Proc. Natl. Acad. Sci. USA
Vol. 82, pp. 8582–8586, December 1985
Genetics

Induction of surface exclusion (entry exclusion) by *Streptococcus faecalis* sex pheromones: Use of monoclonal antibodies to identify an inducible surface antigen involved in the exclusion process

(bacterial conjugation/plasmid/clumping inducing agent/immunoblotting)

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Communicated by R. H. Wasserman, August 12, 1985

ABSTRACT **The *Streptococcus faecalis* plasmid pCF-10 is representative of a class of plasmids that enables its host cells to respond to sex pheromones produced by other *S. faecalis* cells. The pheromone response has been previously shown to result in increased conjugal plasmid transfer, cell clumping, and multiple cell-surface antigenic changes. To test for other effects of pheromone induction, cells carrying pCF-10 were used as recipients in matings with an isogenic donor strain carrying a derivative of pCF-10, tagged with a transposon to provide an additional selective marker. Pheromone induction of the "male recipients" decreased their recipient ability by a factor of 10–300 in comparison to uninduced cells or plasmid-free recipients. These results indicate that an entry exclusion (surface exclusion) function, similar to that described in studies of plasmids in Gram-negative bacteria, is induced during the *S. faecalis* pheromone response process. The exclusion operates only against homologous plasmids. Immunological, biochemical, and genetic experiments using monoclonal antibodies reactive with C130, the predominant protein antigen associated with the pheromone response of cells carrying pCF-10, indicate that this antigen is involved in surface exclusion. The data also support the notion that synthesis of C130 involves a post-translational modification of a precursor of C130 to a final product of higher molecular weight form.**

Conjugal transfer of certain *Streptococcus faecalis* plasmids is enhanced as a result of the response of donor cells carrying these plasmids to peptide pheromones (clumping inducing agents) produced by recipients (1, 2). The pheromone response of cells carrying the tetracycline-resistance plasmid, pCF-10 (3), involves the synthesis of multiple cell-surface antigens (4, 5). Presumably, one result of these antigenic changes is to increase the ability of donor cells to attach to recipients via synthesis of an adhesin termed "aggregation substance" (2). This system represents an attractive bacterial model for the analysis of cell–hormone interactions, and it also provides an opportunity to combine genetic, biochemical, and immunological techniques to analyze the streptococcal cell surface at the molecular level.

Our previous immunoblotting studies (4) indicated that the predominant surface antigenic change associated with the pheromone response is the synthesis of proteinaceous antigenic material that migrates as a group of bands in the 130-kDa range in denaturing polyacrylamide gels. We have termed this group of bands the C130 antigen. A second antigen, SA73, that migrates as a single band of 73 kDa, also appears during pheromone induction. We have obtained monoclonal antibodies that react with at least two bands in the C130 group (5). Preliminary experiments with these antibodies indicated that C130 was not involved in the processes of attachment or transfer of DNA between donor and recipient cells.

Conjugative plasmids in Gram-negative bacteria confer, by at least two different mechanisms, immunity to superinfection of their host cell by related plasmids. Incompatibility is observed between related plasmids within the same cell, whereas entry exclusion or surface exclusion reduces the ability of plasmid-carrying cells to acquire additional copies of related plasmids via conjugation (6, 7). We now present data demonstrating that, in addition to increasing donor potential, the pheromone response of donor cells also involves induction of a surface exclusion function similar to that observed with the *Escherichia coli* sex factor F and related plasmids. Our data also show that the surface exclusion mediated by pCF-10 does not operate against plasmids that respond to different pheromones. The results of biochemical and genetic experiments using conventional and monoclonal antibodies are consistent with the notion that the pheromone-induced surface exclusion exhibited by cells carrying pCF-10 is mediated by the C130 antigen.

