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Sex Pheromone cAD1 in *Streptococcus faecalis*: Induction of a Function Related to Plasmid Transfer

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In short matings between two donor strains with distinguishable isogenic conjugative plasmids (derivatives of pAD1), only the strain preexposed to the sex pheromone cAD1 behaved as a donor.

Recent reports from our laboratory have shown that recipient strains of *Streptococcus faecalis* excrete diffusible compounds (sex pheromones) which induce donor cells, harboring certain conjugative plasmids, to become adherent (7, 8). (Such a phenomenon had not previously been reported among bacteria.) The induction requires RNA and protein syntheses (7) and can be brought about by exposing donors to cell-free filtrates of recipients. Induced donors will not only aggregate with recipients, but also will "clump" in the absence of recipients.

Filtrates which exhibit "clumping inducing" activity also enhance mating. For example, if donor cells harboring the conjugative plasmid pAD1::Tn*917*(Em), conferring erythromycin (Em) resistance, are exposed to a filtrate of plasmid-free recipient cells for 45 min before a short (10-min) mating, the number of plasmid transconjugants appearing is increased by a factor of 10^6 over control experiments in which donors are not previously induced (8).

Recipient cells actually produce multiple sex pheromones, each specific for a different class of donors (8). When a recipient acquires a given conjugative plasmid, the production of the related pheromone is "shut off"; however, other pheromones specific to donors with different plasmids continue to be produced. The different pheromones are designated by their relationship to the plasmid system originally used to resolve them. For example, cAD1, cOB1, cPD1, and cAMγ1 refer to the pheromones to which specific responses occur by strains respectively harboring the plasmids pAD1, pOB1, pPD1, and pAMγ1 (8). All of the pheromones examined thus far are heat stable (resistant to autoclaving) and protease sensitive (8; R. Craig, unpublished data); cPD1 appears to have a molecular weight of less than 1,000 (4; R. Craig, unpublished data).

The *S. faecalis* sex pheromones bring about a modification of the donor cell surface so that contact from random collisions of the donors and recipients (both of which are nonmotile) results in mating aggregates. Whereas it was not heretofore known whether the pheromones also induced other functions related to conjugation, we now present evidence that this is indeed the case. Experiments described below involving donor-donor matings between isogenic strains with differentially marked homologous plasmids show that events associated with plasmid transfer are also pheromone induced. We essentially questioned that if the sole function of the pheromone was to generate mating aggregates, plasmid transfer should still occur equally well in both directions—regardless of which donor was induced before mating. That this is not the case is shown below.

The two donor strains harbor distinguishable derivatives of the conjugative hemolysin plasmid pAD1 (9) (molecular weight, 35×10^6; one to two copies per chromosome). One donor contained pAD1::Tn*916*(Tc) (molecular weight, 44×10^6) conferring tetracycline (Tc) resistance (A. E. Franke and D. B. Clewell, manuscript in preparation), whereas the other harbored pAD1::Tn*917*(Em) (molecular weight, 38×10^6), which confers erythromycin resistance (9, 10). Each host was also distinguishable by chromosomal resistance markers. Thus, using appropriate selective conditions, transconjugants resulting from plasmid transfer in either direction were easily identified. A 20-min mating between the two strains resulted in very little transfer in either direction (Table 1). However, when one of the strains was exposed to a cAD1-containing filtrate of the isogenic plasmid-free strain OG1-10 (8) for 45 min before the mating, the frequency of transfer increased by a factor of about 10^3 to 10^4, but this occurred primarily in the direction from the cAD1-exposed cells to the unexposed cells. It made no difference which strain was induced before mating; plasmid transfer was enhanced primarily in the direction from induced to uninduced cells. In the case where both strains were induced, transfer was enhanced (in this case by a factor of about 10^2) in

TABLE 1. *Pheromone-promoted plasmid transfer between S. faecalis donors*

Strain induced before mating[a]	No. of transconjugants per donor[b] selected on:	
	Sm Tc	Rif Fa Em
Control	8×10^{-8}	3×10^{-7}
OG1RF[pAD1::Tn*916*(Tc)]	2.5×10^{-3}	$<8 \times 10^{-8}$
OG1S[pAD1::Tn*917*(Em)]	1.2×10^{-6}	8.5×10^{-4}
Both	5.1×10^{-5}	2.5×10^{-5}

