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Role of the Pheromone-Inducible Surface Protein Asc10 in Mating Aggregate Formation and Conjugal Transfer of the *Enterococcus faecalis* Plasmid pCF10

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The high transfer frequency of pheromone-inducible conjugative plasmids of *Enterococcus faecalis* in liquid culture is due in part to the formation of mating aggregates. These aggregates result from the interaction of two surface components, aggregation substance (AS), which is plasmid encoded, and the chromosomally encoded binding substance (BS). In the accompanying paper (S.-M. Kao, S. B. Olmsted, A. S. Viksnins, J. C. Gallo, G. M. Dunny, J. Bacteriol. 173:7650–7664, 1991), the sequence of the *prgB* gene encoding the AS molecule (Asc10) produced by pheromone-induced cells carrying plasmid pCF10 is presented. Here we report the results of genetic and immunological experiments which define the role of Asc10 in aggregation and plasmid transfer. These data indicate expression of AS on the surface of an *E. faecalis* cell and its binding to BS expressed on a second cell are required for the formation of a mating pair and the efficient transfer of pCF10 in liquid matings. However, the orientation of the receptors was not critical for transfer; ie., AS expressed on recipient cells could facilitate plasmid transfer via binding to BS on the donor. Our results suggest that additional (as yet unidentified) products are involved in forming the channel that ultimately serves to transfer the DNA, with AS-BS binding serving primarily to generate the initial attachment between cells. The putative *prgC* gene product, identified by DNA sequencing (data presented in the accompanying paper), could be involved in transfer events occurring subsequent to aggregation.

Pheromone-inducible conjugation in *Enterococcus faecalis* is characterized by the formation in liquid culture of aggregates or of clumps of donor and recipient cells under conditions in which efficient plasmid transfer occurs (12, 13). Exposure of pure cultures of donor cells to culture filtrates of recipients (12) or to purified peptide pheromones (30, 31, 33) can induce self-clumping of the donor cells, with kinetics resembling those of the induction of high-frequency transfer ability (30). These observations have been incorporated into a model (6, 10, 13) which postulates that the pheromone response of donor cells includes the synthesis of a plasmid-determined surface adhesin, aggregation substance (AS), which mediates attachment to recipient cells via a chromosomally encoded receptor, binding substance (BS). Because donor cells also express BS, they will aggregate with other donor cells, but the subsequent plasmid transfer between aggregated donors appears to be inhibited by a pheromone-inducible surface exclusion mechanism (16). Although efficient transfer in liquid is dependent on AS-BS binding, there is genetic evidence suggesting that additional pheromone-inducible functions are also necessary for transfer of plasmids between aggregated cells (5).

A combination of genetic, biochemical, and immunological methods has been used to determine the molecular nature of AS and BS. Most of this work has focused on three pheromone-inducible plasmids, including the hemolysin-bacteriocin plasmid pAD1 (17), the bacteriocin plasmid pPD1 (41), and the tetracycline resistance plasmid pCF10 (14). In the case of the former two plasmids, immunoblotting analysis of cell surface extracts prepared with the detergent Zwittergent (6, 20) led to the suggestion that a 78-kDa protein antigen was involved in aggregation. Similar analysis of the pCF10 system, which utilized primarily lysozyme extracts, suggested that a 150-kDa protein (formerly Tra150), now called Asc10, was involved in mating aggregate formation (4, 30). In the accompanying paper (24), we used insertional mutagenesis of the cloned *Eco*RI c and e fragments of pCF10 with Tn*5* to identify the gene, *prgB*, which encodes Asc10 and to show that expression of this protein on the surface of *E. faecalis* cells results in cell aggregation. We also confirmed that a second pCF10-encoded surface protein, Sec10 (formerly Tra130), previously implicated in surface exclusion (16), was not involved in aggregation. Recently, Galli et al. have shown that the 78-kDa protein encoded by pAD1 represents a proteolytic fragment of a 150-kDa protein, Asa1, which serves as the AS in the pAD1 system (19).