MATERIALS AND METHODS

Bacterial Strains, Culture Media, and Conjugation Experiments. For all mating experiments, BYGT medium (3) was used, whereas cells grown for antigenic analysis were cultured in M9/Casamino acids/yeast extract medium as described (8). The strains used in this study are listed in Table 1. Most of these strains have been described in detail (4, 9). Strains OG1SSpP1 and OG1RFP1 were constructed by scoring for conjugal transfer of hemolysin production from *S. faecalis* strain DS-16, as described by Tomich *et al.* (10). The pCF-10:Tn*917* plasmid was constructed by transfer of PCF-10 into a strain carrying the nonconjugative plasmid pAD2 and selecting for transposition of Tn*917* from pAD2 to pCF-10 in a subsequent round of transfer, similar to the methods described by Tomich *et al.* (11).

Pheromone induction and mating conditions have been described in detail (2, 3, 9). Concentrations of antibiotics in selective plates were as follows: tetracycline, 10 μg/ml; rifampicin, 100 μg/ml; fusidic acid, 20 μg/ml; streptomycin, 1000 μg/ml; spectinomycin, 500 μg/ml; erythromycin, 20 μg/ml. To test for surface exclusion, the protocol outlined in Fig. 1 was followed, selecting for the transfer of the erythromycin marker on pCF-10:Tn*917*. Frequencies of transfer were calculated as described (3, 9). Monoclonal antibodies used in these experiments were concentrated from tissue culture fluid by precipitation with 50% saturated ammonium sulfate, followed by dialysis against 0.01 M phosphate-buffered saline (PBS), pH 7.0. The enzyme-linked immunoassay (ELISA) described below was used to assess the potency of monoclonal antibody preparations used in surface exclusion experiments. A 1:2 dilution of an antibody preparation that showed significant ELISA reactivity when diluted 1:500

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Table 1. Bacterial strains used in this study

Strain	Plasmid content	Markers	Ref.
OG1RF	—	Rf, Fs	4, 9
OG1SSp	—	St, Sp	4, 9
OG1SSpP10	pCF-10	St, Sp, Tc	4, 9
OG1SSpP11	pCF-11	St, Sp, Tc	9
OG1SSpP10:Tn*917*	pCF-10:Tn*917*	St, Sp, Tc, Er	This study
OG1SSpP1	pAD1	St, Sp, Hm	10, this study
OG1RFP10	pCF-10	Rf, Fs, Tc	4, 9
OG1RFP11	pCF-11	Rf, Fs, Tc	9
OG1RFP1	pAD1	Rf, Fs, Hm	10, this study

Rf, rifampicin; Fs, fusidic acid; St, streptomycin; Sp, spectinomycin; Tc, tetracycline; Er, erythromycin; Hm, hemolysin.

was used in mating experiments. For the control matings with no antibodies added, tissue culture medium was precipitated, dialyzed, and used in place of monoclonal antibodies.

Immunological Studies. Polyclonal antiserum was raised in rabbits immunized with pheromone-induced OG1SSpP11 cells as described by Tortorello and Dunny (4). Production of monoclonal antibodies reactive with the C130 antigen has been described (5) and will be further discussed in a forthcoming paper (unpublished results). Briefly, spleen cells from rats immunized with induced OG1SSpP11 were fused with mouse myeloma cells (SP2-0) and the hybridoma cells were selected and propagated as described by Galfré and Milstein (12). From each of two independent fusions, a cell line was obtained that secreted monoclonal antibody reactive to the C130 antigen, as verified by immunoblot analysis (5). Antibody from either cell line 66.2 or 2.1 showed identical reactivity, both with *S. faecalis* cells and in immunoblotting assays, as well as having identical effects on mating frequencies.

Immunoblotting analyses were carried out as described (4). For the experiment shown in Fig. 2, the antigen was prepared by ammonium sulfate precipitation of culture supernatants (4), and the antigenic preparations used in the experiment depicted in Fig. 4 were concentrated by immunoprecipitation of culture supernatants with the rabbit antiserum described above and *Staphylococcus aureus* cells coated with protein A (4) prior to electrophoresis and blotting.

