Proc. Natl. Acad. Sci. USA
Vol. 75, No. 7, pp. 3479–3483, July 1978
Microbiology

Induced cell aggregation and mating in *Streptococcus faecalis*: Evidence for a bacterial sex pheromone

(plasmid/gene transfer/clumping/drug resistance)

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Communicated by J. L. Oncley, April 17, 1978

ABSTRACT **Recipient strains of *Streptococcus faecalis* produce a trypsin sensitive, heat resistant, nuclease resistant factor, designated clumping-inducing agent (CIA) which causes strains carrying certain conjugative plasmids to aggregate. RNA and protein synthesis but not DNA synthesis are required for aggregation to occur. Recipient filtrates that contain CIA activity also induce donors to mate at high frequencies. Introduction of a transferable plasmid into strains producing CIA dramatically reduces the amount of CIA activity produced by the strain but allows the strain to respond to exogenously added CIA. Our data suggest that CIA represents a bacterial sex hormone (pheromone).**

Plasmids coding for traits such as antibiotic resistance, bacteriocins, and hemolysins are commonly found in clinical isolates of *Streptococcus faecalis* (1–6). Plasmid-mediated gene transfer in this species was first demonstrated by Jacob and Hobbs (3), and reports from our laboratory have also shown that a number of *S. faecalis* hemolysin plasmids are capable of self transfer, as well as of mobilization of other plasmids and chromosomal genes (6–8). Whereas contact between donor and recipient cells appears necessary for the transfer of DNA, the mechanism by which mating pairs (or aggregates) of these nonmotile organisms are formed has remained a mystery. (Gene transfer by transformation or transduction has, to our knowledge, never been reported in *S. faecalis*.) Unlike the case for Gram-negative bacteria, evidence for pilus structures that might serve to bring donors and recipients together is lacking.

We have observed that mating mixtures of *S. faecalis* strains form aggregates, or "clumps", not usually observed during growth of donors or recipients alone. In following up this observation, we have obtained data, which is presented here, indicating that recipient cells excrete a substance (probably a small protein or peptide) that induces donor cells to become very adherent. The same preparations that induce aggregation also greatly increase frequencies of plasmid transfer.

MATERIALS AND METHODS

Bacteria and Plasmids. All bacterial strains and plasmids used in this study are described in Tables 1 and 2, respectively. Of the three plasmids used in this study, two of them, pAMγ1 and pPD1, determine hemolysin and are conjugative; whereas the third plasmid, pAMα1, determines tetracycline resistance and is nonconjugative. The latter plasmid, however, can be mobilized by either of the other two.

Media, Antibiotics, and Enzymes. All experiments described here were performed in Oxoid nutrient broth no. 2, supplemented with 0.2% glucose and buffered to pH 7.0 with 0.1 M Tris·HCl. Rifampicin, tetracycline, and streptomycin were from Calbiochem; 6-parahydroxyphenylazouracil was a gift from B. W. Langley; chloramphenicol was from Parke-Davis; and soybean trypsin inhibitor (B grade) was from Calbiochem. Pancreatic DNase and RNase were from Worthington Biochemical. Trypsin was from Difco. These enzymes were dissolved in distilled water before using.

Mating and Clumping Experiments. Mating experiments were carried out in broth at 37° as described in the text. Clumping-inducing filtrates were obtained from cell cultures grown to mid-log phase at 37° with shaking. The cells were then pelleted by centrifugation. The supernatant was filtered through a 0.2 μm Millipore filter, and a portion was then added to indicator cells. In some cases the filtrates were given heat or enzymatic treatment, or antibiotics were added to the indicator cells. Unless otherwise indicated, 5 ml of filtrate plus 4 ml of fresh broth plus 1.0 ml of a late log phase culture of indicator cells were mixed, incubated at 37°, and monitored for clumping.

