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Identification and Partial Characterization of a Pheromone-Induced Adhesive Surface Antigen of *Streptococcus faecalis*

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A surface protein antigen that is produced only during the induction of aggregation of *Streptococcus faecalis* was shown to contribute to and perhaps be primarily responsible for aggregation. The antigen is an immunodominant surface component of induced cells. F(ab) fragments of immunoglobulins specific for this antigen prevented aggregation, providing direct evidence that the antigen is an adhesin. Consistent with this proposed association was the coincident timing of appearance of the antigen and the timing of aggregation after induction.

Certain conjugative plasmids of *Streptococcus faecalis* transfer more readily in broth culture (frequency of 10^{-3} to 10^{-1} per donor) than others ($<10^{-6}$ per donor) (3). In those combinations of donors and recipients in which the higher frequency of transfer is observed, aggregation or clumping together of the cells is also observed (3, 7, 8). Some strains, including plasmid-free strains, excrete specific "clumping-inducing agents" (CIA) or sex pheromones which induce other strains carrying certain conjugative plasmids to become self-adherent and adherent to cells of the pheromone-producing strain (7). After aggregation of the "induced" donors and the pheromone-producing recipients, plasmid transfer takes place. A model of pheromone induction of the mating process has been developed (8) and reviewed, along with the current knowledge of the pheromones themselves (3).

Studies on the process of pheromone-induced aggregation have revealed that the response requires RNA and protein synthesis (7) as well as divalent cations and phosphate (22). Aggregation is sensitive to trypsin and pronase but not to nucleic acid hydrolases or mixed glycosidases (22). Immunofluorescence and immunoelectron microscopy studies show evidence of at least one unique immunodeterminant on the surface of induced cells (22). Different plasmids (pPD1, pAD1, pOB1, pAMγ1, pAMγ2 and pAMγ3) in different *S. faecalis* strains that are induced with the appropriate pheromones (i.e., pPD1 with cPD1) all appear to govern production of substances that react with antisera raised against cPD1-induced strain 39-5 cells (which contain pPD1) that had been absorbed with uninduced 39-5 cells.

By crossed immunoelectrophoresis (XIE) of extracts of induced and uninduced cells of strain 39-5, there appears to be a single unique antigen associated with induced cells (11, 22). This antigen has thus been considered a putative adhesin, that is, the aggregation substance referred to in the model (3, 8). The evidence presented here strongly supports this designation and partially characterizes the induced antigen as a protein with an apparent molecular weight of 78,000.

MATERIALS AND METHODS

Bacterial strains and culture conditions. *S. faecalis* 39-5 (obtained from B. Rosan) bears six plasmids: pPD2, pPD3, pPD4, and pPD6 are cryptic; pPD5 is conjugative and determines a hemolysin-bacteriocin activity; and pPD1 is conjugative and determines a bacteriocin activity, UV resistance, and response to a bacterial pheromone, cPD1 (3, 22). *S. faecalis* JH2-2 (obtained from A. Jacob) is plasmid-free and produces several pheromones, including cPD1 (8). Both strains were grown at 37°C in Oxoid nutrient broth no. 2 supplemented with 0.2% glucose and 0.1 M Tris (final pH, 7.4 ± 0.1) (N2GT broth) (8). The culture fluid from static growth of JH2-2 cells was used to prepare crude cPD1 by trichloroacetic acid precipitation (5% [wt/vol]) of cell-free culture fluid, resuspension of the resulting precipitate that was obtained by sedimentation at 6,000 × *g* for 30 min in distilled water at 1/100 the original volume, and neutralization with 6 N sodium hydroxide (R. Craig and D. B. Clewell, personal communication).

Growth was monitored turbidimetrically at 650 nm in 16-mm cuvettes with a Spectronic 21 (Bausch & Lomb, Inc., Rochester, N.Y.). Absorbance was corrected (19) to adjust optical density to a linear function with respect to culture mass (i.e., conform to Beer's law). For experiments in which strain 39-5 was to be induced to clump by the pheromone cPD1, the crude cPD1 was added to exponentially growing strain 39-5 in stirred cultures at adjusted optical densities of 100 to 150 (1 adjusted optical density unit = corrected optical density × 1,000). In most experiments, the amount of

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crude cPD1 added was approximately 16-fold in excess of the amount required for minimum clumping. For example, if a crude cPD1 preparation had a titer of 1,600, 10 ml would be used per liter of 39-5 culture.

Extraction methods. Harvesting of pheromone-aggregated cultures of strain 39-5 before extraction required the addition of EDTA at 0.025 M to dissociate cell aggregates (22). EDTA was removed by washing with 30 mM Tris-hydrochloride (pH 7.5) before extraction.