To test for reactivity of monoclonal antibodies with *S. faecalis* cells, an ELISA was developed. Overnight cultures of cells were centrifuged, washed once in 0.2 M EDTA (pH 7.5), and resuspended in distilled water to an absorbance at 450 nm of 0.25 ± 0.05. The cells were distributed into microtiter plate wells and dried overnight at 65°C. All subsequent steps were carried out at ambient temperature, and after each incubation step the wells of the plates were rinsed with PBS containing 0.05% (vol/vol) Tween 20 (PBS-T). After a brief rinse of the dried plates with PBS-T, a blocking agent of 0.5% ovalbumin in PBS was added, and after 30 min, each well was rinsed with PBS-T. Salt-precipitated monoclonal antibodies were added and incubated for 1 hr, and the wells were rinsed with PBS-T. Anti-rat immunoglobulin conjugated to horseradish peroxidase (Cooper Biomedical, Malvern, PA) was added and incubated for 2 hr. After PBS-T rinsing, a peroxidase substrate consisting of 0.04% (wt/vol) *o*-phenylenediamine and 0.01% (vol/vol) H_2O_2 in 0.05 M phosphate citrate buffer (pH 5.0) was added. The reaction was allowed to proceed for 5 min and was stopped with 2.5 M H_2SO_4, and color development was read in a Dynatech microplate reader at 490 nm.

RESULTS

Evidence for the Modification of a Precursor of the C130 Antigen to a Higher Molecular Weight Form. A plasmid (pCF-11) derived by spontaneous mutation of pCF-10 (9), confers a constitutive high frequency transfer and clumpy phenotype on its host cell and determines constitutive production of the SA73 antigen as well as a group of antigenic bands similar to C130, but this group of bands (denoted C125) migrates slightly faster on $NaDodSO_4$/PAGE (4). Uninduced wild-type donors may also contain a small amount of C125 (4). Pheromone induction of cells carrying pCF-11 causes a "shift" in the migration pattern of this group of bands to a form identical to the C130 pattern observed with induced wild-type donors, as determined by immunodevelopment with polyclonal antiserum.

We recently were able to obtain two independent rat–mouse hybridoma cell lines secreting monoclonal antibodies reactive to some of these antigenic bands (5). Fig. 2 shows the reactivity of one of these monoclonal antibodies (the same results were obtained with both antibodies) to cell-surface antigens from either induced or uninduced cells carrying pCF-11. Reactivity to a pair of bands was evident in both blots, but the antigenic bands from the induced cells migrated more slowly than those from the uninduced cells. Analyses of wild-type donor cells gave similar results, except that very little reactivity was detected in blots prepared from uninduced cells (data not shown). These findings indicated that at least some of the bands in the C125 and C130 groups were antigenically related. One possible interpretation of

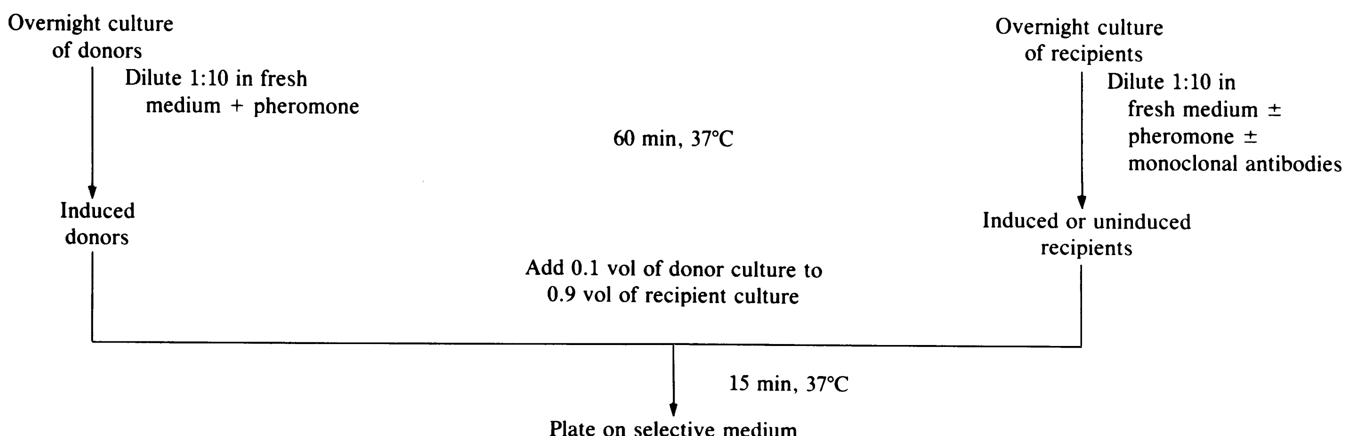

FIG. 1. Protocol for surface exclusion experiments.

