) and were spread (0.1 ml) on antibiotic-containing plates to select for donors (on streptomycin, tetracycline, and erythromycin), recipients (on rifampin and fusidic acid), transconjugates containing pAM714 (on rifampin, fusidic acid, and erythromycin), transconjugates containing pAM351 (on rifampin, fusidic acid, and tetracycline), and transconjugates with both plasmids (on rifampin, fusidic acid, erythromycin, and tetracycline).

Purification of LTA. Lipoteichoic acid (LTA) was obtained by aqueous phenol extraction (21, 23) of strain JH2-2 and by

FIG. 1. SDS-PAGE showing the time course of induction of cells containing pAM714. Cell surface protein extracts (70- μ l samples) were applied to a 10% polyacrylamide gel and stained with Coomassie brilliant blue R-250 (A) or transferred to a nitrocellulose membrane for enzyme-linked immunosorbent assay (B). Primary antisera [raised against induced FA2-2(pAM714)] were diluted 1:100, and the secondary antisera (goat antirabbit) were diluted 1:3,000. For both analyses, protein samples were taken at the following times: lanes a (uninduced) and b (induced), 0 min; lanes c (uninduced) and d (induced), 15 min; lanes e (uninduced) and f (induced), 30 min; lanes g (uninduced) and h (induced), 45 min; lanes i (uninduced) and j (induced), 60 min; lanes k (uninduced) and l (induced), 75 min; lanes m (uninduced) and n (induced), 90 min. Lanes x and y show the molecular size standards 200, 116.5, 92.5, 66.2, and 45 kDa.

chromatography (ACA 22; LKB Instruments Inc., Gaithersburg, Md.) as previously described (13). The details of analytical procedures for determining chemical composition have been previously published (13, 14). The phosphorus concentration was 3.6 μ mol/mg (dry weight) of LTA. The phosphorus-to-glucose-to-alanine molar ratio was 1.0:0.29:0.34. Other than glucose, only trace amounts of glucosamine (<0.03% of dry weight) were detected by gas-liquid chromatography. Total amino acid content, excluding alanine, was <0.5% of the dry weight.

Cell reaggregation assay for testing the effect of LTA. Cultures (20 ml) of OG1X(pAM714) and OG1-10(pAM351) were induced to clump by exposure to equal volumes of the appropriate pheromone for 90 min. Then the cells were dissociated in the presence of 50 mM EDTA, washed, and resuspended in one-half of the original volume in 50 mM Tris, pH 7.5 (25). LTA then was serially diluted by twofold increments through the induced-dissociated cells in the wells of a microtiter plate. Next, solutions containing divalent cations (pH 7.5, final concentrations of 0.25 mM CaCl_2 and 10 mM Na_2HPO_4 or 0.25 mM MgCl_2 and 10 mM Na_2HPO_4) were added to allow reclumping. The cells were shaken at 200 rpm at 37°C for 3 h before aggregate formation was quantified.

RESULTS

Induction of novel surface proteins by *S. faecalis* OG1X(pAM714). The synthesis of pheromone-induced proteins by cells containing pAM714 (a derivative of pAD1 carrying a Tn917 insertion [10]) was analyzed by SDS-PAGE and Western blotting. Zwittergent 3-12 extracts were prepared from cells at 15-min intervals during a 90-min exposure to a cAD1-containing filtrate [from a culture of JH2-

2(pAM351)] or a control filtrate devoid of cAD1 [from JH2-2(pAM714)]. (pAM351 is a pPD1::Tn916 derivative [11]; cells containing it excrete cAD1 but not cPD1.) Extracts from equal quantities of cells were run on polyacrylamide gels and were stained with Coomassie brilliant blue. The major protein synthesized during the induction period was a 74-kDa protein (Fig. 1A). This is slightly smaller than the reported size of the major species of protein induced in pPD1-containing cells (15). Designated AD74, the protein was first visible after 45 min (lane h) and became prominent at 60 min (lane j), when aggregation of the induced cells was first observed. The cells exposed to the culture filtrate without cAD1 neither expressed novel surface proteins nor formed aggregates.

