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Transfer Functions of the *Streptococcus faecalis* Plasmid pAD1: Organization of Plasmid DNA Encoding Response to Sex Pheromone

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The conjugative plasmid pAD1 (59.6 kilobases) of *Streptococcus faecalis* shows a 10,000-fold increase in transfer frequency following induction by the sex pheromone cAD1. Mutagenesis of the plasmid with transposon Tn*917* was undertaken to determine the region(s) of pAD1 required for the mating response. The relevant genetic material was found to be distributed over a 31.2-kilobase contiguous region of the plasmid. Although insertions in two previously identified regions (*traA* and *traB*) exhibited increased transfer frequencies, insertions in five new regions (D, E, F, G, and H) decreased the ability of pAD1 to transfer. Insertions in region H allowed the cells to form visible mating aggregates, but the plasmid transfer frequency was decreased to levels below detection during a 1-h broth mating. Mutants with mutations in region G were able to form aggregates; however, insertions in regions D, E, and F prevented aggregate formation. Insertions in region C decreased the sensitivity of the cell to exogenous cAD1 and exhibited increased activity of the pheromone inhibitor iAD1. Surface protein profiles produced by a number of these mutants were examined, and in some cases were found to be different from those of the wild type. A map showing the various regions is presented, and related aspects of the regulation of the pAD1 mating response are discussed.

Conjugal transfer of certain *Streptococcus faecalis* plasmids is regulated by a system of sex pheromones (2, 7, 8). Potential recipient cells excrete small-peptide pheromones that elicit a specific mating response from plasmid-carrying donor cells (8, 22). Upon exposure to pheromone, donor cells show an approximately 10,000-fold increase in plasmid transfer frequency (11). *S. faecalis* recipients excrete multiple pheromones (five or more), each specific for donor cells harboring a particular class of plasmids (2). Once a recipient cell acquires a plasmid (e.g., pAD1), the production of the related pheromone activity (e.g., cAD1) is shut down, and a second peptide that behaves as a specific competitive inhibitor (e.g., iAD1) is excreted (12, 19). Unrelated pheromones (e.g., cPD1 and cAM373), however, continue to be produced.

The mating response is induced within a 30- to 40-min exposure to pheromone (7). It is characterized by the synthesis of a proteinaceous surface adhesin that facilitates the formation of mating aggregates upon random collisions of donor and recipient cells (29). Functions related to plasmid transfer are also increased (4). The substance that appears on the surface of donor cells has been termed aggregation substance (8). Aggregation substance is postulated to bind to a cell surface receptor referred to as binding substance. Both donor and recipient cells express binding substance, and donor cells can be induced to self-aggregate upon induction with culture filtrate from plasmid-free recipient cells (7). There is evidence that lipoteichoic acid corresponds to binding substance, since low concentrations (0.1 to 1.0 μg/ml) of free lipoteichoic acid are able to inhibit the aggregation of induced cells (10).

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Immunoelectron microscopy studies of cells carrying the bacteriocin plasmid pPD1 reveals a uniformly coating fuzzy material on induced, but not uninduced, cells (29). The induced adhesin was determined to be a protein, since aggregation may be prevented by treatment with trypsin, pronase, sodium dodecyl sulfate (0.05%), or heat but is insensitive to lysozyme, lipase, and the presence of a number of sugars in the medium (29). Aggregation was also shown to be dependent on RNA and protein synthesis and on the presence of divalent cations (e.g., magnesium or calcium) and phosphate ions in the medium (29). Analyses by crossed immunoelectrophoesis demonstrate the presence of novel surface antigens induced upon stimulation by pheromone (10, 16, 29).

pAD1 is a 59.6-kilobase (kb) plasmid (6, 11, 24; see Fig. 1) originally identified in *S. faecalis* DS16. It encodes resistance to UV light (5) and the production of a hemolysin-bacteriocin (24) shown to contribute to pathogenicity in mouse infections (13). An examination of cells carrying pAD1 by Western blot (immunoblot) analyses identified four novel surface proteins that appeared after exposure of cells to the related sex pheromone cAD1 (10). At 30 min after induction a 130-kilodalton (kDa) protein was seen, and at 45 min a 74-kDa protein (the major species present) and a pair of bands at 153 and 157 kDa also became evident (10). The four proteins were designated AD74, AD130, AD153, and AD157. AD74 was shown to be unique to pAD1-containing cells; however, bands corresponding the three higher-molecular-weight proteins were also observed after induction of cells containing pPD1 and pAM373 (10). Cells carrying pCF-10 were shown by Tortorello and Dunny (25) to induce a 74-kDa protein, as well as several additional proteins in the 120- to 130-kDa range. One of these bands (C130) was found to be important in surface (entry) exclusion (9).