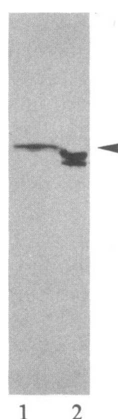

FIG. 2. Shift in antigenic banding pattern of *S. faecalis* cells carrying pCF-11 as a result of pheromone induction. Culture supernatants were concentrated by ammonium sulfate precipitation, separated by $NaDodSO_4$/PAGE, and blotted; the blots were developed with monoclonal antibody 66.2. Lanes: 1, antigen from pheromone-induced OG1SSpP11 cells; 2, antigen from uninduced OG1SSpP11 cells. Arrow denotes position of the C130 antigen.

these data is that synthesis of C130 involves a post-translational modification of a structural gene product. Both the synthesis of the precursor (C125) and the modification activity may be inducible in the wild-type donor cells. In contrast, cells carrying pCF-11 may express the precursor constitutively, while the modification activity remains inducible by sex pheromones. Further support for this notion will be presented below.

An Inducible Surface Exclusion Function Mediated by pCF-10 and pCF-11. Conjugative plasmids in Gram-negative bacteria often encode genes that reduce the ability of their host cells to acquire closely related plasmids by conjugation. This phenomenon is termed surface exclusion or entry exclusion (6, 7). Considerable information has been obtained regarding the genes and gene products that mediate surface exclusion by the sex factor F and related plasmids (6, 7, 13). To test whether pCF-10 determines a surface exclusion function, we constructed a pCF-10 derivative containing an insertion of the erythromycin-resistance transposon Tn*917* (11) as described in *Materials and Methods*. This derivative plasmid (pCF10:Tn*917*) displayed wild-type mating behavior and pheromone response. The extra erythromycin-resistance marker enabled us to use strains carrying pCF-10 or pCF-11 as recipients for pCF10:Tn*917* in mating experiments (Fig. 1).

Table 2*a* illustrates the effects of plasmid content and pheromone induction of recipient cells on their ability to acquire pCF10:Tn*917* by conjugation. Uninduced cells carrying either pCF-10 or pCF-11 displayed an ability to acquire pCF10:Tn*917* that was equal to (or even slightly greater than) that of isogenic plasmid-free cells (matings 1, 3, and 5). However, when plasmid-containing recipient cells were exposed to sex pheromones prior to mating, there was a decrease in the transfer frequency by a factor of 10–100 (matings 4 and 6). Interestingly, strains carrying pCF-11 (and exhibiting constitutive expression of clumping, increased transfer functions, and the SA-73 surface antigen), showed inducible surface exclusion, similar to that of wild-type donors. The expression of surface exclusion by the donors carrying pCF-11 is associated with the shift in antigenic migration pattern from C125 to C130 (Fig. 2).

Inhibition of Surface Exclusion by Monoclonal Antibodies. It might be expected that at least some antibodies directed against pheromone-induced antigens would affect mating frequencies or clumping responses of donor cells. We have observed repeatedly that our monoclonal antibodies to C130 did not affect either clumping of cells carrying this antigen or frequencies of transfer between donors and plasmid-free recipients. However, when the monoclonal antibodies were added to mating mixtures containing pheromone-induced recipients carrying either pCF-10 or pCF-11, there was a 4- to 10-fold enhancement of transfer (Table 2*b*, matings 3, 4, 7, and 8). Thus, the inhibition of transfer by a factor of 50–300 resulting from surface exclusion (Table 2*b*, matings 1–3) was partially relieved by the monoclonal antibody. The presence of the antibodies had no effect on the matings that used uninduced recipients (Table 2*b*, matings 1, 2, 5, and 6). Nonspecific stimulation of transfer by monoclonal antibodies was unlikely because the enhancement was observed with induced but not uninduced cells carrying pCF-11. Since the uninduced cells carried a C125 antigen that was reactive with the monoclonal antibody, it appeared that only a reaction with the C130 form of the antigen on the recipient cell (note that all the donor cells in these experiments were expressing C130) had an effect on transfer. In addition, some monoclonal

