Plasmid Content of *Streptococcus faecalis* Strain 39-5 and Identification of a Pheromone (cPD1)-induced Surface Antigen

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Streptococcus faecalis 39-5 is a haemolytic, bacteriocinogenic strain harbouring six plasmids. One of these plasmids, pPD1 (36·4 MDal) determines a bacteriocin and encodes a conjugative response to the sex pheromone cPD1 excreted by recipient (plasmid-free) strains. The pheromone response is characterized by the formation of mating aggregates of donors (responders) with recipients. Aggregation required the presence of phosphate and divalent cations and was inhibited by agents or conditions that destroy protein structure. Aggregation was postulated to be due to synthesis of a new proteinaceous molecule on the donor cell surface. Referred to as 'aggregation substance', such a material was identified and found to exhibit antigenic properties not associated with uninduced cells; it could be detected by immunoelectron microscopy. Aggregation substance could be extracted from induced cells but not uninduced cells as demonstrated by crossed immunoelectrophoresis. Antibody raised against the aggregation substance controlled by pPD1 cross-reacted with aggregation substance determined by other plasmid systems which respond to pheromones unrelated to cPD1.

INTRODUCTION

Recent reports from our laboratory have dealt with pheromone-induced mating responses in *Streptococcus faecalis* (Dunny *et al.*, 1978; Dunny *et al.*, 1979; Clewell & Brown, 1980; Clewell, 1981). Potential plasmid-free recipient cells excrete several low molecular weight, heat-stable substances which have been referred to as 'clumping inducing agents'. These clumping inducing agents induce donor strains harbouring certain conjugative plasmids to undergo a process involving protein synthesis that leads to the formation of mating aggregates. Once the plasmid is acquired by the recipient, the production of the related pheromone is shut off. Donors are induced to self-aggregate upon exposure to extracts containing clumping inducing agent. This self-clumping generally occurs within 45 min and is useful in quantifying the sex pheromones; a microtitre dilution assay was developed for this purpose (Dunny *et al.*, 1979). It was proposed (Dunny *et al.*, 1979) that induction resulted in the appearance of an adhesive 'aggregation substance' which interacts with a 'binding substance' present on the surface of recipients (and donors) to facilitate aggregation.

Streptococcus faecalis strain 39-5 was involved in many of the original experiments relating to the identification and behaviour of sex pheromones, and one of its plasmids, pPD1, was shown to be responsible for the mating (aggregation) response. Here we present a general characterization of the plasmid content of strain 39-5 as well as a restriction map of pPD1. We also report the identification of a surface antigen whose appearance correlates with aggregation and which is assumed to represent aggregation substance controlled by pPD1. The data show that it is a proteinaceous substance whose activity requires the presence of phosphate and divalent cations. We also show that the aggregation substance induced in donors harbouring

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Table 1. *Strains of Streptococcus faecalis used in this study*

Strain	Plasmid content	Comments and references
39-5	pPD1, pPD2, pPD3, pPD4, pPD5, pPD6	A haemolytic strain from subgingival scrapings of a patient with periodontitis (Rosan & Williams, 1964).
JH2SS	None	Resistant to streptomycin and spectinomycin (Franke & Clewell, 1981).
JH2-2	None	Resistant to rifampin and fusidic acid. Derivative of JH2 (Jacob & Hobbs, 1974).
OG1-10	None	Streptomycin resistant (Dunny <i>et al.</i> , 1978).
OG1-RF1	None	Rifampin, fusidic acid resistant. Mutant of OG1. (Dunny <i>et al.</i> , 1979).
OG1-30	pAD1::Tn917(Em)	Transconjugant from a mating between JH2-2 (pAD1::Tn917) and OG1-10. pAD1::Tn917 had been derived as previously reported (Tomich <i>et al.</i> , 1980).
OG1-31	pOB1	OG1-RF1 transconjugant from mating with 5952 (Dunny <i>et al.</i> , 1979).
OG1-32	pPD1	OG1-10 transconjugant from mating with 39-5.
YA101	pAM γ 1, pAM α 1	JH2-2 transconjugant. Plasmids were from DS5C1 (Clewell <i>et al.</i> , 1982). pAM α 1 is a non-conjugative 6 Mdal plasmid encoding tetracycline resistance.
YA102	pAM γ 2, pAM α 1	JH2-2 transconjugant. Plasmids were from DS5C1 (Clewell <i>et al.</i> , 1982).
YA103	pAM γ 3, pAM α 1	JH2SS transconjugant. Plasmids were from DS5C1 (Clewell <i>et al.</i> , 1982).
DT11	pAM α 1	A JH2-2 transconjugant (Dunny & Clewell, 1975).