RESULTS

Appearance of Clumped Cells. Fig. 1 shows photographs of clumped and nonclumped *S. faecalis* cells. As can be seen, a 4-hr broth mating mixture of strains JH2-S (recipient) and 39-5 (donor) exhibited extensive clumping (Fig. 1*C*), in contrast to recipient and donor cells grown separately (Fig. 1 *A* and *B*). Clumping reactions of this sort were observed when several different recipient and donor strains were used. Strain 39-5, which carries a self-transferable hemolysin plasmid called pPD1 and 3 cryptic plasmids (unpublished data), was routinely used in the clumping assays reported here, because it showed the greatest amount of clumping of all of our strains.

Induction of Clumping. We have found that a 40- to 120-min incubation period (depending on the strain used, cell concentration, etc.) is required after the donors and recipients are mixed for visible clumping to occur. We originally carried out several experiments to test whether a 1-hr pre-growth of donors (39-5 cells) or recipients (JH2-S cells) in the presence of growth-medium filtrates of the opposite mating type would result in immediate clumping when donors and recipients were mixed. In carrying out such experiments, we found that growth of donor cells in the presence of a filtrate from recipient cells would cause the donors themselves to clump—(i.e., in absence of recipient cells)—(Fig. 1*D*) within 30–60 min. Thus, there appeared to be a "clumping-inducing agent" (CIA) produced by recipients, to which donors responded by becoming very adherent. Supernatants from donor cells had no effect on recipients.

Nature of CIA. Table 3 shows the results of experiments testing the sensitivity of CIA to various treatments. A CIA preparation was divided into portions that were treated with

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Abbreviation: CIA, clumping inducing agent.

Table 1. Strains used in this study

Strain	Source	Presence of a self-transferable plasmid	Comment
39-5	B. Rosan (9)	+	Carries transferable hemolysin plasmid pPD1 and 3 other cryptic plasmids (unpublished).
DS-5C1	Derived here	+	Obtained by curing erythromycin resistance plasmid pAMβ1 from strain DS-5 (1). Carries plasmids pAMα1 & pAMγ1.
JH2	A. Jacob (3)	−	Plasmid-free strain.
JH2-S	Derived here	−	Spontaneous mutant, resistant to streptomycin at >1 mg/ml.
JH2-2	A. Jacob (3)	−	Rifampicin, fusidic acid resistant mutant of JH2.
DT4	Derived here	+	JH2-2 transconjugant carrying pAMγ1 and pAMα1 (6).
DT-11	Derived here	−	JH2-2 transconjugant carrying pAMα1 (6).
HD1	Derived here	+	JH2-2 transconjugant carrying pAMγ1 (6).
HJ30-2	Derived here	+	Obtained by mating 39-5 with DT-11. Carries pAMα1 and pPD1.
HJ30-10	Derived here	+	Obtained in same way as HJ30-2.
OG1	O. Gold (10)	−	Plasmid-free, formerly called 2SaR.
OG1-10	Derived here	−	Spontaneous mutant resistant to streptomycin at >1 mg/ml.
OG1-11	Derived here	−	OG1-10 transconjugant carrying pAMα1.
OG1-12	Derived here	+	OG1-10 transconjugant carrying pAMα1 and pAMγ1.
OG1-13	Derived here	+	OG1-10 transconjugant carrying pAMγ1.
OG1-RF1	Derived here	−	Carries spontaneous mutations to rifampicin and fusidic acid resistance.
OG1-20	Derived here	+	OG1-RF1 transconjugant carrying pAMα1 and pAMγ1. Formerly RT-12 (8).

enzymes or heat, and each treated portion was then added to 39-5 cells to test for clumping activity. Treatment of CIA with nucleases or heat had no effect on its activity, whereas trypsin removed all activity. This inactivation by trypsin could be eliminated by the simultaneous addition of trypsin inhibitor or by immediate exposure to heat. These results lead us to believe that CIA may be a relatively small protein or peptide. CIA will pass through an Amicon filter with a pore size designed to retain molecules of 10,000 molecular weight. It is retained on a filter with a 1000 molecular weight limit.