Table 2. Effects of plasmid content, pheromone induction, and monoclonal antibodies on ability of *S. faecalis* cells to acquire pCF10:Tn*917* by conjugation

Plasmid content of recipient	Induction of recipients	Presence of monoclonal antibody 66.2	Transconjugants per donor	Transconjugants per recipient
(*a*) Effects of plasmid content				
1. None	−	−	2.7×10^{-2}	2.3×10^{-3}
2. None	+	−	3.6×10^{-2}	3.1×10^{-3}
3. pCF-10	−	−	5.5×10^{-2}	6.0×10^{-3}
4. pCF-10	+	−	8.6×10^{-4}	1.8×10^{-4}
5. pCF-11	−	−	1.1×10^{-2}	2.8×10^{-3}
6. pCF-11	+	−	9.6×10^{-4}	2.8×10^{-4}
(*b*) Effects of monoclonal antibody to C130 antigen				
1. pCF-10	−	−	2.0×10^{-1}	3.0×10^{-2}
2. pCF-10	−	+	1.2×10^{-1}	1.8×10^{-2}
3. pCF-10	+	−	6.9×10^{-4}	3.4×10^{-4}
4. pCF-10	+	+	4.8×10^{-3}	1.3×10^{-3}
5. pCF-11	−	−	6.2×10^{-2}	1.3×10^{-2}
6. pCF-11	−	+	6.3×10^{-2}	7.6×10^{-3}
7. pCF-11	+	−	1.0×10^{-3}	3.0×10^{-4}
8. pCF-11	+	+	4.0×10^{-3}	1.3×10^{-3}

Pheromone-induced OG1SSpP10:Tn*917* cells were used as donors in all matings. The recipient strain was OG1RF carrying the plasmid indicated.

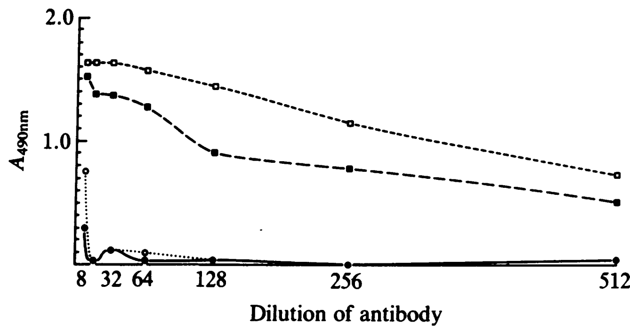

FIG. 3. Reactivity of isogenic *S. faecalis* cells carrying either pCF-10 or pAD1 with monoclonal antibodies. Whole-cell ELISAs were carried out with monoclonal antibody 2.1 as described. ●, Uninduced OG1SSpP1 cells; ○, pheromone-induced OG1SSpP1 cells; ■, uninduced OG1SSpP10 cells; □, pheromone-induced OG1S-SpP10 cells. Isogenic plasmid-free cells showed the same reactivity as the cells containing pAD1.