different classes of plasmids cross-reacts immunologically with antiserum raised against the aggregation substance synthesized by cells harbouring pPD1.

METHODS

Bacteria and media. The bacterial strains used in this study are listed in Table 1. *Streptococcus faecalis* strain 39-5, the primary strain used in this study, is a haemolysin-bacteriocin-producing strain originally given to us by B. Rosan (University of Pennsylvania). Media used were N2GT (Dunny *et al.*, 1979) [Oxoid nutrient broth no. 2 supplemented with Tris buffer (pH 7.0) and glucose (0.2%, w/v)] or Difco Brain-Heart Infusion. *Streptococcus faecalis* strains harbouring the various plasmids were constructed in 4 or 18 h matings in broth or on filters as previously described (Franke & Clewell, 1981). Pheromone assays of culture filtrates were as previously described (Dunny *et al.*, 1979).

Reagents and enzymes. These materials and their sources were as follows: Zwittergent 3-12, *N*-acetyl D-galactosamine, *N*-acetyl D-glucosamine, L-fucose, pronase, lysozyme and lipase (pancreatic) from Calbiochem; trypsin from Sigma; glutaraldehyde, D-glucose and D-lactose from Baker (Phillipsburg, New Jersey); horseradish peroxidase-conjugated goat anti-rabbit IgG from Cappel Labs (Downingtown, Pa., U.S.A.); fluorescein-labelled goat anti-rabbit IgG from Sylvania (Millburn, N.J., U.S.A.); D-galactose, D-mannose and fluorescent antibody mounting fluid from Difco. Restriction enzymes used in the mapping of pPD1 were obtained from Bethesda Research Laboratories (Gaithersburg, Md., U.S.A.). Seakem HGT agarose, used in crossed immunoelectrophoresis, was from Marine Colloids Inc. (Rockland, Me., U.S.A.).

Bacteriocin assays. Strain JH2-2 was used as an indicator strain. The bacteriocin activity associated with pPD5 was easily detected using a previously reported procedure involving stabs of producer strains into soft agar overlays containing the indicator strain (Franke & Clewell, 1981). The bacteriocin associated with pPD1 was not as easily resolved by this method. In this case, plates containing fresh colonies of cells harbouring pPD1 (but not pPD5) were overlaid with soft (0.75%) agar containing the indicator strain. Zones of inhibition were visible after overnight incubation at 37 °C.

Isolation and electrophoretic analysis of plasmid DNA. Plasmid DNA from strains 39-5 and DS16 was prepared as follows. Cells grown to mid-exponential or stationary phase in 250 ml of Brain-Heart Infusion medium were harvested, washed with TES (0.03 M-Tris, 0.005 M-Na₂EDTA, 0.05 M-NaCl) and resuspended in 15 ml of 25% glucose in TES. Lysozyme [5 ml of an 8 mg ml⁻¹ solution in TES/glucose (25%)] was added and the suspension was allowed to incubate at 37 °C for 1 h. The cells were then lysed by the addition of 1.1 ml of 20% (w/v) SDS (in TES); the container was inverted several times to obtain uniform mixing. Six ml of 5 M-NaCl was then added; the lysate was kept on ice for 3 h, after which it was centrifuged at 20000 r.p.m. (4 °C) for 1 h in the SS34 (Sorvall) rotor to remove chromosomal DNA selectively. The supernatant was then removed, mixed with 2 vol. ethanol and placed at -70 °C for 40 min. The precipitated DNA was collected by centrifugation at 5000 r.p.m. (SS34 rotor), dried in a desiccator for 15 min and redissolved in TES. The sample was then centrifuged to equilibrium in an ethidium bromide/CsCl buoyant density gradient (Beckman, 50Ti rotor) as previously described (Franke & Clewell, 1981). The plasmid band was removed by needle and syringe from the side of the tube and recentrifuged as before. Plasmid DNA was then removed (as before), dialysed against SSC (0.05 M-NaCl, 0.015 M-sodium citrate, pH 7.3)