[a] Strain OG1RF[pAD1::Tn*916*(Tc)] has chromosome-determined resistances (mutations) to rifampin (Rif) and fusidic acid (Fa) and carries pAD1::Tn*916*(Tc); strain OG1S[pAD1::Tn*917*(Em)] has a chromosome-determined resistance to streptomycin (Sm) and carries pAD1::Tn*917*(Em). Using fresh overnight cultures grown in N2GT broth (8), donors and recipients were separately diluted 1:20 in fresh broth and grown to late log phase (about 5×10^8 cells per ml). The culture to be induced was then diluted 1:10 into a 1:1 mixture of fresh broth and culture filtrate (prepared as previously described [8]) of the plasmid-free strain OG1-10. In parallel, the other culture (uninduced) was diluted 1:10 into fresh broth. Both cultures were then incubated (37°C) for 45 min (shaking), at which time equal volumes of each were mixed together to give a 1:1 ratio of induced to uninduced cells. The mixture was incubated (shaking) for 20 min at 37°C and then blended on a Vortex mixer and plated on selective medium (Penassay medium [8] containing drugs). The selective medium contained either 1 mg of streptomycin and 5 μg of tetracycline per ml or 25 μg of rifampin, 25 μg of fusidic acid, and 50 μg of erythromycin per ml.

[b] These values represent the averages of two identically performed experiments carried out at different times. (A number of additional experiments showing similar results have also been done but were not averaged in because of differences in the experimental design.)

both directions. The latter experiment implies that the directionality effect observed in the case where only one donor was induced could not have been due simply to a prevention of plasmid uptake by induced cells. However, the fact that the frequencies of plasmid transconjugants are somewhat lower than the case where only one strain was induced suggests that entry (or surface) exclusion functions could be enhanced during induction.

The observed transfer frequencies that occurred upon induction are 1 to 2 orders of magnitude lower than cases where the recipient was plasmid free (8). This is assumed to be due to surface exclusion or perhaps a preference for the resident plasmid over the newly introduced plasmid with respect to incompatibility functions. Insofar as the selection of each type of transconjugant did not involve coselection of the recipients' resident plasmid, the majority of each type of transconjugant lost the resident plasmid (marker) due to plasmid incompatibility. In the case where pAD1::Tn*916*(Tc) was transferred from induced cells, 10 of 12 transconjugants examined had become sensitive to erythromycin, whereas in the case where pAD1::Tn*917*(Em) was transferred from induced cells, 7 of 8 were sensitive to tetracycline. The remaining cells were recombinants resistant to both drugs; the observed plasmid-plasmid recombination frequency here is consistent with previous observations (11). (In cases where recombinants were tested as donors in subsequent matings, the two markers were found to be linked.)

Induction experiments involving the two donors mentioned above have also been done under conditions where the cells were diluted up to 10^5-fold during the 45-min induction to eliminate self-aggregation before mixing with the recipient strain. (This was to rule out the possibility that self-contact, rather than the pheromone itself, might be responsible for inducing the directionality phenomenon.) In this type of experiment the cells were concentrated by filtration (membrane filters [Millipore Corp.]) and resuspended in a small volume immediately before mating. The results (not shown) were essentially the same as those in Table 1 with regard to preferred directionality of plasmid transfer. This was true regardless of whether the two strains were mixed before or after the filter-concentration step, or whether recipients were in great excess (up to 10^4-fold) of donors.

Thus, whereas the response of plasmid-containing cells to cAD1 results in a modification of the cell surface, making it adherent, it also results in a preparation step for plasmid transfer. The nature of the preparation event is unknown at this point, and we can only speculate that it may involve: (i) setting up a particular plasmid-membrane (or plasmid-protein) configuration necessary for initiating DNA transfer; (ii) synthesis of new copies of the plasmid, which may be necessary for transfer; or (iii) nicking or RNA priming, or both, of the circular plasmid DNA molecule in preparation for transfer. With regard to the latter two points, we have performed experiments with radioactive thymidine which show that the amount of plasmid [pAD1::Tn*917*(Em)] that can be isolated as covalently closed circular DNA (measured as a percentage of the total DNA in the cell) remains essentially unchanged after a 1-h exposure to cAD1. Whereas this tends to argue against an enhanced replication or nicking as part of the preparation process occurring before cell contact, it is possible that newly replicated molecules are all nicked (or linear) and are, thus, not

detected (5) as covalently closed circular plasmid DNA. An additional argument against replication, however, is the observation that the presence of the replication inhibitor 6-(*p*-hydroxyphenylazo)uracil (2, 6) (50 μg/ml) during induction had no or little effect on the preparation phenomenon. However, it should be noted that this drug is specific for only certain DNA polymerases (6); thus, a resistant polymerase could be involved.