Large batches of pheromone-induced 39-5 cells were extracted at 1 to 2% cell (wt/vol) concentrations in 1% Triton X-100 (Research Products International, Mount Prospect, Ill.) in barbital-hydrochloride buffer (2.5 mM; pH 8.6) or, in later experiments, 0.2% Zwittergent 3-12 (Zw312) (Calbiochem, La Jolla, Calif.) in phosphate-buffered saline (PBS) (0.01 M phosphate, 0.85% NaCl). These extractions were carried out at ambient temperature for 30 to 60 min with constant agitation. Cells were removed from the extraction mixture by sedimentation. In most experiments, the cells were extracted again, and the two extracts were combined.

For comparison of extraction with various detergents, buffers, and buffer concentrations, the equivalent of approximately 5 mg of cells (dry weight) from a 39-5 culture that had been incubated in the presence of crude cPD1 for 90 min was resuspended in 0.9 ml of each buffer. While the extractions were vortexing, an additional 0.1 ml of buffer or 10-times-concentrated detergent in buffer was added dropwise. Each extraction mix was kept at ambient temperature (18°C) for 30 min and vortexed at 4- to 5-min intervals. After separation of the cells from the extraction liquids by sedimentation at 10,000 × *g* for 5 min at 4°C, the extracts were dialyzed extensively against distilled water and then stored at −20°C until analyzed.

Preparation of antisera. New Zealand white rabbits were injected intramuscularly with glutaraldehyde-fixed, pheromone-induced or uninduced 39-5 cells. Glutaraldehyde fixation was accomplished by adding 0.25 ml of 5% filter-sterilized glutaraldehyde to approximately 5×10^{10} cells in 1.6 ml of 30 mM Tris-hydrocholoride (pH 7.5) and 3.15 ml of PBS. After 40 min of incubation at ambient temperature, the cells were washed free of glutaraldehyde with PBS. The cells were resuspended in 10 ml of PBS, yielding approximately 5×10^9 cells per ml. Rabbits were injected intramuscularly with approximately 0.5 ml (2.5×10^9 cells) of fixed bacteria four times at 3-day intervals. After 2 weeks, and periodically thereafter (at 2- to 3-month intervals), they were boosted with the same inoculum. Blood was taken by venipuncture, checked by XIE, and, if satisfactory, pooled. In the present study two pools of serum from rabbits injected with pheromone-induced cells were used. Pool A consisted of sera from three rabbits, and pool B consisted of later bleedings from the same three rabbits plus sera from two additional rabbits. A pool of serum from two rabbits inoculated with uninduced cells was also used. The immunoglobulin fraction of each pool of serum that had been raised against pheromone-induced cells was prepared (9), concentrated approximately threefold, dialyzed against 0.1 M NaCl—15 mM NaN_3, and stored in 2.5-ml portions at −70°C. Each portion was thawed once and stored at 4°C thereafter.

Absorption of anti-pheromone-induced cell immunoglobulins. A 10-ml portion of anti-pheromone-induced cell immunoglobulins (pool A) was absorbed three times in succession with approximately 5×10^8 uninduced 39-5 cells and then two times in succession with approximately 2×10^8 uninduced 39-5 cells. This preparation of immunoglobulins reacted with pheromone-induced 39-5 cells but not with uninduced 39-5 cells by agglutination and by the indirect fluorescent antibody technique (22).

Preparation of F(ab) fragments. F(ab) fragments of absorbed anti-pheromone-induced cell immunoglobulins and a preimmune immunoglobulin pool were prepared by papain cleavage (17). Papain (Sigma Chemical Co., St. Louis, Mo.) was added 10% (wt/wt) to immunoglobulins in 0.05 M cysteine-hydrochloride—0.5 M sodium phosphate—0.1 M EDTA (pH 7) and incubated for 2 h at 37°C. After dialysis against 0.01 M borate buffer containing 0.9% NaCl (pH 9) in the cold (4°C) for 2 days, Fc crystals were removed by sedimentation. The papain was subsequently removed by filtration on Bio-Gel P-60 (1.5 by 25 cm) (Bio-Rad Laboratories, Richmond, Calif.), since it did not appear to be completely inactivated (see below). Undiluted, immune F(ab) fragments contained 3 mg of protein per ml. Undiluted, preimmune F(ab) fragments contained 1.4 mg of protein per ml before absorption with 39-5 cells and 0.5 mg of protein per ml after absorption with uninduced 39-5 cells.