The induction of AD74 was also seen in a Western blot (Fig. 1B) of an identical gel with antisera raised against induced FA2-2(pAM714) cells. In addition to AD74, three other novel bands appeared during the induction period. The first, a 130-kDa band (AD130), was detected at 30 min (lane f, barely visible), and at 45 min a pair of bands at 153 (AD153) and 157 kDa (AD157) became apparent (lane h). At the later time points there seemed to be a slight smearing of protein at molecular weights below 74,000, which may be attributed to degradation products of the AD74 band (lanes l and n).

Comparison of pAM714 and pAM351 with regard to inducible surface proteins. *S. faecalis* OG1X(pAM714), OG1-10(pAM351), and OG1-10(pAM714, pAM351) were exposed to each of the following culture filtrate pheromone preparations: cAD1, cPD1, cAD1 + cPD1 (see Materials and Methods), and uninoculated broth. After 2 h, the surface proteins were extracted and analyzed by SDS-PAGE.

The first gel (Fig. 2A), stained with Coomassie brilliant blue, showed the AD74 protein band from pAM714-

FIG. 2. SDS-PAGE of induced proteins from pAM714- and pAM351-containing cells. Cell surface protein extracts (50- μ l samples) applied to a 10% polyacrylamide gel and stained with Coomassie brilliant blue (A) or analyzed by enzyme-linked immunosorbent-assay with antisera raised against cells carrying pAM714 (B) or pPD1 (C). In panel A the AD74 bands (lanes b, d, j, and l) are marked (●). In panels B and C primary antisera were diluted 1:100, and secondary antisera were diluted 1:3,000. Extracts from OG1X(pAM714) were exposed to plain broth (lane a), cAD1 + cPD1 (lane b), cPD1 (lane c), and cAD1 (lane d). Extracts from OG1-10(pAM351) were exposed to plain broth (lane e), cAD1 + cPD1 (lane f), cPD1 (lane g), and cAD1 (lane h). Extracts from OG1-10(pAM714, pAM351) were exposed to plain broth (lane i), cAD1 + cPD1 (lane j), cPD1 (lane k), and cAD1 (lane l). Lane m (panel A) shows molecular size standards 94, 67, 43, and 30 kDa.

containing cells induced with cAD1 (lanes b and d, band marked with a dot), but the band was absent when the cells were exposed to cPD1 or pheromone-free media (lanes c and a, respectively). Exposure of OG1-10(pAM351) to cPD1 (lanes f and g), but not to cAD1 (lane h), stimulated the production of the previously described 78-kDa protein (PD78). In the strain which contained both plasmids, exposure to the single pheromones induced the expected AD74 or PD78 bands (lanes l and k, respectively), and when a filtrate containing both cPD1 and cAD1 was added (lane j), both AD74 and PD78 were observed.

Figure 2B represents a gel identical to that in Fig. 2A that was transferred to a nitrocellulose membrane and was incubated with antisera raised against pheromone-induced FA2-2(pAM714). As in Fig. 1B, Fig. 2B shows a prominent 74-kDa band and three minor bands in the 130- to 157-kDa range for extracts of pAM714-carrying strains induced with cAD1 (lanes b and d). This strain, when exposed to the filtrate devoid of cAD1, did not give rise to these bands (lanes a and c). Lanes e and h show the pAM351-carrying strains when uninduced and exposed to cAD1, respectively; as expected, no new bands were seen. However, when the pAM351 strain was exposed to cPD1, PD78 (the major species in the Coomassie-brilliant-blue-stained gel) was not visible, but the 130- to 157-kDa bands were present (lanes f and g). This pattern held true when both plasmids were present in the same cell. If pAM714 was induced, both the high-molecular-weight bands and the lower-molecular-weight band (AM74) were present (lanes j and l), but when cPD1 was used to induce, only the high-molecular-weight bands were visible (lane k).