CIA Appears to be a Signal. Before visible clumping is observed, an incubation period of at least 30 min is necessary after the addition of CIA, regardless of the concentration of cells or CIA. It also appears that cells exposed to CIA must undergo RNA and protein syntheses in order to clump. In Table 4, it can be seen that addition of chloramphenicol or rifampicin to 39-5 cells along with CIA prevented clumping; whereas 6-parahydroxyphenylazouracil,which blocks DNA synthesis (12–14) and also halts the growth of 39-5 cells, did not prevent clumping. Table 4 also shows that the addition of chloramphenicol or rifampicin 15 min after the addition of CIA resulted in clumping, indicating that aggregation could occur in the presence of these drugs when the necessary proteins had been synthesized.

Relationship of CIA to Plasmid Content and Mating. We have observed that a number of clinical isolates of *S. faecalis* that lack transferable plasmids produce a CIA activity capable of inducing clumping of certain independently isolated strains that carry transferable plasmids. Isogenic strains differing in plasmid content also exhibited differences in CIA production as well as response to exogenous CIA. In Table 5 it is shown in two isogenic systems that the acquisition of the conjugative plasmid pAMγ1 resulted in a great reduction in the amount of CIA activity observed in medium filtrates. Cells lacking pAMγ1 produced up to 30-fold more CIA than cells harboring this plasmid. In addition, the presence of pAMγ1 sensitized the cell to exogenous CIA. The presence or absence of the nonconjugative tetracycline-resistance plasmid pAMα1 had no effect on the host's ability to produce or respond to CIA.

It is not clear whether the presence of pAMγ1 results in a repression of synthesis of CIA or in a modification of it. The low level of activity produced by cells harboring the plasmid could reflect an incomplete shut-off of synthesis or a residual activity of a modified form of CIA; however, other explanations are conceivable. Relating to this point, we also emphasize that the cells used for the quantitation of CIA (i.e., strain 39-5) represent a different plasmid system involving a conjugative plasmid

Table 2. Relevant plasmids involved in this study

Plasmid	Molecular weight	Related phenotype	Comments and source
pAMγ1	3.5×10^7	Hemolysin, conjugative	DS-5C1 (1, 6)
pAMα1	6×10^6	Tetracycline resistance, nonconjugative	DS-5C1 (1,6,11)
pPD1	3.5×10^7	Hemolysin, conjugative	39-5

Table 3. Effect of heat and degradative enzymes on CIA*

Treatment	Clumping inducing activity
None	+
100°, 10 min	+
DNAse, 10 min, then 100°, 10 min	+
RNase, 10 min, then 100°, 10 min	+
Trypsin, 10 min, then 100°, 10 min	−
Trypsin, 10 min, then trypsin inhibitor, 10 min	−
Trypsin + 100° (immediately) 10 min	+
Trypsin + trypsin inhibitor, simultaneously, 10 min	+

* A filtrate from a JH2-S culture was examined for its ability to clump 39-5 cells in a standard assay after various treatments. Portions (5 ml) of the filtrate were treated for 10 min either with an enzyme or with heat (boiling water bath). The nuclease-treated samples were subsequently also treated with heat, whereas the trypsin-treated samples were subsequently treated with either heat or trypsin inhibitor for 10 min. Trypsin-inactivation controls were carried out by a 10 min simultaneous incubation with trypsin plus either heat or trypsin inhibitor. All enzymatic treatments were at 37°. Concentrations of enzymes were as follows: trypsin, 20 μg/ml; trypsin inhibitor, 200 μg/ml; DNase and RNase, 500 μg/ml.