Immunoelectrophoresis techniques. XIE (2) is a modification of the Laurell electroimmunoassay technique (15). Barbital-hydrochloride buffer (2.5 mM; pH 8.6) was used throughout the system. One percent agarose gels were prepared from Seakem HGT (Marine Colloids, Rockland, Maine) and cast on glass plates (50 by 50 mm) to give a volume-to-surface-area ratio of 0.132 ml/cm^2. Samples were applied in wells of minimal diameter, and unless otherwise noted, first-dimension electrophoresis was at 6 V/cm for 45 min. Second-dimension electrophoresis into antibody-containing agarose was at 2 V/cm for 16 to 18 h. The amount of antibody used for individual immunoplates varied and is given for each in the figure legends.

The methods of tandem XIE (12), XIE with intermediate gel (1), fused rocket immunoelectrophoresis (18), and rocket-line immunoelectrophoresis (13) have been described in detail elsewhere. All were performed in the buffer system described above without modification, except that an intermediate gel rather than a trough was used for rocket-line immunoelectrophoresis. XIE with sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) for first-dimension separation was performed as previously described (10).

All gels were alternately pressed and washed twice with 0.1 M NaCl. After a final press, a brief wash with a stream of distilled water, and drying, they were stained with Coomassie brilliant blue R-250 (21).

SDS-PAGE. The SDS-PAGE system used was that of Laemmli (14). Electrophoresis was carried out on 10% slab gels at 70 V until the dye marker entered the running gel and at 100 V until it reached within 4 mm of the gel bottom. Standards were purchased (low molecular weight kit) from Pharmacia Fine Chemicals, Piscataway, N.J. Gels were fixed and stained with Coomassie blue R-250 as described previously (20).

Microtiter plate assay of pheromone. A previously

TABLE 1. Comparison of extraction procedures

Extraction medium	Protein (μg/ml)	Carbohydrate (μg/ml)	Rocket ht[a] (mm)	Antigen: protein	Antigen: CHO
PBS (pH 7.3) (buffer A)	50	27	2.6	0.05	0.10
Barbital-hydrochloride (2.5 mM; pH 8.6) (buffer B)	77	14	3.0	0.04	0.21
1% Triton X-100 in buffer B	110	34	5.7	0.05	0.17
0.1% Triton X-100 in buffer B	98	38	4.1	0.04	0.11
2% Zw312 in buffer A	280	ND[b]	18.0	0.06	ND
2% Zw314 in buffer A	271	32	17.0	0.06	0.53
2% Zw316 in buffer A	204	36	7.2	0.04	0.20
0.1% LiDS in buffer A	367	58	20.0	0.05	0.34
4 M LiCl	19	7	0.8	0.04	0.11

[a] Rocket height after rocket immunoelectrophoresis of 5 μl of extract.
[b] ND, Not determined.

described microtiter plate assay (8) was used to determine pheromone titers and to assay the blocking ability of F(ab) preparations and different pheromone titers. For the latter, an additional control of borate buffer [in which F(ab) fragments were stored] was used. In general, the titer of pheromone diluted in the borate buffer was within one well of pheromone diluted in N2GT broth. However, the growth rate of strain 39-5 in the resulting N2GT-borate buffer was somewhat slower; plates required 3 h of incubation rather than the usual 2 h for clumping to be discernible.

Chemical assays. Protein was measured by the method of Lowry et al. (16), using the modification of Dulley and Grieve (6) when necessary. Carbohydrate was estimated by the phenol-sulfuric acid method (5).