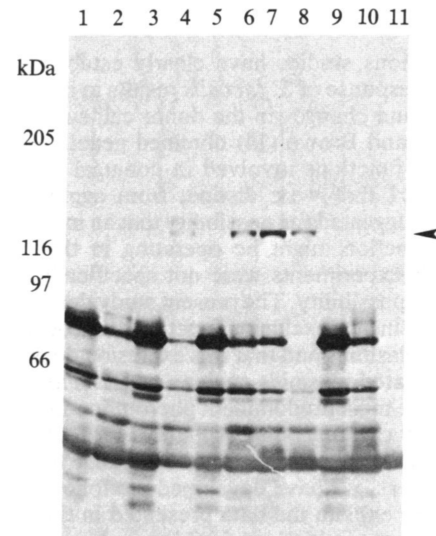

FIG. 4. Antigenic profile of *S. faecalis* cells carrying various plasmids. Immunoblotting analysis was carried out on surface antigen preparations (concentrated by immunoprecipitation as described in *Materials and Methods*), using for immunodevelopment whole rabbit antiserum raised against induced OG1SSpP11 cells. Lanes: 1, uninduced OG1SSp cells; 2, pheromone-induced OG1SSp cells; 3, uninduced OG1SSpP10 cells; 4, pheromone-induced OG1S-SpP10 cells; 5, uninduced OG1SSpP10:Tn*917* cells; 6, pheromone-induced OG1SSpP10:Tn*917* cells; 7, uninduced OG1SSpP11 cells; 8, pheromone-induced OG1SSpP11 cells; 9, uninduced OG1SSpP1 cells; 10, pheromone-induced OG1SSpP1 cells; 11, no antigen control. Arrow denotes position of the C130 antigen.

antibodies that react with common antigens of *S. faecalis* actually inhibited conjugation (unpublished results).

Specificity of Surface Exclusion. *S. faecalis* cells produce a number of different pheromones (2), and it is not uncommon to isolate strains carrying several conjugative plasmids, each determining a response to a different pheromone (2, 3). Therefore, it might be predicted that the surface exclusion mediated by pCF-10 would not operate against a plasmid that determined a response to a different pheromone. Furthermore, if C130 is involved in surface exclusion, a plasmid with an exclusion mechanism distinct from that of pCF-10 would not be expected to encode an inducible antigen cross-reactive with C130. We decided to test these predictions by comparing the surface exclusion mechanisms and surface antigens encoded by pCF-10 to those determined by the hemolysin plasmid pAD1 (10). This plasmid has been studied by Clewell and coworkers (14, 15), and the pheromone (cAD1) to which cells carrying pAD1 respond has been purified and sequenced (16). D. B. Clewell (personal communication) has determined that cells carrying pCF-10 do not respond to the purified cAD1 pheromone, and our unpublished data indicate that the two plasmids are compatible.

The antigenic relatedness of C130 to inducible antigens encoded by pAD1 was examined by ELISA and by immunoblotting. Fig. 3 shows that monoclonal antibody to C130 reacted very strongly with the cell surface of induced cells carrying pCF-10, and somewhat less strongly with uninduced cells of the same strain. However, isogenic cells carrying pAD1 showed no reactivity regardless of their exposure to pheromone preparations. As shown in Fig. 4 (lanes 9 and 10), immunoblotting analysis (using rabbit antiserum raised against pheromone-induced cells carrying pCF-11) also indicated that pAD1 does not encode an antigen related to C130. These results were confirmed by developing a blot identical to that shown in Fig. 4 with monoclonal antibody (data not shown).

The data presented in Table 3 indicated that surface exclusion against pCF10:Tn*917* did not seem to occur in cells carrying pAD1. As expected, induction of recipient cells carrying pCF-10 caused a marked decrease in the ability of these cells to acquire pCF10:Tn*917*, and this decrease was partially overcome by the monoclonal antibody. In contrast, induction of isogenic recipient cells carrying pAD1 actually resulted in higher frequencies of transfer (probably due to increased aggregation of donors and recipients), and the monoclonal antibody had no effect. These data indicate that surface exclusion is specific for plasmids related to the resident plasmid of the recipient and provide additional evidence for the involvement of C130 in surface exclusion mediated by pCF-10. Additional unpublished experiments, similar to those shown in Table 3, indicated that pAD1 also determines a surface exclusion function specific for related plasmids, and not operating against pCF-10.

Table 3. Specificity of surface exclusion

Plasmid content of recipient	Induction of recipient	Presence of monoclonal antibody	Transconjugants per donor	Transconjugants per recipient
1. pCF-10	−	−	7.0×10^{-2}	2.2×10^{-2}
2. pCF-10	−	+	1.7×10^{-2}	2.3×10^{-2}
3. pCF-10	+	−	2.8×10^{-4}	4.0×10^{-4}
4. pCF-10	+	+	7.4×10^{-4}	2.0×10^{-3}
5. pAD-1	−	−	1.9×10^{-2}	5.3×10^{-3}
6. pAD-1	−	+	2.9×10^{-2}	3.3×10^{-3}
7. pAD-1	+	−	1.6×10^{-1}	1.5×10^{-2}
8. pAD-1	+	+	1.3×10^{-1}	1.1×10^{-2}

Pheromone-induced OG1SSpP10:Tn*917* cells were used as donors in all matings. The recipient strain was OG1RF carrying the plasmid indicated.