and then concentrated by ethanol precipitation (Franke & Clewell, 1981) and resuspended in 50 mM-Tris pH 7.2. Plasmid DNA was then analysed by agarose gel electrophoresis as previously described (Franke & Clewell, 1981). Plasmid DNAs from *S. faecalis* strain DS16 (Tomich *et al.*, 1979) and *Escherichia coli* strain VA517 (Macrina *et al.*, 1978) (generously supplied by F. Macrina), were used as molecular weight markers.

The isolation of pPD1 for the purpose of restriction mapping, the electrophoresis procedures, and strategy used in restriction mapping were similar to those reported elsewhere for pAD1 (Franke & Clewell, 1981; Franke, 1980).

Preparation of induced-dissociated cells. A culture (10 ml) of responder cells was grown to mid-exponential phase (70 Klett Units, using the no. 54 filter, on a Klett-Summerson colorimeter). Filtered supernatant (5 ml; Millipore 0.22 µm) of a culture of JH2-2 containing cPD1 (pheromone) was added. Using 39-5 cells as responders, the filtrate showed a cPD1 activity with a titre of 64. After incubation for 40 min, significant clumping generally occurred. The cells were then dissociated by the addition of EDTA (25 mM, pH 7.0), sedimented, washed with 30 mM-Tris (pH 7.0) and resuspended in 5 ml of the same buffer. The cells remained well suspended (i.e. dissociated) in this state.

Enzyme-treatment of induced-dissociated cells. Dissociated cells (1 ml in each case) were treated in the following ways: trypsin (20 µg ml⁻¹) in 30 mM-Tris, pH 8.0, for 20 min; pronase (20 µg ml⁻¹) in 30 mM-Tris, pH 7.0, for 20 min; lysozyme (100 µg ml⁻¹) in 30 mM-Tris, pH 7.0 for 20 min; lipase (10 µg ml⁻¹) in 1 mM-CaCl₂, 30 mM-Tris, pH 7.0 for 20 min. After treatment, the cells were washed twice by centrifugation in 30 mM-Tris and suspended in 1 ml 30 mM-Tris; CaCl₂ (to 0.1 mM) and phosphate (to 20 mM) were then added.

Preparation of antiserum against induced 39-5 cells. A 30 ml mid-exponential phase culture of pheromone-induced 39-5 cells was harvested and washed in PBS (0.14 M-NaCl, 1.5 mM-KH₂PO₄, 2.7 mM-KCl, pH 7.4). The cells were resuspended in 2 ml PBS and fixed with 0.5% (w/v) glutaraldehyde at 37 °C for 1 h; they were then washed several times by centrifugation with PBS. After obtaining pre-immunization serum, approximately 10⁸ cells were injected intramuscularly into each rabbit four times over a period of 10 d at 2 d intervals and then again at 30 d and 37 d. Two weeks later, the rabbits were bled from the marginal ear veins. In some experiments, sera (2 ml) were absorbed twice at 37 °C for 20 min with uninduced 39-5, JH2-2 and OG1-30 cell pellets (from 250 ml mid-exponential cultures). Cells were removed by centrifugation.

Fluorescent-antibody staining. Ten µl of cell suspension (approximately 10⁸ cells ml⁻¹ in PBS) was placed on a glass microscope slide and air-dried. The cells were heat-fixed by two passages through a flame. The fixed specimens were covered with 10 µl of a series of twofold dilutions of antisera and were incubated at room temperature in a moist chamber for 1 h. Following three washes with PBS, the slides were flooded with a 1:20 dilution of fluorescein-labelled goat anti-rabbit IgG. After incubation for 1 h at room temperature, they were washed three times with PBS and then counterstained with eriochrome black solution. Finally, a drop of buffered glycerol saline mounting fluid (pH 7.5) and a cover slip was placed on the stained specimen. The slides were then examined with a microscope equipped with transmission UV illumination.