RESULTS

Detection of a unique antigen in induced cells. The extraction of an antigen from cPD1-induced 39-5 cells but not uninduced 39-5 cells was demonstrated previously (11, 22) by XIE of detergent extracts of induced and uninduced cells. We subsequently compared various extraction procedures for their relative efficacy in removal of the induced antigen (Table 1). Extraction of induced antigen with low-ionic-strength buffers appeared to yield more induced antigen than did extraction at high salt concentration. The degree of extraction with buffer alone was variable from experiment to experiment, however, and detergents not only improved the yield but also the consistency of the yield. The potential problem of apparent differences in rocket height caused by detergent binding or differences in buffer concentration was ruled out in preliminary experiments. Lithium dodecyl sulfate is included for comparison in Table 1, but was not routinely used because of problems with denaturation and difficulty in removal that are usually associated with this ionic detergent. Triton X-100, rather than any of the Zwittergents, was used in early experiments since the antigen-to-protein ratio was as good, and the antigen-to-carbohydrate ratio was better with the former than the latter. It was subsequently determined that Zw312 was as efficient as Zw314 in extraction of antigen and could be used at 0.2% rather than 2%. The result of XIE of a Zw312 extract is shown in Fig. 1A. The induced antigen is contained within the prominent immunoprecipitate marked a. Immunoprecipitates b and c were very faint and did not reproduce well. Immunoprecipitate a was the major one seen after XIE of a variety of extracts into sera from six of six rabbits immunized with fixed, induced 39-5 cells and bled at different times after immunization. No extract of uninduced cells, including ionic detergent extracts, yielded this immunoprecipitate. Whether this antigen was unique to induced cells or was simply not extracted in detectable quantity was determined by the combined results of several approaches. First, an anti-induced-cell immunoglobulin preparation was used in XIE of detergent extracts in an intermediate gel (Fig. 1B) and in a main gel (Fig. 1E). The only immunoprecipitate remaining when absorbed pool A was used in the main gel (Fig. 1E) and the only one removed completely from a main gel containing unabsorbed pool A by an intermediate gel containing absorbed pool A (Fig. 1B) was the major immunoprecipitate seen in the control (Fig. 1A). The baseline immunoprecipitate contiguous with c in Fig. 1B indicates that absorbed pool A contains c that was released during absorption. Second, the results of crossed-line immunoelectrophoresis (Fig. 1C) and tandam XIE (Fig. 1D) confirmed that no induced antigen was contained in uninduced cell extracts. That is, in Fig. 1C there was no baseline immunoprecipitate formed that was contiguous with the major immunoprecipitate, but all other immunoprecipitates exhibited contiguous baselines. This is not easily seen in the photograph; however, it can be seen that the feet of all immunoprecipitates are splayed outward and do not extend as far down as in the control. By tandem XIE