The converse expression of protein-antibody complexes was visible (Fig. 2C) when the primary antisera used in Western blotting were raised against an induced strain containing pPD1 (39-5). OG1X(pAM351) induced with cPD1 showed the characteristic PD78 band (lanes f and g) as well as the higher-molecular-weight bands (PD130, PD153, and PD157). When cells harboring only pAM714 were induced, the 130- to 157-kDa bands were seen as well as a light 74-kDa band, which we believe was due to partial cross-reactivity with the antisera (lanes b and d). The expression of only the high-molecular-weight bands was seen on blots done at lower antiserum concentrations (data not shown).

Thus, the three high-molecular-weight bands were seen whenever either of the plasmids was induced with its specific pheromone, and these proteins cross-reacted indistinguishably with antisera made against pAM714- or pPD1-induced cells. A third conjugative *S. faecalis* plasmid, pAM378, a pAM373::Tn918 derivative (3) present in strain FA2-2, also was tested for the induction of novel surface proteins. This strain produced the three 130- to 157-kDa bands upon stimulation with a specific pheromone and analysis with antisera raised against induced pAM714-containing cells (data not shown).

Comparison of surface proteins extracted with different detergents. Cell surface extracts of pAM714- and pAM351-containing cells were prepared using three different detergents. SDS (anionic), Zwittergent 3-12 (dipolar ionic), and Triton-X (nonionic) were used in each case at a concentration of 0.2% in phosphate-buffered saline to extract cell surface proteins after a 2-h induction period. Figure 3 shows a Coomassie-brilliant-blue-stained PAGE of the preparations. Comparison of the induced preparations for SDS (lane b), Zwittergent 3-12 (lane d), and Triton-X (lane f) for pAM714 showed that, in reference to the background bands (those expressed at similar levels throughout), AD74 was

extracted significantly better with Zwittergent 3-12 or Triton-X. In contrast, preparations of pAM351-containing cells showed higher levels of the analogous protein, PD78, when extracted with SDS or Zwittergent 3-12 (lanes i and k, respectively). This difference in extraction with different detergents further distinguished the AD74 and PD78 proteins.

Conjugative transfer from induced cells containing two plasmids. OG1-10(pAM714, pAM351) cells were induced for 1 h with pheromone preparations containing cAD1, cPD1, cAD1 + cPD1, or no activity, before a short (15 min) mating with the plasmid-free recipient strain FA2-2. Table 2 shows the results from two independent experiments (trials 1 and 2). Increased levels of transfer of pAM714 occurred only after pretreatment with cAD1, and only transfer of pAM351 was stimulated after induction of the donor strain with cPD1. pAM351 transfer was also increased after exposure to cAD1; the frequency, however, was at least 2 orders of magnitude below that which occurred upon cPD1 exposure. This induction to an intermediate level of transfer was reflected in the cotransfer frequency after cAD1 induction in trial 2 but not in trial 1. Cotransfer of the plasmids was seen at elevated frequencies only when a pheromone preparation containing activity for both plasmids was used.

LTA as a possible binding substance. To determine whether LTA might represent the proposed binding substance, this material was tested for the ability to competitively inhibit aggregate formation in clumping assays of pheromone-

TABLE 2. Transfer of pAM714 and pAM351 from strain OG1-10 to FA2-2

Trial	Pheromone activity	Frequency ^a		
		pAM714/donor	pAM351/donor	pAM714 + pAM351/donor
1	None	2.6×10^{-5}	5.1×10^{-8}	$<5.1 \times 10^{-9}$
	cAD1 + cPD1	1.8×10^{-1}	8.8×10^{-3}	9.3×10^{-5}
	cPD1	2.7×10^{-5}	1.8×10^{-2}	1.5×10^{-7}
	cAD1	2.2×10^{-1}	1.3×10^{-5}	$<1.8 \times 10^{-8}$
2	None	2.5×10^{-3}	9.8×10^{-7}	$<4.3 \times 10^{-9}$
	cAD1 + cPD1	5.1×10^{-1}	1.8×10^{-1}	1.4×10^{-2}
	cPD1	4.8×10^{-4}	1.3×10^{-1}	2.0×10^{-5}
	cAD1	1.1×10^{-1}	7.0×10^{-5}	1.7×10^{-6}