DISCUSSION

Several previous studies have clearly established that the pheromone response of *S. faecalis* results in induction of cell aggregation and changes in the donor cell surface (4, 5, 17, 18). Clewell and Brown (14) obtained genetic evidence for induction of functions involved in conjugal transfer of the plasmid pAD1 that were distinct from aggregation. Their results also suggested the possibility that an inducible surface exclusion function might be operating in that system, although their experiments were not specifically designed to examine this possibility. The present study demonstrates that an inducible surface exclusion function is encoded by pCF-10 and related plasmids and that this exclusion does not operate against unrelated plasmids such as pAD1. The data suggest that C130, the most predominant pheromone-induced antigen expressed by cells carrying pCF-10, is involved.

To design further experiments analyzing the surface exclusion process, we have developed the following hypothetical model to explain the data presented in this paper.

(*i*) Pheromone induction of wild-type donor cells carrying pCF-10 results in simultaneous expression of a structural gene product (possibly the C125 antigen) and a modification activity that converts this product to the C130 antigen. Cells expressing C130 on their cell surface exhibit surface exclusion.

(*ii*) Cells carrying pCF-11 constitutively express the precursor (C125) of C130, which is inactive until it is modified to the C130 form. Since the modification results in a product that migrates more slowly in $NaDodSO_4$ gels, it presumably involves a process such as glycosylation, phosphorylation, or addition of lipid moieties, rather than proteolysis.

(*iii*) Because monoclonal antibodies 66.2 and 2.1 bind to both C125 and C130, but only C130 appears to be active, the antibodies probably do not bind to the active site of C130 that is directly involved in surface exclusion. The inhibition of surface exclusion caused by these antibodies might result from a conformational change in C130 molecules that have bound to the antibody.

(*iv*) Although the plasmid pAD1 presumably determines a surface antigen analogous to C130, it is not closely related to C130, because *S. faecalis* cells carrying pAD1 are indistinguishable from plasmid-free cells with regard to expression of antigens cross-reactive with C130. The cells carrying pAD1 actually were better recipients for pCF-10. Thus, acquisition of one pheromone-dependent conjugative plasmid may increase the chance for the host cell to acquire additional plasmids.

To test this model, we are currently conducting biochemical purification and isotopic labeling studies of C130 and attempting to isolate mutant derivatives of pCF-10 that show altered surface exclusion phenotypes. This type of analysis in the F plasmid system in *E. coli* has revealed the existence of two genes, *traS* and *traT*, that seem to be involved in two independent surface exclusion functions (6, 7). The *traS* gene product is found on the inner membrane (6) whereas the *traT* product is a major outer membrane protein whose synthesis involves post-translational modification steps (13). Surface exclusion in the F system seems to act after the initial attachment of donors to recipients, possibly at steps involving stabilization of mating aggregates or DNA transfer (6, 7).

It is noteworthy that surface exclusion in *S. faecalis* must also occur after the initial attachment event, since pure cultures of donors aggregate after pheromone induction (1, 2). It also seems quite logical that there would be mechanisms to prevent wasteful donor–donor transfer events, since donor cells are certainly in close proximity to one another during donor–recipient matings. The fact that surface exclusion is inducible rather than constitutive supports the notion that most transfer events in nature that involve these plasmids result from response of donors to pheromones.

We thank Peter Christie and Don Clewell for providing bacterial strains and for helpful discussions, and Jean Adsit and Debra Krug for excellent technical assistance. This project was supported by Public Health Service Grant 1RO1 AI 19310 from the National Institute of Allergy and Infectious Diseases and by U.S. Department of Agriculture Grant 82-CRSR-2-2023.

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