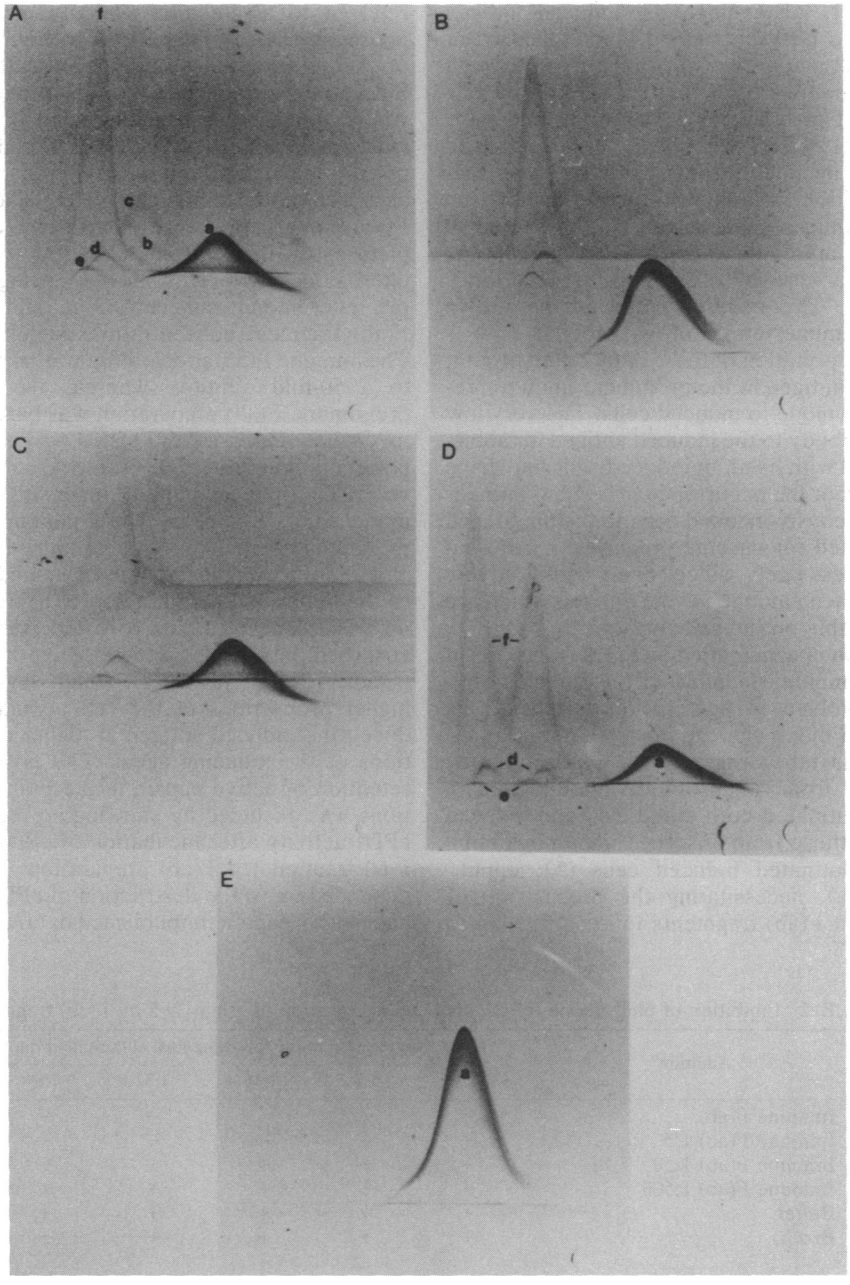

FIG. 1. XIE analysis to demonstrate that the induced antigen was unique to cPD1-induced cells. Zw312 (0.2%) extracts of uninduced and induced 39-5 (39-5I) cells (approximately 0.2 and 0.3 mg of protein per ml, respectively) were used. (A) Control. 5 μl of 39-5I extract, intermediate gel of agarose only, and a main gel of 18 mg of protein per ml of anti-39-5I immunoglobulin pool B were used. (B) 5 μl of 39-5I extract, an intermediate gel of 14 mg of protein per ml of absorbed pool A, and a main gel of 18 mg of protein per ml of pool B were used. (C) 5 μl of 39-5I extract, an intermediate gel of 100 μl of Zw312 extract per ml of uninduced 39-5 cells, and a main gel of 18 mg of protein per ml of pool B were used. (D) Front well, 10 μl of uninduced 39-5 extract; rear well, 5 μl of 39-5I extract and a main gel of 18 mg of protein per ml of pool B. (E) 5 μl of 39-5I extract and a main gel of 14 mg of protein per ml of absorbed pool A were used. The anode was at the top and left.

(Fig. 1D), only the major immunoprecipitate lacked an identical counterpart in the uninduced cell extract. Also, since the induced antigen appeared to be such a good immunogen, relatively small amounts of this antigen on the cell surface (amounts too small to be directly detectable) might give rise to an antibody response. The sera of rabbits immunized with uninduced cells were included in intermediate gels so that we could look for antibody formed against the induced antigen. Evidence of a very small amount of antibody to the induced antigen, as shown by a very slight "inward feet reaction," was found. This reaction could not be shown with preimmune sera from two rabbits.

Our interpretation of these data, taken in sum, is that the antigen in the prominent immunoprecipitate is unique to induced cells. The very low titer of antibody to the induced antigen in rabbits immunized with fixed, uninduced cells may have resulted from the occurrence of a small number of spontaneously induced cells in the uninduced cultures used for vaccine preparation. Alternatively but less likely, all cells may constitutively produce trace amounts of the antigen which are not detectable on the cell surface.

Inhibition of aggregation by F(ab) fragments of absorbed immunoglobulins. If this induced antigen is involved in aggregation, it should be possible to block aggregation with antibody directed against the antigen alone. Whole antibody molecules from unabsorbed immunoglobulin pools agglutinated both uninduced and induced cells, and those from absorbed immunoglobulin pools agglutinated induced cells (22; unpublished data), necessitating the preparation of monovalent F(ab) fragments to avoid having to distinguish agglutination from aggregation. F(ab) fragments were prepared from absorbed pool A, referred to as immune F(ab), and a pool of preimmune sera obtained before immunization of the same rabbits used to generate pool A. Since the preimmune serum pool agglutinated whole uninduced cells, as previously observed with preimmune sera of other rabbits (22), a portion of the preimmune F(ab) preparation was absorbed with uninduced cells. These three F(ab) preparations were tested in the microtiter plate assay that is routinely used to measure pheromone titers. This allowed testing at multiple pheromone concentrations and multiple F(ab) fragment concentrations simultaneously. The immune F(ab) preparation was inhibitory up to a 50-fold dilution, whereas the absorbed preimmune F(ab) preparation was barely inhibitory when undiluted and tested against the endpoint dilution of cPD1 (Table 2). It was not surprising that undiluted, unabsorbed preimmune F(ab) fragments were inhibitory, since preimmune sera may have contained immunoglobulin directed against the presumed receptor of the adhesin. The inhibition of aggregation at different concentrations of F(ab) fragments of absorbed pool A also depended on the concentration of cPD1 (Table 2), which suggests that higher proportions of the cell population produced the induced antigen at higher concentrations of the inducing agent. The possibility of retention of active papain in the F(ab) preparations was excluded by showing no reduction in cPD1 activity after incubation of cPD1 with the P-60 fractionated F(ab) preparation. The assay depended on (i) the destruction of cPD1 and the adhesin by papain (unpublished data) and (ii) the