^a Number of transconjugants containing the indicated plasmid per total number of donor cells. Induction was for 60 min and was followed by a 15-min mating.

treated cells. Cells containing pAM714 or pAM351 were induced with the respective pheromone. After 90 min, the clumped cells were exposed to EDTA to chelate the divalent cations needed for cell aggregation (25). The dissociated donor cells were washed and resuspended in 50 mM Tris (pH 7.5) and then were placed in the wells of a microtiter plate. LTA, extracted from *S. faecalis* JH2-2, was serially diluted through the cells from a concentration of 5.0×10^2 to 1×10^{-1} μ g/ml before one of the two divalent cation-phosphate solutions was added to allow reclumping. In the presence of Mg^{2+} , the cells reassociated in the presence of LTA only at a concentration below 0.12 μ g/ml for pAM351-containing cells and below 1.0 μ g/ml for pAM714-containing cells. The cells reassociated in the presence of Ca^{2+} only at an LTA concentration below 4.0 μ g/ml for cells containing pAM351 and below 16.0 μ g/ml for those cells with pAM714. In the absence of the divalent cation-phosphate solution no reclumping was observed.

DISCUSSION

Exposure of pAM714-containing cells to the sex pheromone cAD1 stimulated the production of at least four novel proteins on the cell surface. These proteins were detected in cell surface protein extracts 45 to 60 min after the addition of the pheromone to the culture. The protein with the lowest molecular weight, AD74, appeared to have the highest relative concentration. Three higher-molecular-weight proteins, AD130, AD153, and AD157, were detected only when immunoblotting techniques were used.

Comparison of proteins produced by induced pAM714-containing cells with induced cells carrying pAM351 showed differences and similarities between the two systems. Induction of pAM351-containing cells gave rise to a major 78-kDa band (PD78), similar in size to the AD74 band produced by cells containing pAM714. Under the conditions of extraction, the PD78 protein yield was considerably higher than that of AD74. Some antigenic cross-reactivity between these two bands was detected in Western blotting, but only if high concentrations of antisera were used. Both proteins were produced by cells containing both plasmids when exposed to a filtrate containing both cAD1 and cPD1. It is possible that the functions of these two proteins are analogous, and since they differ in size and other characteristics (e.g., antigenic determinants and extractability with different detergents), their genetic determinants are likely to be plasmid borne. Tortorello and Dunny (19) reported that induced cells carry-

FIG. 3. SDS-PAGE comparing different protein extractions of cells containing pAM714 or pAM351. Detergent extracts of cell-surface proteins applied to a 10% polyacrylamide gel and stained with Coomassie brilliant blue. Lanes show proteins from OG1X(pAM714): a (uninduced) and b (induced), extracted with SDS; c (uninduced) and d (induced), extracted with Zwittergent 3-12; e (uninduced) and f (induced), extracted with Triton-X. Proteins from OG1-10(pAM351) are shown in lanes h to m in the same order of induction and extraction as lanes a to f. Lane g shows molecular size standards 200, 116.5, 92.5, 66.2, and 45 kDa.

ing pCF10 produce a 73-kDa protein (seen in Zwittergent 3-12 extracts), which may also be analogous to AD74.

The higher-molecular-weight bands were produced by the induced cells harboring either pAM714 or pAM351. The two sets of bands were of the same molecular weight and appeared essentially identical. These bands were also visible when a third plasmid (pAM378), unrelated to the other two, was induced to clump in a different cell background. The high-molecular-weight bands were not evident in the initial report on protein induction involving pPD1 (15). However, the cross-reaction of induced cells containing pPD1 and pAD1 revealed by fluorescent antibody staining (25) may be related to these proteins.