With regard to BS, Ehrenfeld et al. (18) have presented evidence that purified lipoteichoic acid, a major component of the *E. faecalis* cell envelope, inhibits aggregation of *E. faecalis* cells, indicating that lipoteichoic acid might function as BS. Subsequently, a genetic analysis of BS was carried out by Trotter and Dunny (37). Conjugation-negative (Con^-) mutants, defective as recipients in liquid matings with donors carrying the pheromone-inducible plasmid pCF10 or pAD1, were generated by multiple insertions of Tn*916* into

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TABLE 1. *E. faecalis* strains and plasmids

Strain	Plasmid	Description[a]	Reference
OG1RF	None	Chromosomal Rif^r Fus^r Con^+	12
	pCF500	Ery^r Con^+ pCF10 containing a Tn*917* insertion in the Tn*925* element of the plasmid	37
	pINY1825	*Eco*RI c fragment (7.5 kb) of pCF10 cloned into pWM402	4
OG1SSp	None	Chromosomal Str^r Spc^r Con^+	12
	pCF11	Tet^r pCF10 derivative, constitutive expression of Asc10 and Sec10	14
INY3000	None	Str^r Spc^r Tet^r (contains four Tn*916* chromosomal insertions) Con^- BS^-	37
INY3071	pWM401	INY3000 containing *E. coli*/*E. faecalis* shuttle vector; Cat^r Tet^r (*E. coli*); Cat^r (*E. faecalis*)	This study
INY3072	pINY1801	INY3000 containing *Eco*RI c and e fragments (11.958 kb) of pCF10 cloned into pWM402	This study
INY3073	pINY4515	INY3000 containing pINY1801 containing a Tn*5* insertion within the *prgA* structural gene of the *Eco*RI c fragment	This study
INY3074	pINY4561	INY3000 containing pINY1801 with a Tn*5* insertion in the *prgB* structural gene of the *Eco*RI e fragment	This study

[a] Abbreviations: Rif, rifampin; Fus, fusidic acid; Ery, erythromycin; Str, streptomycin, Spc, spectinomycin; Tet, tetracycline; Cat, chloramphenicol; Con, conjugation.

the *E. faecalis* chromosome. The best characterized of the mutants, INY3000, has four different Tn*916* inserts. Recent cloning studies indicate that two of these inserts are required to produce the Con^- phenotype (3). The behavior of these mutants as recipients and donors in mating experiments indicated that the Con^- phenotype resulted from the failure of these mutants to express BS. Biochemical analysis of these mutants indicated that they had alterations in the chemical composition of their surface. This work (37) also demonstrated that AS-BS binding is required for plasmid transfer in liquid, but not on solid surfaces, that alteration of BS has no effect on the donor ability of a strain carrying a pheromone-inducible plasmid, that the AS proteins of pAD1 and pCF10 utilize the same BS receptor, and that aggregate formation cannot occur via AS-AS binding.

In this report, we describe studies which utilize the BS^- strain INY3000 in mating experiments which focus on the role of the AS. These experiments demonstrate directly the requirement for AS-BS binding for pCF10 transfer in liquid. In a variation of the normal mating situation, we employ the INY3000 strain, along with nonconjugative chimeric plasmids, to create a scenario in which transfer is dependent on the binding of BS on the donor cell to AS on the recipient, reversing the normal orientation of these receptors. The restoration of recipient ability of a BS^- mutant in these experiments shows that AS acts as a type of "grappling hook" that mediates donor-recipient cell attachment but argues against the notion that the Asc10 protein is an integral component of a channel for DNA transfer. The actual transfer process is probably mediated by as yet unidentified plasmid-encoded products. The possibility that the putative *prgC* gene product identified by DNA sequencing (24) could possess such a function is discussed.

MATERIALS AND METHODS

Bacterial strains, media, and reagents. The bacterial strains and plasmids used in this study are described in Table 1. Strain INY3000 (37) was transformed with chimeric plasmids by electroporation (15). *E. faecalis* strains were grown in BYGT (35) or in M9-YE medium (35). Antibiotics (Sigma Chemical Co., St. Louis, Mo.) were added to agar plates for plasmid selection in the following concentrations: tetracycline (10 μg/ml), chloramphenicol (10 μg/ml), rifampin (50 μg/ml), fusidic acid (10 μg/ml), streptomycin (1000 μg/ml), and spectinomycin (500 μg/ml). For all pheromone inductions, we used synthetic cCF10 as described previously (30).

Preparation of rabbit polyclonal antiserum and anti-Asc10 antibody. Rabbit polyclonal antiserum was prepared by using whole, pheromone-induced OG1RF(pCF11) cells as previously described (35); although pCF11 is a mutant derivative of pCF10 constitutively expressing the normally inducible surface antigens, it is also responsive to induction with cCF10, resulting in extremely high levels of these antigens. To prepare a monospecific, polyclonal rabbit antibody specific for Asc10, lysozyme extracts from strain OG1SSp(pCF11) were separated by preparative sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE). The region of the gel corresponding to Asc10 was cut out and homogenized into a slurry. A New Zealand White rabbit was immunized with three successive subcutaneous injections of this material at 5-day intervals. The animal was bled, and the serum was collected. This serum was diluted 1:50 in phosphate-buffered saline (PBS; 50 mM NaH_2PO_4, 150 mM NaCl), and 2 ml of the mixture was mixed with approximately 10^{10} OG1RF(pINY1825) cells expressing Sec10 but not Asc10 (4). The mixture was incubated for 30 min at ambient temperature and centrifuged at 10,000 × *g*, and the supernatant was collected. This procedure was repeated five additional times, and the final supernatant was used in Western immunoblotting and for preparation of F'ab fragments as described below. Monoclonal antibodies (MAb) were prepared against pheromone-induced OG1RF(pCF11) cells according to the method of Köhler and Milstein (26) as previously described (34).