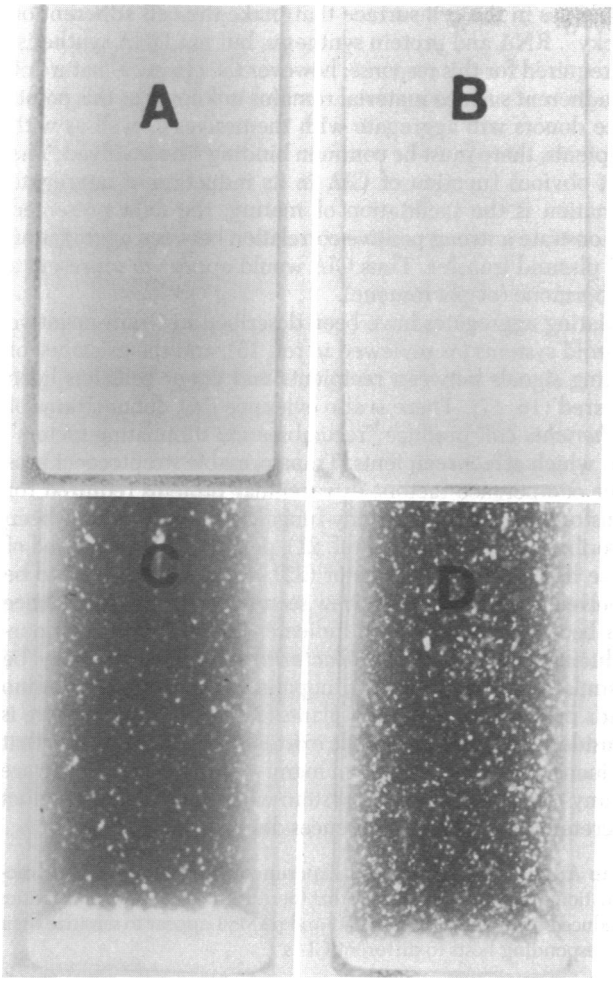

FIG. 1. Appearance of clumped and nonclumped cells. (*A*) Overnight culture of JH2-S diluted 1:10 into fresh broth and incubated 4 hr at 37°. (*B*) Similar culture of 39-5 cells. (*C*) 4 hr mating mixture of 39-5 and JH2-S. (Overnight cultures of JH2-S cells were diluted 1:10 and 39-5 cells were diluted 1:100 into 5 ml of fresh medium). (*D*) 39-5 cells diluted 1:10 into mixture containing equal volumes of fresh medium and cell-free filtrate from a JH2-S log-phase culture, and incubated 2 hr at 37°. After incubation, the cell suspensions were placed in quartz spectrophotometer cuvettes, shaken by hand to disperse the clumps evenly, and photographed 5 min after shaking. The growth medium and the approximate cell concentration is the same in all four cultures. The photographs of cultures A and B appear light colored, whereas C and D are dark with white clusters of cells. This is due to the fact that virtually all of the cells in cultures C and D are clumped (note the large mass of cells at the bottom of cuvettes C and D) and the supernatant fluid is clear, whereas in A and B, the cells are uniformly suspended in the medium and it appears turbid (OD_{660} of A and B $\cong$ 1.75; OD_{660} of C and D $\cong$ 0.100).

(pPD1) similar, but not necessarily identical, to pAMγ1. It is important to note that the cells that harbor pAMγ1 and produce this low level of activity do not themselves clump during growth, although stationary cultures occasionally exhibit a "granular" appearance that suggests some degree of aggregation.