A number of high-molecular-weight surface antigens have been identified in concentrated culture supernatant of induced cells carrying the conjugative plasmid pCF10 (19). These proteins are in the range of 125 to 130 kDa. Although induction of pAM714 resulted in the production of one protein about this size (AD130), the other two proteins identified were larger. These differences in induced proteins produced by isogenic strains carrying pAM714 or pCF10 suggests that the high-molecular-weight bands may also be plasmid encoded. However, the differences also could be explained by variations in the techniques used to extract and identify the proteins.

The transfer of two plasmids (pAM714 and pAM351) from a common host cell was also examined. The transfer of the individual plasmids was dependent on induction by the specific pheromone; there was little apparent *trans* complementation of transfer functions between the two plasmids. The low-level complementation of mating functions that did occur (in both trials) increased the transfer frequency of pAM351 after induction by cAD1. This increase in transfer frequency occurred unidirectionally, as there was no corresponding increase in pAM714 transfer after treatment with cPD1. However, for all pheromone preparations (except cAD1 induction in trial 1), the frequency of cotransfer occurred at a level corresponding to the product of the individual transfer frequencies, implying that the transfer of each plasmid occurred independently.

The pheromone response model (9) predicts that an induced aggregation substance will bind specifically to a binding substance present on both recipient and donor cells. LTA is present on the cell surface of many gram-positive bacteria, including *S. faecalis* (24). LTA is important in the binding of Lancefield group A streptococci to epithelial cells because of the ability of free LTA or anti-LTA antibody to inhibit binding (1). Specific receptors for bacterial LTA have been found on a number of types of eucaryotic cells (2).

Inhibition of aggregation by relatively low concentrations of LTA (less than 1 µg/ml) in this study suggests a role for cell-associated LTA in aggregation, possibly as a binding substance. The LTA of *S. faecalis* and the other group D streptococci is the group antigen and thus differs serologically from other LTAs (22). Further studies with LTAs of different chemical structures and with deacylated LTA will be required to probe the specificity of this inhibition and to strengthen the apparent identification of a binding substance as LTA.

Other data consistent with LTA as a putative binding substance come from an earlier report (25) that, at pH 2.5, aggregation of induced pPD1-containing cells occurs in the absence of divalent cations and phosphate. Conceivably this could relate to a significant reduction of the negative charge of the phosphate residues present in the LTA backbone. At physiological pHs the phosphate and divalent cations might

act to stabilize the association of aggregation substance and highly negatively charged LTA.

The concentration of LTA needed to inhibit clumping of pAM714-containing cells was at least fourfold higher than the concentration needed for cells with pAM351. Regarding the dichotomy in response to competitive inhibition of LTA, it is also noted that pheromone-induced clumping of pAM351-containing cells generally gives rise to larger flakes of aggregated cells, compared with the more grainy appearance of the pAM714-containing aggregates. One could speculate that these differences relate to the two proteins AD74 and PD78 that distinguish the two systems and that these proteins represent two similar aggregation substances.