Preparation of F'ab fragments. F'ab fragments of the adsorbed anti-Asc10 polyclonal antibody were prepared by using immobilized papain and immobilized protein A agarose beads (Pierce Chemical Co., Rockford, Ill.) as instructed by the manufacturer. Briefly, 1 ml of adsorbed serum was mixed with the immobilized papain beads (suspended in 20 mM NaH_2PO_4–20 mM cysteine-HCl–10 mM EDTA · Na_4 [pH 6.2]) for 6 h at 37°C with gentle rocking. After the addition of 3 ml of 10 mM Tris (pH 7.5), the mixture was centrifuged briefly in a microcentrifuge (Fisher Scientific Co., Pittsburgh, Pa.), and the supernatant was applied to an immobilized protein A column. The F'ab fragments were eluted by washing with 10 mM Tris buffer (pH 7.5), and the

mixture was lyophilized. It was resuspended in Tris buffer for use in the mating experiments.

Inhibition of aggregation by specific antibody. Pheromone assays were conducted as previously described (14, 30). Briefly, a 1:10 dilution of an overnight OG1RF(pCF10) culture was made in M9 medium containing pheromone and antibody at the concentrations indicated, using 96-well plates. These plates were then incubated for 90 min at 37°C with shaking.

Mating experiments. Matings were conducted as previously described (30). Briefly, overnight cultures were diluted 1:10 in fresh medium (medium for donor cells containing cCF10). After 120 min of incubation at 37°C, 1 volume of donor cells was added to 10 volumes recipient cells, and the cells were incubated for 15 min 37°C. Serial 10-fold dilutions were plated on BYGT agar plates containing the appropriate antibiotics. The frequencies reported are based on viable counts calculated from the average of at least four replicate plates per determination. The standard deviations of all viable counts were less than 25%.

Preparation of bacterial cell surface protein extracts. *E. faecalis* cell surface protein extractions were performed as previously described (4). Briefly, a 10-ml exponential-phase culture of *E. faecalis* cells was washed twice with PBS (pH 7.2) and resuspended in 200 μl of PBS containing the protease inhibitors EDTA, phenylmethylsulfonyl fluoride (Sigma), leupeptin (Boehringer Mannheim Biochemicals), pepstatin A (Boehringer Mannheim Biochemicals), and aprotinin (Sigma) at final concentrations of 1.5 g/liter, 87.1 mg/liter, 0.5 mg/liter, 0.7 mg/liter, and 0.02 mg/liter, respectively. For extractions of wild-type OG1 *E. faecalis* strains, lysozyme (7.5 mg/ml) was added to the suspension, with an incubation period of 1 h at 37°C with shaking. For INY3000 strains and derivatives, 2.5 mg of lysozyme per ml and an incubation period of 40 min at room temperature were used. At the end of the incubation period, the bacterial suspension was centrifuged for 5 min (10,000 × *g*) in a microcentrifuge (Fisher Scientific), and the supernatant was removed.

ELISA for Asc10 production. The binding of MAb 8.3c10 to *E. faecalis* cells was assessed by enzyme-linked immunosorbent assay (ELISA) as previously described (16). Briefly, *E. faecalis* were fixed to microtiter plates overnight at 65°C. The dried plates were blocked with 0.5% ovalbumin and then treated with the MAb supernatant. After washing with PBS containing 0.05% (vol/vol) Tween 20, a secondary, peroxidase-conjugated goat anti-mouse antibody was added. The plates were then developed with *o*-phenylenediamine and read at 490 nm on a Dynatech microplate reader.