To gain more direct evidence linking CIA to mating, we performed mating-induction experiments. OG1-10 cells (plasmid free) were mixed with OG1-20 cells (harboring pAMα1 and pAMγ1) for 10 min (an amount of time during which little transfer is normally observed); prior to mixing, donor cells were exposed to a culture filtrate (CIA preparation) from recipient cells or, as a control, from a separate donor culture. The frequency of mobilization of pAMα1 by pAMγ1 was measured. (Under the conditions of this experiment, visible clumping in the donor culture had not yet occurred at the time they were mixed with recipients.) As can be seen in the upper part of Table 6, treatment of donor strains with recipient CIA increased the transfer frequency approximately 200- to 300-fold. Similar experiments using DS-5C1 (the original host harboring pAMα1 and pAMγ1) as a donor with JH2-2 (plasmid free) as a recipient have also shown that CIA preparations can greatly enhance transfer (data not shown). This induction of donors to high mating potential could be blocked by rifampicin. However, if the donors were treated with CIA preparations in the absence of this drug, addition during mating did not block transfer. [As shown above (Table 4), rifampicin blocks induction of clumping by exogenous CIA.] These experiments show that filtrates of recipients that induced clumping in donors also greatly facilitated plasmid transfer.

We obtained additional data that add support to the notion that clumping and mating are directly related. By mating strain

Table 4. Effect of drugs on cellular response to CIA*

CIA source	Drug added	Clumping induction
	Addition of drug and CIA together	
JH2-S	None	+
JH2-S	Cam†	−
JH2-S	Rif‡	−
JH2-S	HPUra§	+
39-5	None	−
	Drug added 15 min after CIA	
JH2-S	Cam†	+
JH2-S	Rif‡	+

* CIA preparations from JH2-S or 39-5 (negative control) were mixed with 39-5 cells and fresh broth. In the cases noted, drugs were added either immediately after or 15 min after mixing of cells + CIA. The occurrence of clumping was then monitored after 1 hr.
† Chloramphenicol–50 μg/ml.
‡ Rifampicin–100 μg/ml.
§ 6-Parahydroxyphenylazouracil–50 μg/ml.

Table 5. CIA production and response to exogenous CIA by isogenic strains differing in plasmid content

Strain	Plasmid content	Relative CIA activity* produced	Clumping response to CIA† produced by
			OG1-10
OG1-10	−	64	−
OG1-11	pAMα1	64	−
OG1-12	pAMα1, pAMγ1	2	+
OG1-13	pAMγ1	2	+
			JH2-2
JH2-2	−	64	−
DT-11	pAMα1	64	−
DT4	pAMα1, pAMγ1	4	+
HD1	pAMγ1	4	+

* Activity represents the maximum dilution (in a series of 2-fold dilutions) of CIA which induced clumping of *S. faecalis* strain 39-5. Clumping was observed after 90 min. Response to CIA in these assays is generally all or none. While there is occasional overlap of the two highest dilutions in the series of 2-fold dilutions, end points are usually very distinct.
† The CIA used in these assays was diluted 2-fold.

Table 6. Induction of mating by CIA preparations*

Mating system	Conditions of donor induction	Trans-conjugants/ donor
OG1-20 × OG1-10	20 min with recipient filtrate	8.4×10^{-4}
	50 min with recipient filtrate	1.1×10^{-3}
	20 min with donor filtrate	3.6×10^{-6}
	50 min with donor filtrate	3.6×10^{-6}
HJ30-10 × JH2-S†	90 min with recipient filtrate‡	1.4×10^{-4}
	90 min with donor filtrate	5.4×10^{-7}
HJ30-2 × JH2-S§	90 min with recipient filtrate	2.9×10^{-7}
	90 min with donor filtrate	2.8×10^{-7}

* For the experiments shown in this table, donor cells were grown to late log phase (approximately 5×10^8 cells/ml) and diluted 1:10 into 2 ml of a 1:1 mixture of fresh broth and filtrate of a log culture of either donors or recipients. The cells were then incubated at 37° for the specified incubation period. A late log culture of recipient cells was diluted 1:10 into fresh broth and incubated at 37° during induction of the donors. At the end of the incubation period an aliquot of the donor culture was diluted 1:10 into the recipient culture (total volume 2.5 ml). The final mixture resulted in a ratio of approximately 10 recipients per donor. After 10 min at 37° the cells were Vortex mixed and plated on selective media. [Plates contained streptomycin (1 mg/ml) and tetracycline (8 μg/ml).]