Horseradish peroxidase staining of 39-5 cells. Immunoelectron microscopy employing horseradish peroxidase-labelled goat anti-rabbit IgG was as described by Lai *et al.* (1975). Prior to staining with labelled antibody, 39-5 cells, uninduced or induced by cPD1, were incubated with antiserum against induced 39-5 cells which had previously been absorbed with uninduced 39-5 cells. (Absorbed antiserum no longer reacted with uninduced cells as determined by indirect immunofluorescence). The final preparations were examined in a Hitachi HS-8 electron microscope.

Crossed immunoelectrophoresis. Crossed immunoelectrophoresis was carried out using a modification (Clark & Freeman, 1967) of the method of Laurell (1965). Barbital/HCl buffer (2.5 mM, pH 8.6) was used throughout the system. One percent agarose in buffer was cast on glass plates (50 mm × 50 mm) to give a volume to surface area ratio of 0.132 ml cm⁻². Pheromone (cPD1)-induced and uninduced cells of strain 39-5 (approximately 0.2 g dry weight of each) were extracted with 10 ml 0.2% Zwittergent 3-12 (ZW312) in PBS. First dimension electrophoresis of 5 µl of each extract was carried out at 6 V cm⁻¹ for 45 min. Second dimension electrophoresis into agarose containing anti-induced 39-5 immunoglobulins (75 µl ml⁻¹) was at 2 V cm⁻¹ for 16 h. The anti-induced 39-5 immunoglobulin fraction was prepared as described by Harboe & Ingild (1973), omitting the DEAE-cellulose chromatography step. The final volume of this fraction was one-third that of the original serum pool. All gels were alternately pressed and washed twice with 0.1 M-NaCl. After a final press, brief wash with a stream of distilled water and drying, they were stained with Coomassie blue R-250 (Weeke, 1973).

RESULTS

Plasmid content of S. faecalis strain 39-5

As shown in Table 2, strain 39-5 harboured at least six plasmids (pPD1, 2, 3, 4, 5, and 6) ranging in mass from 3.4 to 38.5 MDal. Haemolytic-negative derivatives obtained at a frequency of 2% after growth overnight in the presence of the curing agent novobiocin

Table 2. *Plasmids in Streptococcus faecalis strain 39-5*

All but two of the molecular weight values are based on plasmid migration rate during agarose (0.7%) gel electrophoresis of plasmid DNA isolated from strain 39-5. Molecular weight markers consisted of plasmid DNA from *S. faecalis* strain DS16, representing pAD1 (37.8 MDal) and pAD2 (17.1 MDal), and plasmid DNA from *Escherichia coli* strain VA517. The latter contained plasmids with molecular masses of 1.4, 1.8, 2.0, 2.6, 3.4, 3.7, 4.8 and 35.8 MDal. The values for pPD1 and pPD5 were based on the sum of the masses of corresponding *EcoRI* restriction fragments. Using *EcoRI* fragments of lambda phage DNA as a standard, the pPD1 fragments A through E measured 14.5, 8.6, 5.8, 4.4 and 3.1 MDal, respectively. *EcoRI* fragments for pPD5 measured 8.9, 7.7, 6.6 (doublet), 6.1, 1.6 and 1.0 MDal.