SDS-PAGE and Western blot analysis. Bacterial extracts were separated on 7.5% SDS-polyacrylamide slab gels (Hoefer Scientific Instruments, San Francisco, Calif.) according to the method of Laemmli (27). Protein standards consisting of myosin 200 (kDa), phosphorylase (97.4 kDa), bovine serum albumin (68 kDa), ovalbumin (43 kDa), carbonic anhydrase (29 kDa), β-lactoglobulin (18.4 kDa), and lysozyme (14.3 kDa) (Life Technologies, Inc., Gaithersburg, Md.) were used for molecular weight determination. Bacterial samples and molecular weight standards were diluted in loading buffer consisting of 0.5 M Tris, 1% SDS, 10% (vol/vol) glycerol, and 0.001% bromphenol blue. Gels were fixed with a solution of 50% methanol and 10% acetic acid, washed with 7.5% methanol and with 5% acetic acid, then treated with 10% glutaraldehyde (Fisher Scientific, Rochester, N.Y.), and stained with ammoniacal silver solution. Western blots were prepared as described by Towbin et al. (36). SDS-polyacrylamide gels containing separated bacterial extracts and prestained protein standards (Life Technologies) were washed for 15 min twice with transfer buffer, and the proteins were electrophoretically transferred to nitrocellulose sheets (Bio-Rad, Richmond, Calif.) in a TE 50 transfer apparatus (Hoefer) at 1 A for 2 h in cold 20 mM PBS transfer buffer (pH 6.5) or a carbonate-methanol buffer (10 mM $NaHCO_3$, 3 mM Na_2CO_3, 20% methanol [pH 8.8]) (8). The nitrocellulose blots were incubated in NET buffer (8.76 g of NaCl, 1.86 g of EDTA, 2.5 g of gelatin, and 0.5 ml of Triton X-100 [Sigma] per liter of 0.5 M Tris [pH 7.4]) for 30 min and then incubated overnight in either a 1:200 dilution of rabbit polyclonal immune serum or a 1:30 dilution of the adsorbed polyclonal anti-Asc10 antibody, with NET as the diluent. After a 1-h wash in NET buffer, the blot was incubated in goat anti-rabbit horseradish peroxidase conjugate (Zymed, San Francisco, Calif.) for 90 min. Finally, the blot was washed in PBS and developed with 4-chloro-1-naphthol.

RESULTS

Effects of anti-Asc10 antibodies on aggregation and plasmid transfer. Pheromone-induced *E. faecalis* cells carrying pCF10 express two surface proteins, Sec10 (130 kDa) and Asc10 (150 kDa) (4, 16, 35); the Sec10 protein is involved in a surface exclusion process inhibiting conjugal acquisition of pCF10 by cells which already contain the plasmid (16). Analysis of isogenic strains of *E. faecalis*, differing only in plasmid content, indicated that only strains which expressed the 150-kDa protein as determined by Western blot exhibited a clumpy phenotype in broth. As shown in the accompanying paper (24), Tn*5* insertion into a region of pCF10 designated the *prgB* gene abolished expression of this protein as well as the expression of the clumpy phenotype. These results led to the conclusion that the 150-kDa Asc10 antigen was responsible for aggregation.

To obtain immunological evidence for the role of Asc10 in aggregation and conjugal transfer of pCF10, specific antibodies and F'ab fragments to Asc10 were prepared. Figure 1 illustrates the specificity of mouse MAb 8.3c10 against various isogenic strains of *E. faecalis* in an ELISA. The binding of the MAb to cells containing either the pWM402 vector or a recombinant plasmid (pINY1825) conferring only Sec10 expression was compared to that observed with an isogenic strain expressing both Asc10 and Sec10. The antigenic profiles of these strains on Western blots developed with polyclonal antibody against pheromone-induced *E. faecalis* donor cells have been presented previously (4). MAb 8.3c10 bound only to those which express Asc10. However, this antibody failed to react to the denatured form of Asc10 on a Western blot (data not shown).

We also prepared a polyclonal, monospecific anti-Asc10 antibody by immunizing a rabbit with gel-purified Asc10 and adsorbed the serum with isogenic cells expressing Sec10. The Western blot in Fig. 2 illustrates that this adsorbed rabbit antiserum reacts specifically with Asc10. (Preimmune serum from the same rabbit did not react with any *E. faecalis* antigens.) The data presented in Table 2 demonstrate the ability of both the monoclonal and polyclonal antibodies against Asc10 to inhibit aggregation of *E. faecalis* cells expressing Asc10. The polyclonal anti-Asc10 antibody inhibited aggregation at dilutions of 1:320 and 1:640. MAb 8.3c10 inhibited aggregation at all dilutions tested up to 1:128. In contrast, neither a rat anti-Sec10 MAb nor normal rabbit serum affected cell aggregation. The rat MAb also served as a control for nonspecific interference with aggregation by hybridoma supernatants.

FIG. 1. ELISA of the reactivity of MAb 8.3c10 with *E. faecalis* cells. Hybridoma culture supernatant from 8.3c10 cells was diluted 1:10 and added to the first row of microtiter plates containing attached *E. faecalis* cells as indicated. The MAb was further diluted serially through seven twofold dilutions, and its binding to the cells was assessed by ELISA as described in Materials and Methods. As a negative control, the eighth row of the assay was developed without MAb 8.3c10. Open circles indicate antibody binding to OG1RF(pINY1801); closed circles indicate binding to OG1SSp (pCF11). When plasmid-free OG1 cells or OG1RF(pINY1825), which produce Sec10 but not Asc10 as determined by Western blot, were used in this ELISA, all readings were less than 0.05, as indicated by the triangles.