† Donor strain shows clumping response to CIA from recipient.

‡ Longer incubation periods were used because HJ30-10 requires a longer period of incubation (~2.5 hr) to clump when exposed to CIA, as opposed to OG1-20 (~1 hr).

§ Donor strain does not show clumping reponse to CIA from recipient.

39-5 with DT-11, it was possible to obtain hemolytic transconjugants carrying pPD1 and pAMα1 that differed in their response to CIA (unpublished data). All such transconjugants were greatly reduced in the production of CIA activity, but some of these strains clumped when exposed to exogenous CIA, while others did not. The reason for these differences is not yet clear, but it may reflect alterations in the pPD1 plasmid as a result of its transfer from 39-5 to DT-11. The important feature to note here is that the differences in clumping response observed with these strains is paralleled by differences in transfer frequency. Strains HJ30-2 and HJ30-10 are two hemolytic, tetracycline-resistant transconjugants obtained by mating 39-5 (donor) with DT-11 (recipient). As shown in Table 6, strain HJ30-10, which exhibits a clumping response, can be induced to a high mating potential by CIA preparations from recipient strains. In contrast HJ30-2, which does not exhibit a clumping response to CIA, also does not exhibit increased donor potential.

DISCUSSION

The data presented here indicate that certain *S. faecalis* strains produce an extracellular, heat-stable, trypsin-sensitive substance designated CIA, that will induce donor strains to become adherent in liquid media. CIA is altered or its production is repressed in donor cells due to the presence of the transferable plasmid; however, the plasmid provides the cells with a mechanism by which exogenous CIA can trigger events leading to changes in the cell surface that make the cell adherent or "sticky". RNA and protein syntheses, but not DNA synthesis, are required for this response; however the chemical nature of the adherent surface material remains unknown at this point. Since donors will aggregate with themselves as well as with recipients, there must be common binding sites involved. The most obvious function of CIA in its induction of aggregate formation is the facilitation of mating; the data presented demonstrate a strong positive correlation between aggregation and plasmid transfer. Thus CIA would appear to represent a sex hormone (or pheromone).

Mating aggregates have been described in Gram-negative plasmid systems (as reviewed in ref. 15), and the existence of mating signals between recipients and donor cells has been inferred (16, 17). There is also evidence that donor strains of *Escherichia coli* produce "recombination stimulating factors" (18) which affect recipients. Transformable streptococci produce competence factors, extracellular proteins required for transformation (19, 20). Many fungal sex hormones have been described (for a review see ref. 21), perhaps the best studied of these being the yeast α factor (22), a peptide believed to be involved in mating (for a review see ref. 23). Recently evidence has been presented which indicates that transfer of tumor-inducing plasmids in *Agrobacterium tumefaciens* may be stimulated by octopine and nopaline, tumor-specific amino acids produced by infected plants (24, 25). Although there is considerable evidence for the existence of soluble factors that influence genetic exchange in many systems, we are not aware of any report of a bacterial system where a diffusible product excreted by recipients influences donors.

Note Added in Proof. Recent experiments involving column fractionation of filtrates have shown that there are at least two CIA activities produced by recipient cells. pPD1 and pAMγ1 appear to sensitize their corresponding hosts to different CIAs.

We thank Paul Tomich for helpful discussions and Margaret Mikus for excellent technical assistance on certain aspects of this work. This work was supported by Public Health Research Grants AI10318 from the National Institute of Allergy and Infectious Diseases and DE02731 from the National Institute of Dental Research and by a grant from the Upjohn Company. D.B.C. is the recipient of Research Career Development Award K04 AI00061 from the National Institute of Allergy and Infectious Diseases. G.D. was supported by a National Science Foundation Graduate Fellowship and an F. G. Novy Fellowship from the Department of Microbiology, University of Michigan.

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