Plasmid	$10^{-6} \times$ Mol. wt	Properties
pPD1	36.4	Conjugative; determines response to cPD1; determines bacteriocin
pPD2	9.4	Cryptic
pPD3	5.0	Cryptic
pPD4	3.4	Cryptic; incompatible with pAM α 1
pPD5	38.5	Conjugative; determines haemolysin-bacteriocin
pPD6	35	Cryptic

(5 μ g ml $^{-1}$) were devoid of pPD5. Haemolytic transconjugants arising from matings between 39-5 and the plasmid-free strain JH2-2 or JH2SS were found to harbour pPD5 or both pPD5 and pPD1. Cured, haemolytic-negative derivatives of the latter type carried only pPD1; these cells were found to produce a bacteriocin. We thus concluded that haemolysin activity is determined by pPD5, whereas a bacteriocin activity is conferred by pPD1. However, pPD5 also conferred a bacteriocin activity which was independent of that determined by pPD1, and it is likely that the haemolysin and bacteriocin activities represent the same protein, as in other haemolysin-bacteriocin systems in *S. faecalis* (Brock & Davie, 1963; Granato & Jackson, 1969). The frequent cotransfer of pPD1 and pPD5 and the inability to resolve these two plasmids by sedimentation analysis led to the earlier erroneous belief (Dunny *et al.*, 1978) that haemolysin was determined by pPD1.

Both pPD1 and pPD5 were capable of independent conjugation but, while transconjugants harbouring only pPD1 exhibited a characteristic clumping response, strains with only pPD5 were unresponsive. Filtrates of cells carrying only pPD1 did not induce 39-5 cells to clump, whereas filtrates from strains with pPD5 alone exhibited a pheromone titre similar to that of filtrates from an isogenic plasmid-free strain (about 64–128). The tetracycline resistance element Tn916 was introduced on to both pPD1 and pPD5 by a method described elsewhere (Franke & Clewell, 1981); the resistance marker facilitated the quantification of conjugation frequencies. The ability of pPD1 to confer an aggregation response correlates with its ability to transfer efficiently in broth ($\geq 10^{-2}$ per donor); however, cells harbouring pPD5 alone (no aggregation response) did not transfer plasmid well in broth ($< 10^{-5}$), yet transfer occurred efficiently if matings were performed on filter membranes ($\geq 10^{-4}$).

Despite the presence of several other plasmids in strain 39-5, the aggregation response exhibited by this strain appeared to relate only to pPD1. (The related pheromone is referred to as cPD1.) The possibility, however, that 39-5 cells have the potential to respond to pheromones other than cPD1, has not been completely ruled out. Conceivably, pPD1 could affect production of pheromones other than cPD1 and, while pPD5 did not express a detectable aggregation response in a different host, it is possible that because of different host influences, it could control a response in the 39-5 background. It is also possible that pPD5, and even pPD6, could have pheromone-response systems that are suppressed in the presence of other plasmids (e.g. pPD1). Construction of strains having different combinations of these plasmids in the 39-5 background could lead to further insight into these possibilities, although the lack of selectable markers makes this a laborious task. *Streptococcus faecalis* strain DS5 was recently shown to harbour three plasmids that encoded three specific pheromone responses (Clewell *et al.*, 1982).

A restriction map of pPD1 is shown in Fig. 1. The restriction map has no resemblance to that of the recently mapped pheromone-plasmid, pAD1 (Clewell, 1981; Franke, 1980).

Fig. 1. Restriction map of pPD1. Plasmid DNA (pPD1) isolated from *S. faecalis* strain OG1-32 was mapped with respect to sites cleaved by *Bam*HI, *Eco*RI, *Ava*I, *Bgl*II, *Kpn*I and *Sal*I. Mapping was based on double and partial digestion analyses using appropriate enzymes. The fragments were named alphabetically according to size, beginning with the largest fragments. The single *Bam*HI site was arbitrarily taken to mark the beginning and end of the map.

Table 3. Phosphate and calcium ion concentrations required for aggregation of induced 39-5 cells

+, Aggregation; -, no aggregation.

(a) Phosphate			(b) Calcium		
Na ₂ HPO ₄ (mm)	CaCl ₂ (mm)	Aggregation	Na ₂ HPO ₄ (mm)	CaCl ₂ (mm)	Aggregation
0	0	-	0	0	-
0	0.1	-	20	0	-
1	0.1	-	20	0.05	-
5	0.1	+	20	0.1	+
20	0.1	+	20	0.5	+

No information is available with regard to determinants on the other plasmids in 39-5. Transconjugants harbouring pPD1 or pPD5 (or both) occasionally also carried the small cryptic plasmid pPD4. The latter was probably mobilized by one (or both) of the larger plasmids. pPD4 was incompatible with the small (6.0 MDal) tetracycline-resistance plasmid pAM α 1 of strain DS5 (Clewett *et al.*, 1975). Mobilization of pAM α 1 into 39-5S, a streptomycin-resistant mutant of 39-5, always resulted in the loss of pPD4 (data not shown).