The MAb as well as F'ab fragments of the polyclonal, anti-Asc10 antibody were tested for their ability to affect the transfer of pCF10 (Table 3). The presence of the MAb during mating reduced the frequency of transfer by approximately 1,000-fold. The F'ab fragments reduced the mating frequency by a factor of about 100. It should also be noted that antibodies against Sec10 (16), and a number of other *E. faecalis* surface antigens (34), have no effect on plasmid transfer. These data support the results of the insertional mutagenesis studies reported in the accompanying paper (24) that indicated a critical role for Asc10 in aggregation.

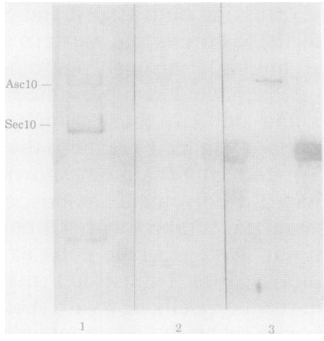

FIG. 2. Western blot of the reactivity of adsorbed polyclonal rabbit antiserum to Asc10. As described in Materials and Methods, Asc10 was purified by SDS-PAGE and used to immunize a rabbit. The serum from this animal was collected and adsorbed extensively with *E. faecalis* OG1RF(pINY4561) to remove antibodies against Sec10 and other common *E. faecalis* surface antigens. An immunoblot of surface antigen extracts from pheromone-induced strain OG1SSp(pCF11) was then developed with this adsorbed serum. Lanes: 1, preadsorbed serum; 2, preimmune serum; 3, adsorbed serum. The bands corresponding to Asc10 and Sec10 proteins are indicated.

Asc10-BS binding mediates the formation of mating aggregates in broth. To further characterize the role of Asc10-BS binding in plasmid transfer, a series of mating experiments between strains expressing various combinations of Asc10 and BS was performed. Derivatives of the BS$^-$ mutant strain INY3000 (37) expressing all possible combinations of Asc10 and Sec10 were constructed by transformation of INY3000 with nonconjugative chimeric plasmids encoding these proteins. Immunoblotting analysis of cell surface extracts of these strains can be seen in Fig. 3. The insertion of a recombinant plasmid (pINY1801 or pINY4515) conferring expression of Asc10 into strain INY3000 enabled us to carry out mating experiments in which plasmid transfer would be dependent on donor-recipient attachment mediated by Asc10 on the recipient cell and BS on the donor cell, a reversal of the natural orientation of these receptors. The INY3000 derivatives were mated with a wild-type *E. faecalis* donor strain (OGIRF) carrying a pCF10 derivative (pCF500) which is wild type with regard to pheromone response but contains an insertion of Tn*917*, enabling the use of erythromycin selection to monitor plasmid transfer in a Tet^r recipient. After induction by pheromone, the OGIRF(pCF500) donor expressed both Asc10 and BS, while the recipient expressed only Asc10. Previous analysis has shown that mating aggregates cannot be formed by AS-AS binding (37), and thus any aggregates formed in the present experiments would be via the binding of Asc10 on the recipient to BS on the donor.

The results of these matings are shown in Table 4. Donor cells were induced with pheromone for 1 h prior to a 15-min broth mating. Pheromone-induced, wild-type donor cells exhibited self-aggregation but very little plasmid transfer to INY3000 recipient. When the BS$^-$ recipient strain carried either pINY1801 (INY3072) or pINY4515 (INY3073), both of which confer constitutive expression of Asc10, plasmid transfer from donor to recipient occurred at a fairly high frequency. Interestingly, the expression of Sec10 on the surface of the recipient cells also expressing Asc10 seemed to cause a slight decrease in the frequency of transfer, consistent with the putative role of this protein in surface exclusion (compare the frequencies of transfer by induced donors to recipients carrying pINY4515 versus pINY1801). Very little plasmid transfer was observed to recipients expressing only Sec10.