TABLE 2. Inhibition of pheromone (cPD1)-induced aggregation of strain 39-5 by F(ab) fragments

Expt[a]	Addition[b]	Aggregation at the following final culture fluid dilutions[c]:					
		1:4	1:8	1:16	1:32	1:64	1:128
1	Immune F(ab)	−	−	−	−	−	−
	Immune F(ab) 1:5	+	−	−	−	−	−
	Immune F(ab) 1:50	+	+	+	+	−	−
	Immune F(ab) 1:500	+	+	+	+	+	−
	Buffer	+	+	+	+	+	−
	Broth	+	+	+	+	+	−
2	Immune F(ab) 1:2	−	−	−	−	−	−
	Preimmune F(ab)	+	−	−	−	−	−
	Preimmune F(ab), absorbed[d]	+	+	+	−	−	−
	Buffer	+	+	+	+	−	−
	Broth	+	+	+	+	−	−

[a] Each experiment was carried out in duplicate and repeated.

[b] Immune F(ab) was prepared from anti-39-5I immunoglobulins which had been absorbed with uninduced 39-5 cells (see text).

[c] Mid-exponential-phase culture fluid of strain JH2-2 (cDP1 source) serially diluted in each solution listed under Addition. −, No aggregation in microtiter plate assay; +, aggregation in microtiter plate assay.

[d] Preimmune F(ab) absorbed with uninduced 39-5 cells.

resistance of cPD1 to boiling (no reduction in cPD1 titer after boiling for 5 min), with concomitant destruction of papain or F(ab) fragments by boiling [boiled papain or F(ab) preparations showed no interference with aggregation whereas unboiled samples did interfere]. Thus, a reduction of cPD1 titer after incubation of cPD1 with F(ab) preparations containing residual papain activity could be distinguished from the inhibition of aggregation by F(ab) fragments simply by boiling the sample before testing. Although unfractionated, dialyzed F(ab) preparations exhibited destruction of cPD1 and thus residual papain activity, P-60 fractionated preparations did not.

Time course of induction. In 1-liter batch cultures, visible clumping or aggregation was seen at approximately 45 to 60 min of induction in the presence of a cPD1 concentration corresponding to a titer of 8 to 16. Uninduced cells showed no evidence of production of induced antigen (Fig. 2A), and little if any induced antigen was extracted after 30 min of exposure to pheromone (Fig. 2A and B). Uninduced cell extracts and extracts of induced 0- and 30-min samples were loaded at a 10-fold-higher concentration in the immunoplate shown in Fig. 2A to enhance detection. The concentrated extracts from 0-min induced samples did not deflect the baseline during crossed-line immunoelectrophoresis to any greater extent than unconcentrated samples, whereas there was a slight increase with the 30-min samples. Thus, significant amounts of induced antigen were not found until the time of visible clumping. Although the overall amount per unit culture volume increased with time of induction (Fig. 2A), the amount per cell mass remained rather constant from 60 to 180 min postinduction (Fig. 2B). In the experiment shown in Fig. 2B, the cells remained in the exponential phase of growth for nearly 120 min postinduction, with a doubling time of 50 min, compared with 45 min for uninduced cells in the same batch of medium. Thus, growth for nearly a full generation was required before both induced antigen and aggregation were observed and synthesis paralleled the overall increase in cell mass.

Some variation in extraction of other antigens can be seen in Fig. 2, especially comparing the last well of Fig. 2A to the remaining seven wells. However, in the experiment shown in Fig. 2B, a faint immunoprecipitate of nearly equal height (approximately 2.7 to 3.0 cm) for each sample was visible on the original immunoplate but does not show up well in the photographs.

Each sample from the experiment shown in Fig. 2B was examined by SDS-PAGE (Fig. 3). The 30-min sample in lane 2 appears to contain a band with an approximate molecular weight of 78,000 (78K) (arrow), which was not present at 0 time. The 78K band became a major polypeptide by 60 min (lane 3). Confirmation that the 78K band was the induced antigen was carried out by XIE with SDS-PAGE for first-dimension separation of extracts (Fig. 4). Other proteins, such as