Requirements for aggregation

The large aggregates of 39-5 cells that arose upon induction with a filtrate of JH2-2 (plasmid-free) were readily dissociated with 25 mM-EDTA. If dissociated cells were pelleted, washed and resuspended in buffer (0.03 M-Tris, pH 7.0), reaggregation did not occur. As shown in Table 3, reaggregation required a minimum of 5 mM-phosphate and 0.1 mM-CaCl₂. In place of calcium ions, Mg²⁺, Mn²⁺, or Co²⁺ also promoted aggregation. Studies on the effect of pH on aggregation led to the surprising observation that phosphate and divalent cations were not required for reaggregation if the pH was lowered to 2.5 (an aqueous cell suspension adjusted with HCl). The range in which this type of clumping was observed was pH 2.0-3.0. The fact that uninduced cells failed to aggregate at the low pH and that 39-5 cells pre-exposed to a filtrate of JH2-2(pPD1) (i.e., a strain which does not excrete cPD1) also failed to aggregate at this pH implied this phenomenon was specifically related to a cPD1-induced response.

Fig. 2. New surface antigen appearing on the surface of pheromone-induced *S. faecalis* 39-5 cells. The immunoelectron microscopic procedure employed a peroxidase stain as described in Methods. (a) Uninduced cells; (b) induced cells.

Biochemical properties

To examine the nature of the cell surface component involved in aggregation, dissociated cells were subjected to various conditions known to alter macromolecular structure. Exposure to trypsin, pronase, SDS [0.05% (w/v), 30 min], LiCl (4 M, 90 min) or heat (98 °C, 5 min) destroyed the ability of the cells to reaggregate, whereas treatment with lysozyme, lipase, or glutaraldehyde [0.5% (w/v), 30 min] had no effect. Similar results were obtained when the treated cells were exposed to pH 2.5 in the absence of phosphate and calcium ions. These data imply that aggregation substance is a protein.

Because the adherence of certain Gram-negative bacteria to specific tissues involves binding to a carbohydrate moiety and because such adherence may be inhibited in the presence of

Table 4. *Binding of antiserum against induced 39-5 cells to strains harbouring different plasmids and induced by different pheromones*

The titre was determined by fluorescent-antibody staining (see Methods). Rabbit antiserum was prepared against pheromone-induced 39-5 and was subsequently absorbed by uninduced 39-5 cells as well as OG1S and JH2SS (see Methods).

Strain (relevant plasmid)	Titre	
	Uninduced	Induced
39-5 (pPD1 through pPD6)	<1	160
OG1-30 (pAD1::Tn917)	<1	40
OG1-31 (pOB1)	<1	40
OG1-32 (pPD1)	<1	80
YA101 (pAM γ 1, pAM α 1)	<1	40
YA102 (pAM γ 2, pAM α 1)	<1	40
YA103 (pAM γ 3, pAM α 1)	<1	20
JH2-2 (pAM α 1)	<1	<1

specific sugars (Jones, 1977), we examined the effect of sugars on reaggregation. The following sugars all had no effect on reaggregation if present with phosphate and calcium ions: D-lactose (0.05 M), D-galactose (0.05 M), sucrose (0.02 M), D-glucose (0.05 M), D-mannose (0.05 M), L-fucose (0.05 M) and a mixture of D-galactose, *N*-acetyl D-galactosamine and *N*-acetyl D-glucosamine (each at a concentration of 0.002 M).