DISCUSSION

During the past 15 to 20 years, a large number of conjugation systems have been identified and studied in gram-positive bacteria (11). Although many of these systems can function in a wide variety of bacterial hosts, the frequencies of transfer are generally quite low and DNA transfer often requires incubation of the mating mixtures on solid surfaces. In contrast, the pheromone-inducible plasmids of *E. faecalis* seem to have a limited host range, but they are capable of mediating transfer in liquid at frequencies comparable to those observed with conjugative plasmids in gram-negative enteric bacteria such as sex factor F (29). Electron microscopic studies have clearly shown that the sex pili are not involved in enterococcal plasmid transfer (9, 38). The data reported here and elsewhere (20, 24, 40) support the notion that the pheromone-inducible plasmids utilize cell surface protein adhesins, the AS molecules, to form the initial contact between donor and recipient cells that leads to generation of a mating pair. A complementary receptor, BS,

TABLE 2. Inhibition of aggregation by specific antibody

1/pheromone dilution (10^4)	Aggregate formation[a] at given 1/antibody dilution																	
	MAb 8.3c10								Rat anti-Sec10 MAb, 10	Anti-Asc10				Normal rabbit serum				
	Undiluted	2	4	8	16	32	64	128		160	320	640	1,280	5	10	20	40	80
2	−	+−	+−	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
4	−	+−	+−	+−	+	+	+	+	+	+−	+−	+	+	+	+	+	+	+
8	−	+−	+−	+−	+	+	+	+	+	−	+−	−	−	+	+	+	+	+
16	−	+−	+−	+−	+−	+−	+−	+−	+	−	−	−	+	+	+	+	+	+
32	−	−	−	−	−	−	−	−	+	−	−	−	+	+	+	+	+	+
64	−	−	−	−	−	−	−	−	+	−	−	−	−	−	−	−	−	−
128										−	−	−	−	−	−	−	−	−

[a] +, visible aggregate formation; +−, presence of smaller granular aggregates as distinguished from the large flocculent aggregates in the wells scored as +. Visible aggregate formation was observed in positive control wells (those without antibody) to a pheromone dilution of 1:64 × 10^4, which was equivalent to the results obtained with the rat anti-Sec10 MAb.

common to most if not all *E. faecalis* strains, is required to bind AS.

The data presented show that specific anti-Asc10 antibodies or F'ab fragments block the formation of visible mating aggregates. The decrease in the frequencies of plasmid transfer is probably a direct consequence of the lack of this aggregate formation. The simplest interpretation of these results is that the AS of pCF10 is Asc10. By binding to Asc10, the specific antibody prevents the interaction of AS and BS. However, it may be argued that the inhibition of aggregation and plasmid transfer could be a result of steric hindrance from Asc10 being located near a different and distinct AS. A definitive demonstration of the involvement of Asc10 in mating aggregate formation was obtained by carrying out genetic experiments reported here and in the accompanying paper (24). Single Tn*5* insertions into *prgB* or *prgA* inactivated the expression of these genes individually while allowing for the expression of all others. By cloning these constructs into *E. faecalis*, analysis could then be performed on isogenic strains varying in only the expression of one or the other of the corresponding proteins.

The generation of transposon insertional mutants which inactivate *prgA* or *prgB* individually and the construction of the Con^- strain, INY3000, allowed for the design of reversed-receptor mating experiments. With the wild-type (AS^+ BS^+) strain OG1RF(pCF500) acting as the donor and the BS^- strain INY3000 as the recipient, high-frequency plasmid transfer occurred in liquid only when Asc10 was expressed on the INY3000 recipient cell. Though plasmid transfer in the normal direction from donor to recipient was observed, the formation of mating aggregates occurred by a reversal of the normal AS-BS orientation. These data, taken along with the results of the antibody inhibition experiments and previous genetic data (4, 24), confirm that Asc10 comprises a vital component of the AS in the transfer of pCF10 by maintaining close contact between the mating pair. It is possible that other components could be involved as well.

TABLE 3. Inhibition of conjugal plasmid transfer by anti-Asc10 antibodies and F'ab fragments[a]

Expt	Antibody or F'ab	Transconjugants/donor
1[b] (effect of mAb 8.3c10)	−	2.6×10^{-4}
	+	3.1×10^{-7}
2[c] (effect of F'ab fragments of polyclonal anti-Asc10)	−	5.2×10^{-1}
	+	1.6×10^{-3}

[a] Each experiment involved a 15-min broth mating between OG1SSp (pCF10) donor cells and OG1RF recipients. The donor cells were induced with pheromone for 60 min prior to the matings.

[b] The cells used in these matings were grown in BYGT medium as described in Materials and Methods. Just prior to mating, the cells were harvested in the microcentrifuge and resuspended to the same cell density in a 1:1 mixture of BYGT and either Dulbecco modified Eagle tissue culture medium (−) or culture supernatant from hybridoma cell line 8.3c10 (+).

[c] The cells used in these matings were grown in BYGT. Just prior to preparation of the mating mixtures, they were centrifuged and resuspended in BYGT containing approximately 30 μg of F'ab fragments per ml from the polyclonal anti-Asc10 antibody.