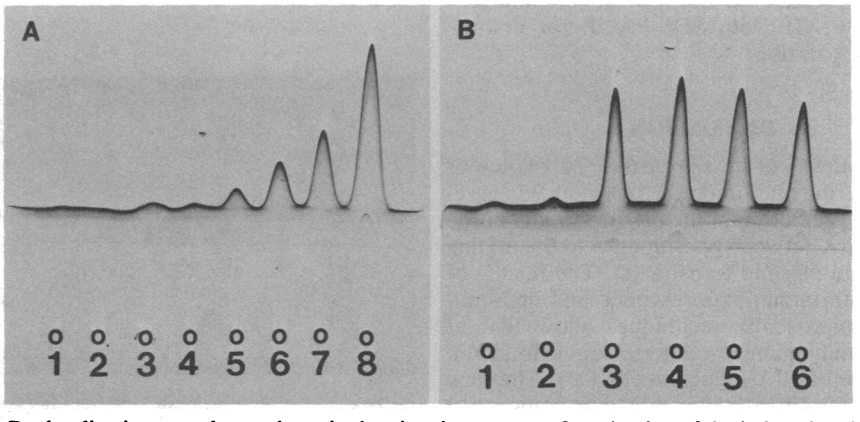

FIG. 2. Rocket-line immunoelectrophoresis showing time course of production of the induced antigen. (A) 5-μl samples of Zw312 extracts of 200-ml culture samples of uninduced and induced 39-5 were used. The original extraction volume was 1 ml. Wells 1 and 2, 10-times-concentrated extracts of uninduced 39-5 cells, exponential phase and stationary phase, respectively; wells 3 and 4, 10-times-concentrated extracts of induced 39-5 cells at 0 and 30 min postinduction, respectively; wells 5 to 8, extracts of induced 39-5 cells at 60, 90, 120, 180 min postinduction. (B) 5-μl samples of Zw312 extracts of 200-ml culture samples of induced 39-5 cells. $T = 0$ extraction volume was 1 ml; all others were adjusted to keep the extraction-volume-to-cell-mass ratio constant. Wells 1 to 6, $T = 0$, 30, 60, 90, 120, 180 min postinduction. In both (A) and (B) the main gel contained 10 mg of anti- 39-5I immunoglobulin pool B per ml, and the intermediate gel contained 40 μl of the 180-min sample shown in (A) per ml. The anode is at the top.

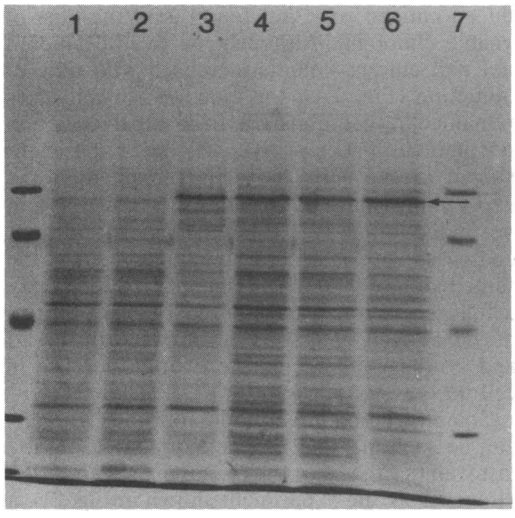

FIG. 3. SDS-PAGE of extracts of 39-5 cells during induction of aggregation response. Lanes 1 to 6: 0, 30, 60, 90, 120, 180 min after induction with cPD1. Samples from the experiment described in Fig. 2B were run on a 7.5% gel. The extraction volume-to-cell mass ratio was kept constant for each sample, and 60 μl of each extract was electrophoresed. Total protein applied: lanes 1 and 2, 13 μg; lane 3, 15 μg; lanes 4 to 6, 20 μg. Lane 7, Pharmacia low-molecular-weight standards 94K, 67K, 43K, 30K, and 20.1K (from top to bottom) were used. The unlabeled lane to the left of lane 1 also contained the same standards.

the very faint one at approximately 150K, may also be induced proteins, but they were neither consistently observed nor yielded immunoprecipitates by XIE with SDS-PAGE for first-dimension separation.