Immunoelectron microscopic analyses

Using a preparation of induced 39-5 cells that were dissociated with EDTA and fixed with glutaraldehyde, we prepared a rabbit antiserum which reacted readily in an indirect immunofluorescence assay with induced cells (titre 4096) as well as uninduced cells (titre 1024). Antisera absorbed with uninduced cells no longer reacted with uninduced cells, but had a titre of 80–160 against induced cells. This serum was used in an immunoelectron microscopic examination of induced and uninduced 39-5 cells. Figure 2 shows the surface of uninduced cells to be relatively 'clean', whereas induced cells exhibited an amorphous material, presumably representing aggregation substance, coating their surface. It is conceivable that this represents a microfibrillar substance, although fibrillae, *per se*, were not clearly resolved here. Earlier studies using negative-staining techniques as well as scanning electron microscopy, found no detectable differences between induced and uninduced cells (Dunny, 1978).

Similarities of aggregation substance associated with different conjugative plasmids

Table 4 shows that antiserum specifically prepared against induced 39-5 cells cross-reacted readily with the aggregation substance induced in strains harbouring different conjugative plasmids. pAM γ 1, pAM γ 2 and pAM γ 3 make use of pheromones distinct from cPD1 and from each other (Clewell *et al.*, 1982; R. Craig, personal communication). pAM γ 1 and pAD1, however, make use of the same pheromone (Clewell *et al.*, 1982). The pOB1 plasmid responds to a pheromone (cOB1) distinct from cAD1 and cPD1 (R. Craig, personal communication); its relationship to pAM γ 2 and pAM γ 3, however, has not been tested. Because of the significant degree of cross-reaction observed in all cases, it would appear that, despite differences in pheromone specificity, the aggregation substances have significant structural similarity.

Extraction of aggregation substance and detection by crossed immunoelectrophoresis

A substance was extracted from induced, but not uninduced, 39-5 cells which was readily detectable by the method of crossed immunoelectrophoresis. The data of Fig. 3 illustrate the use of an unabsorbed antiserum which gave rise to several immunoprecipitate arcs. A prominent immunoprecipitate is seen in the case of the extract from induced, but not uninduced cells.

Fig. 3. Crossed immunoelectrophoresis of extracts of *S. faecalis* 39-5 cells. Zwittergent 3-12 extracts (5 μ l) of uninduced cells (a) and induced cells (b) were placed in sample wells of minimal diameter (marked by circles in the lower right-hand corners). Electrophoresis at 6 V cm^{-1} for 45 min was carried out with the anode to the left. Second dimension electrophoresis with the anode at the top was into agarose containing a concentrated immunoglobulin preparation (75 μ l ml^{-1} ; see Methods).

DISCUSSION

Streptococcus faecalis strain 39-5 has been shown to harbour six plasmids. Three of the plasmids, pPD1, pPD5 and pPD6, are relatively large in size, and at least two of these, pPD1 and pPD5, are conjugative. Only pPD1, however, appeared to control the pheromone-induced appearance on the cell surface of a proteinaceous macromolecular substance with unique immunological properties. Believed to represent aggregation substance, this material was found to be immunologically similar to the aggregation substance induced in different plasmid systems responding to different sex pheromones.

Phosphate and divalent cations were required for aggregation. It is interesting, however, that these factors were not necessary at pH 2.5. At physiological pHs the phosphate and divalent cations may act to stabilize the conformation of aggregation substance or its receptor and/or modulate the repulsive effects of neighbouring cell surface molecules. The net negative charge on the bacterial surface may be significantly reduced at pH 2.5. It is not likely that there is any physiological significance to aggregation at this low pH.

The location of the structural gene(s) for aggregation substance remains to be determined. It could reside on either the plasmid or a plasmid-controlled chromosomal locus. A characterization of the pPD1-related aggregation substance is in progress (Kessler *et al.*, 1982; R. E. Kessler & Y. Yagi, unpublished observations) and the use of specific antiserum has been, and will continue to be, extremely useful in monitoring aggregation substance in the course of purification and subsequent analyses. Studies on the structure of aggregation substance and the nature of its binding to the, as yet, unidentified 'binding substance' should lead to a better understanding of the biochemical processes involved in pheromone-induced conjugation in *S. faecalis*.

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