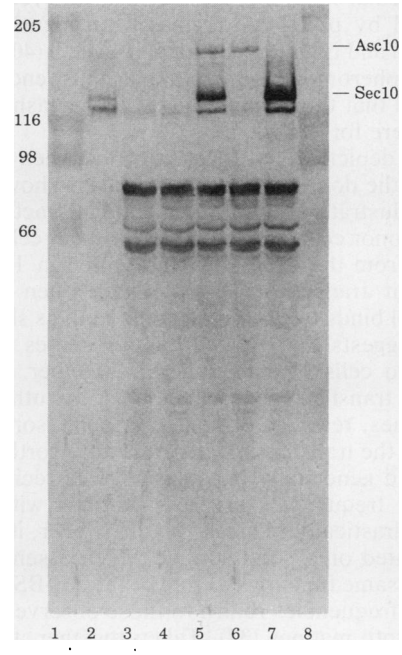

FIG. 3. Antigenic profile of *E. faecalis* strains expressing various combinations of Asc10, Sec10, and BS. Lysozyme extracts of *E. faecalis* surface antigens from the strains indicated below were subjected to SDS-PAGE, transferred to nitrocellulose, and developed with a rabbit polyclonal, anti-OG1SSp(pCF11) antiserum. This antibody reacts with all known surface antigens involved in pheromone-inducible conjugation. Lanes: 1, molecular weight standards (positions indicated in thousands); 2, pheromone-induced OG1RF(pCF10); 3, INY3000; 4, INY3000(pWM401); 5, INY3000 (pINY1801); 6, INY3000(pINY4515); 7, INY3000(pINY4561). The Asc10 and Sec10 proteins are identified.

TABLE 4. Effects of the expression of Asc10 and Sec10 on recipient cell surfaces on plasmid transfer[a]

Plasmid content of recipient	Pheromone induction	Antigens expressed on recipient	Transconjugants	
			Per donor	Per recipient
None	−	None	$<4.3 \times 10^{-8}$	$<5.6 \times 10^{-9}$
	+	None	9.9×10^{-6}	2.9×10^{-7}
pWM402	−	None	$<1.0 \times 10^{-7}$	6.5×10^{-8}
	+	None	1.6×10^{-5}	9.7×10^{-7}
pINY1801	−	Asc10, Sec10	1.1×10^{-6}	3.6×10^{-7}
	+	Asc10, Sec10	2.3×10^{-4}	7.3×10^{-5}
pINY4515	−	Asc10	2.9×10^{-6}	1.3×10^{-6}
	+	Asc10	2.5×10^{-3}	1.7×10^{-4}
pINY4561	−	Sec10	2.4×10^{-8}	1.0×10^{-8}
	+	Sec10	3.2×10^{-5}	2.9×10^{-6}

[a] Fifteen-minute broth matings were carried out between pheromone-induced or uninduced BS^+ *E. faecalis* donor cells containing pCF500 (a pCF10::Tn*917* derivative showing a wild-type pheromone response) and various derivatives of strain INY3000 (a BS^- derivative of *E. faecalis* OG1SSp). Under the conditions used in these experiments, the frequency of transfer to a wild-type (BS^+) recipient strain by pheromone-induced donors is normally about 10^{-2}. The BS^- recipient strains carried plasmid pWM402 or chimeric pWM402 derivatives containing cloned fragments of pCF10 encoding constitutive expression of either the Asc10 aggregation substance protein, the Sec10 surface exclusion protein, or both. The donor cell expressed both Asc10 and BS, but the Asc10 on the donor cell could not promote binding to recipients since the recipients did not express BS.