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LITERATURE CITED

1. Beachey, E. H., and I. Ofek. 1976. Epithelial cell binding of group A streptococci by lipoteichoic acid on fimbriae denuded of M protein. *J. Exp. Med.* **143**:759-771.
2. Beachey, E. H., W. A. Simpson, I. Ofek, D. L. Hasty, and J. B. Dale. 1982. Role of surface polysaccharides in bacterial adhesion, p. 327-330. In D. Schlessinger (ed.), *Microbiology*—1982, American Society for Microbiology, Washington, D.C.
3. Clewell, D. B., F. Y. An, B. A. White, and C. Gawron-Burke. 1985. *Streptococcus faecalis* sex pheromone (cAM373) also produced by *Staphylococcus aureus* and identification of a conjugative transposon (Tn918). *J. Bacteriol.* **162**:1212-1220.
4. Clewell, D. B., and B. L. Brown. 1980. Sex pheromone cAD1 in *Streptococcus faecalis*: induction of a function related to plasmid transfer. *J. Bacteriol.* **143**:1063-1065.
5. 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 Tn917. *J. Bacteriol.* **152**:1220-1230.
6. Clewell, D. B., B. A. White, Y. Ike, and F. An. 1984. Sex pheromones and plasmid transfer in *Streptococcus faecalis*, p. 133-149. In R. Losick and L. Shapiro (ed.), *Microbial development*. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
7. Clewell, D. B., Y. Yagi, Y. Ike, R. A. Craig, B. L. Brown, and F. An. 1982. Sex pheromones in *Streptococcus faecalis*: multiple pheromone systems in strain DS5, similarities of pAD1 and pAMy1, and mutants of pAD1 altered in conjugative properties, p. 97-100. In D. Schlessinger (ed.), *Microbiology*—1982. American Society for Microbiology, Washington, D.C.
8. 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.
9. Dunny, G. M., R. A. Craig, R. L. Carron, and D. B. Clewell. 1979. Plasmid transfer in *Streptococcus faecalis*. Production of multiple sex pheromones by recipients. *Plasmid* **2**:454-465.
10. Ike, Y., and D. B. Clewell. 1984. Genetic analysis of the pAD1 pheromone response in *Streptococcus faecalis*, using transposon Tn917 as an insertional mutagen. *J. Bacteriol.* **158**:777-783.
11. Ike, Y., R. Craig, B. White, Y. Yagi, and D. Clewell. 1983. Modification of *Streptococcus faecalis* sex pheromones after acquisition of plasmid DNA. *Proc. Natl. Acad. Sci. USA* **80**:5369-5373.
12. Jacob, A. E., and S. J. Hobbs. 1974. Conjugal transfer of plasmid-borne multiple antibiotic resistance in *Streptococcus faecalis* var. *zymogenes*. *J. Bacteriol.* **117**:360-372.

13. Kessler, R. E., and B. H. Thivierge. 1983. Effects of substitution on polyglycerol phosphate-specific antibody binding to lipoteichoic acids. *Infect. Immun.* **41**:549-555.
14. Kessler, R. E., I. van de Rijn, and M. McCarty. 1979. Characterization and localization of the enzymatic deacylation of lipoteichoic acid in group A streptococci. *J. Exp. Med.* **150**:1498-1509.
15. Kessler, R. E., and Y. Yagi. 1983. Identification and partial characterization of a pheromone-induced adhesive surface antigen of *Streptococcus faecalis*. *J. Bacteriol.* **155**:714-721.
16. Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature (London)* **227**:680-685.
17. Rosan, B., and N. Williams. 1964. Hyaluronidase production by oral enterococci. *Arch. Oral Biol.* **9**:291-298.
18. 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.
19. Tortorello, M. L., and G. M. Dunny. 1985. Identification of multiple cell surface antigens associated with the sex pheromone response of *Streptococcus faecalis*. *J. Bacteriol.* **162**:131-137.
20. Towbin, H., T. Staehelin, and J. Gordon. 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proc. Natl. Acad. Sci. USA* **76**:4350-4354.
21. Westphal, O., O. Luderitz, and F. Bister. 1952. Über die extraktion von bakterien mit phenol/wasser. *Z. Naturforsch. Teil B* **7**:148-155.
22. Wicken, A. J., S. D. Elliott, and J. Baddiley. 1963. The identity of streptococcal group D antigen with teichoic acid. *J. Gen. Microbiol.* **31**:231-239.
23. Wicken, A. J., J. W. Gibbens, and K. W. Knox. 1973. Comparative studies on the isolation of membrane lipoteichoic acid from *Lactobacillus fermenti*. *J. Bacteriol.* **113**:365-372.
24. Wicken, A. J., and K. W. Knox. 1974. Liptoteichoic acids: a new class of bacterial antigen. *Science* **187**:1161-1167.
25. Yagi, Y., R. E. Kessler, J. H. Shaw, D. E. Lopatin, F. An, and D. B. 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.