DISCUSSION

The synthesis of an aggregating substance or adhesin during pheromone (CIA)-induced aggregation is a prediction of the original model of the relationships between pheromones to the mating process and plasmid transfer (8). The results of studies with immunofluorescence and immunoelectron microscopy techniques show that at least one unique immunodeterminant is found on the surface of CIA-induced cells (22). The new immunodeterminant(s) appears to be common to several CIA-plasmid systems, since different *S. faecalis* strains carrying different plasmids (pPD1, pAD1, pOB1, pAMγ1, pAMγ2, pAMγ3) all react by indirect immunofluorescence with absorbed antisera raised against cPD1-induced *S. faecalis* 39-5 (containing pPD1) cells (22). Comparison of extracts of induced and uninduced 39-5 cells by XIE reveals a single unique antigen in the induced cell extract (22). We have now shown by several variations of XIE that this antigen was indeed unique to the induced cell surface and therefore apparently is at least proteinaceous, with an apparent molecular weight of 78,000. It appeared to be a highly immunogenic, dominant antigen on the cell surface of induced cells. Sera from six of a total of six immunized rabbits, which had been bled at different times from 6 weeks to 2 years after the initial immunization, all yielded the same dominant immunoprecipitate after XIE of induced cell extracts. The only conflicting evidence was the presence of a detectable but very low titer of antibody to the antigen contained in the unique immunoprecipitate in a serum pool from rabbits immunized with uninduced 39-5 cells. This probably reflected an immune response to a few spontaneously induced cells in the uninduced cell population that was used for immunization rather than constitutive expression of very low levels of antigen. A few spontaneously induced cells in an otherwise uninduced cell culture could remain undetected by indirect immunofluorescence or by XIE of extracts but could still be enough to elicit antibody response upon immunization. The possibility that pheromone induction resulted in the modification of a previously existing but non-immunogenic surface component rather than synthesis of a new surface antigen is not compatible with the combined results of SDS-PAGE of samples from time course experiments (Fig. 3) and XIE (Fig. 1). That is, there did not appear to be a concomitant disappearance of one antigen or protein band with the appearance of the induced antigen. All

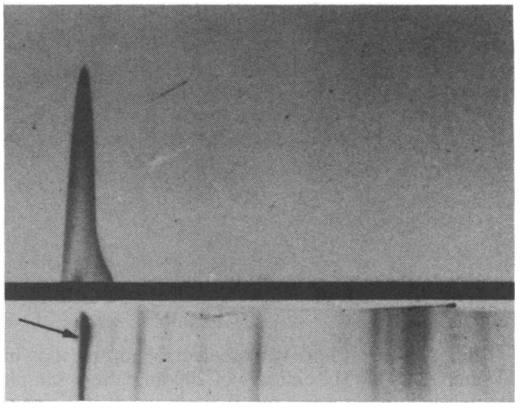

FIG. 4. XIE with SDS-PAGE for first-dimension separation. Zw312 extract was electrophoresed in 10% SDS-PAGE for the first dimension, and then electrophoresis of approximately ¼ strip width was carried out at right angles to the gel strip into agarose containing 10 mg of protein per ml of pool B. The anode is at the top. The 78K protein band is indicated by arrow.

evidence to date is consistent with the induced antigen's serving an adhesive function in aggregation. It appears to be located and evenly distributed on the cell surface, as shown by immunofluorescence and immunoelectron microscopy (22). The timing of aggregation coincided well with the timing of the appearance of induced antigen, as judged by SDS-PAGE and XIE of Zw312 extracts. Growth for nearly a full generation was required before aggregation could be observed and induced antigen could be detected in detergent extracts. Once antigen was detected in extracts, the amount of induced antigen that was synthesized appeared to parallel the overall increase in cell mass. However, it appeared that higher proportions of the cell population produced induced antigen at higher concentrations of inducing agent, since inhibition of aggregation at different F(ab) concentrations also depended on cPD1 concentration. This finding is consistent with that of higher frequencies of transconjugation at higher pheromone concentrations (4; unpublished data).

Consistent with the concept of the induced antigen as an adhesin, F(ab) fragments of absorbed immunoglobulins that reacted only with the induced antigen blocked aggregation. Absorbed F(ab) fragments from preimmune sera inhibited aggregation only weakly or not at all. Absorption of preimmune F(ab) fragments with uninduced cells was necessary, since preimmune sera agglutinated uninduced cells and F(ab) fragments from preimmune sera blocked aggregation at lower cPD1 levels. However, it should be noted that since the receptor for the adhesin is likely to be a common surface antigen on at least most *S. faecalis* strains (3), it is likely that preimmune sera that agglutinate uninduced cells contain immunoglobulins directed against the receptor. Thus, these controls negated nonspecific interference by F(ab) fragments or contaminating serum proteins in general but not immunocoating of the cells by F(ab) fragments. However, depending on the density of the induced antigen on the surface, the use of F(ab) fragments rather than whole antibody molecules greatly reduced the possibility of nonspecific steric hindrance by immunocoating rather than specific blockage of the active site for binding on the adhesin. Thus, the existing evidence strongly supports the identity of the adhesin as the pheromone-induced antigen.

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