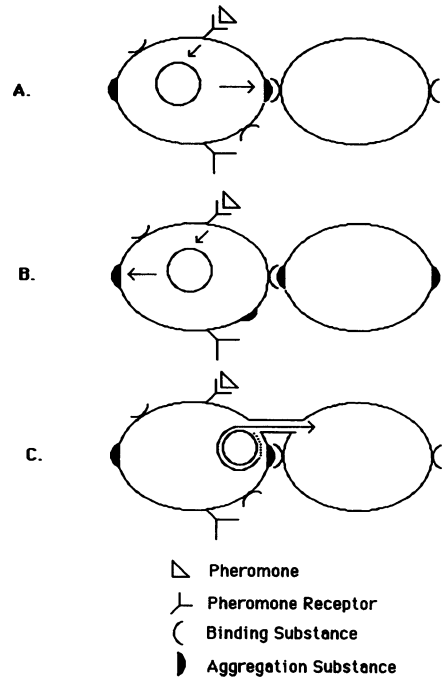

FIG. 4. Models for mating pair formation via Asc10-BS binding. (A) Normally, the attachment of cells in mating aggregates is mediated by the binding of Asc10 produced on the surface of the pheromone-induced donor cells to the BS receptor on recipients. Note that induced donor cells can also self-aggregate since they express both antigens, but that surface exclusion prevents plasmid transfer between donor cells (37). (B) In this situation, the recipient cells are BS^- mutants carrying a chimeric plasmid conferring constitutive production of Asc10. The Asc10 could promote attachment to the donor cells by binding to the chromosomally encoded BS on the donor. Aggregation via Asc10-Asc10 binding has been shown not to occur (37). Thus, the cells are attached but the normal orientation of the receptors mediating attachment is reversed. (C) A refined model, based on the results shown in Table 4, indicating a hypothetical channel for the transfer of plasmid DNA from the donor to the recipient which is formed by gene products other than Asc10 and BS. See text for further discussion.

The genetic, molecular, and immunological analysis of the pCF10 and pAD1 systems reported here and elsewhere (4, 10, 17, 19, 35) provide a considerable amount of information on the molecular nature of these cell surface receptors. The AS encoded by pAD1, Asa1, has been identified and sequenced (19) and is almost identical to Asc10 (40). It is likely that other pheromone-inducible plasmids encode similar proteins and that they function in a similar fashion as those described here for Asc10.

A model depicting the interaction between the surface proteins on the donor and recipient cells is shown in Fig. 4. Figure 4A illustrates the normal mating interaction between AS on the donor cell and BS on the recipient cell. However, the results from the experiment presented in Table 4 demonstrate that transfer can also occur when AS on the recipient cell binds to BS on the donor cell, as shown in Fig. 4B. This suggests that AS-BS binding serves primarily to hold the two cells of a mating pair together. Presumably subsequent transfer events, mediated by other plasmid-encoded genes, result in formation of some sort of channel allowing for the transfer of DNA. It is noteworthy that if BS is inactivated genetically in a plasmid-free recipient strain, the transfer frequencies in broth matings with wild-type donors are drastically reduced (37). However, if these same cells are mated on a solid surface which essentially would perform the same functions as that of the AS-BS interaction, the transfer frequencies return to those observed in normal, wild-type broth matings (37). Taken together, these results suggest that AS and BS are not directly involved in channel formation. Figure 4C illustrates the plasmid transfer process mediated by cellular components distinct from those involved in the initial formation of a mating pair via AS-BS binding.

While the biochemical nature of BS is not completely clear, lipoteichoic acid and protein may both be involved (18, 37). As noted above, our data suggest that additional plasmid-specified gene products may mediate transfer once close cell-cell contact has been established by AS-BS binding. Sequencing studies (24) resulted in identification of a putative gene, *prgC*, which was highly similar to an open reading frame identified in the broad-host-range plasmid pAMβ1 (7). Thus, the *prgC* product could be involved in a plasmid transfer function common to many conjugative plasmids of gram-positive bacteria which have different mechanisms of mating-pair formation. Analysis of the phenotypic effects of insertional inactivation of *prgC*, combined with identification of additional genes of pCF10, should enable us to define all of the genes required for plasmid transfer.

Studies of conjugation in *E. faecalis* were originally initiated because of the clinical importance of the horizontal transfer of antibiotic resistance and other virulence factors such as hemolysin production. These traits are often encoded by pheromone-inducible conjugative plasmids such as pCF10 and pAD1. In addition to serving as a reservoir for the dissemination of antibiotic resistance in gram-positive bacterial pathogens (14), *E. faecalis* is currently one of the three most frequently isolated organisms associated with nosocomial infections (39). It has been isolated from cases of endocarditis (1, 2, 21) and urinary tract infections (21) and

from the intestinal tract (39). With the discovery of the RGD motifs on Asc10 and Asa1, the possiblity arises that these proteins could also act as adhesins for the *E. faecalis* cell by binding to eukaryotic cells via the integrin class of receptors (22, 23, 32). Interestingly, serum samples from patients with enterococcal endocarditis contain antibodies reactive with a 73-kDa enterococcal surface protein (1, 2). Other data published (1, 2, 28) regarding this protein have shown it to be expressed when the bacteria are either grown in horse serum or implanted in the peritoneum of rabbits. This protein exhibits a molecular weight similar to that of the proteolytic product of AS commonly isolated by using the Zwittergent surface antigen extraction methods (20, 25). Thus, it is possible that the AS protein is expressed by *E. faecalis* cells colonizing mammalian hosts. Studies of the role of AS proteins in the adherence of *E. faecalis* cells to mammalian cells should help to elucidate the potential role of this protein in colonization.

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