It is noteworthy that plasmid transfer was not inhibited by rifampin (50 μg/ml) when present during a 10-min mating between induced OG1S [pAD1::Tn*917*(Em)] donors and OG1RF (resistant to rifampin) plasmid-free recipients. This leaves open the possibility that the preparation process could involve or include RNA priming.

Conceivably, cAD1 induces a polycistronic operon with several functions related to transfer. This would be, to some extent, analogous to the Tra operon of certain sex factors in gram-negative bacteria (for a review, see 1 and 3). Whereas there is no substantial evidence for sex pheromones in these organisms, the Tra operon of the *Escherichia coli* F plasmid confers the synthesis of pilus structures (important in initiating contact with recipients) as well as other functions such as surface exclusion and plasmid transfer (1, 3). It is conceivable that surface exclusion could also be involved in the cAD1 induction phenomenon, since the experiments of Table 1 showed that when both donors were induced, the transfer frequencies were significantly lower than the cases where only one strain was induced.

Much is yet to be learned about the nature of *S. faecalis* sex pheromones and the details of their mechanism of action. Pheromones of this type have still not been reported in other bacteria, yet they are widespread in *S. faecalis* (a member of the normal human intestinal flora) and appear to be involved in the evolution of drug resistance in this species (8).

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LITERATURE CITED

1. **Achtman, M., and R. Skurray.** 1977. A redefinition of the mating phenomenon in bacteria, p. 223–279. *In* J. L. Reissig (ed.), Microbiol interactions, series B. Receptors and recognition, vol. 2. Chapman and Hall, London.
2. **Brown, N. C., and R. E. Handschumacher.** 1966. Inhibition of the synthesis of deoxyribonucleic acid in bacteria by 6-(p-hydroxyphenylazo)-2,4-dihydroxypyrimidine. I. Metabolic studies in *Streptococcus faecalis*. J. Biol. Chem. **241**:3083–3089.
3. **Clark, A. J., and G. J. Warren.** 1979. Conjugal transmission of plasmids. Annu. Rev. Genet. **13**:99–125.
4. **Clewell, D. B., G. M. Dunny, B. L. Brown, R. A. Craig, and Y. Yagi.** 1979. Sex pheromones in *Streptococcus faecalis*, p. 284–285. *In* M. J. Parker (ed.), Pathogenic streptococci. Reedbooks, Chertsey, Surry, England.
5. **Clewell, D. B., Y. Yagi, G. M. Dunny, and S. K. Schultz.** 1974. Characterization of three plasmid deoxyribonucleic acid molecules in a strain of *Streptococcus faecalis*: identification of a plasmid determining erythromycin resistance. J. Bacteriol. **117**:283–289.
6. **Cozzarelli, N.** 1977. The mechanism of action of inhibitors of DNA synthesis. Annu. Rev. Biochem. **46**:641–668.
7. **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. U.S.A. **75**:3479–3483.
8. **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.
9. **Tomich, P. K., F. Y. An, and D. B. Clewell.** 1978. A transposon (Tn*917*) in *Streptococcus faecalis* that exhibits enhanced transposition during induction of drug resistance. Cold Spring Harbor Symp. Quant. Biol. **43**: 1217–1221.
10. **Tomich, P. K., F. Y. An, and D. B. Clewell.** 1980. Properties of erythromycin-inducible transposon Tn*917* in *Streptococcus faecalis*. J. Bacteriol. **141**:1366–1374.
11. **Yagi, Y., and D. B. Clewell.** 1980. Recombination-deficient mutant of *Streptococcus faecalis*. J. Bacteriol. **143**:966–970.