Conjugative functions of pAD1 have been examined by isolation of Tn*917* insertion derivatives expressing variant

TABLE 1. *S. faecalis* strains and plasmids

Strain or plasmids	Relevant marker	Comment or reference
Strains		
FA2-2	*rif fus*	6
JH2-2	*rif fus*	15
OG1X	*str gel*	12
DS16	*tet*	Original isolate of pAD1-carrying strain (24)
39-5		Original isolate of pPD1-carrying strain (20, 29)
Plasmids		
pAD1	Hemolysin-bacteriocin	59.6-kb conjugative plasmid from *S. faecalis* DS16 (11, 24)
pPD1	Bacteriocin	54-kb conjugative plasmid from *S. faecalis* 39-5 (29)
pAM714	Hemolysin-bacteriocin *erm*	pAD1::Tn*917*, wild-type transfer (11, 12)
pTV1-ts	Temperature-sensitive replicon, *cat erm*	Delivery vector for Tn*917* (P. Youngman)

phenotypes (11). Insertions in two loci, designated *traA* and *traB*, gave rise to constitutively aggregating cells (constitutive clumpers) that transferred plasmid DNA at high frequencies in short matings. Derivatives with insertions in *traA* were also characterized by the formation of flocculent larger-than-normal clumps when grown in broth; in addition, colonies on agar media appeared "dry" (11). Two derivatives with insertions in the middle of the *traA* region transferred plasmid DNA at reduced levels and were shown to produce increased levels of the peptide inhibitor iAD1. Insertion mutants of pCF-10 have also been generated, and regions similar to *traA* and *traB* have been identified (1). In addition, a ca. 1.5-kb region was shown to be important for transfer, as transposon insertions reduced or blocked the ability of the plasmid to transfer (1).

In this report we examine further the conjugative transfer functions of pAD1. Transposon mutagenesis of pAD1 with Tn*917* has allowed the identification of a 31.2-kb region of the plasmid that is required for normal transfer. A number of mutants of pAD1 were isolated that were defective in one or more properties associated with pheromone induction. These include the ability to transfer plasmid DNA, formation of mating aggregates, and the production of inducible cell surface proteins.

MATERIALS AND METHODS

Bacteria, media, and reagents. The bacterial strains and plasmids used in this study are listed in Table 1. All strains were grown in antibiotic medium no. 3 (Difco Laboratories, Detroit, Mich.) for DNA isolation; for all other experiments, we used N2GT (nutrient broth no. 2 [Oxoid Ltd., London, England] supplemented with 0.1 M Tris buffer [pH 7.5] and 0.2% glucose). Cultures were incubated at 37°C unless otherwise noted and monitored by using a Klett-Summerson colorimeter (Klett Manufacturing Co., Long Island City, N.Y.) with a no. 54 filter. Antibiotics were used at the following concentrations: tetracycline, 8 μg/ml; chloramphenicol, 10 μg/ml; erythromycin, 25 μg/ml (10 μg/ml in experiments involving pTV1-ts); streptomycin, 1 mg/ml; rifampin, 25 μg/ml; fusidic acid, 25 μg/ml. For hemolysin detection we used Todd-Hewitt plates (Difco) containing 4% horse blood (Colorado Serum Co., Denver, Colo.). The sources of reagents used in this study have been given previously (6, 7, 10, 11, 29).

Generation of pAD1::Tn*917* derivatives. The temperature-sensitive plasmid pTV1-ts (P. Youngman, *in* K. Hardy, ed., *Plasmids, a Practical Approach*, in press) was used as a delivery vehicle in the generation of Tn*917* insertions into pAD1. In addition to Tn*917*, pTV1-ts bears a chloramphenicol resistance determinant. Its temperature sensitivity allows it to replicate at 30°C but not at 42°C. pTV1-ts was established in OG1X cells by protoplast transformation (cells obtained from K. Weaver; 28), and pAD1 was subsequently introduced by conjugation. The strain containing both plasmids was maintained on plates containing chloramphenicol.

Overnight cultures of OG1X(pTV1-ts, pAD1) were grown at 30°C (the permissive temperature) in 10 μg of chloramphenicol per ml and 10 μg of erythromycin per ml (to induce transposition [23]) and then diluted 1:1,000 in drug-free broth. The cells were incubated for 10 h at 42°C (the nonpermissive temperature) and then rediluted and allowed to grow for a further 10 h before being plated on selective media. About 0.5% of the colonies were erythromycin resistant (containing Tn*917*), and approximately 99% of these were sensitive to chloramphenicol (devoid of pTV1-ts).

Chloramphenicol-sensitive, erythromycin-resistant colonies were screened for transfer potential by one of two mating assays. Transfer potential was initially assessed by cross-streak matings (3); donors [OG1X(pAD1::Tn*917*)] and recipients (FA2-2) were cross-streaked on nonselective media (Todd-Hewitt plates), and following incubation overnight, a loopful of the region where streaks crossed was moved to selective media containing horse blood. Subsequently, transfer potential was assessed by mini-broth matings in wells of microtiter plates. A 1-μl sample from an overnight culture of donor cells and a 5-μl sample of recipient cells were added to 50 μl of N2GT broth and incubated at 37°C for 1 h before 10 μl of the mating mixture was spotted on selective media containing horse blood. In both cases, recipient cells were examined for the presence of pAD1 (by hemolysin production), and Tn*917* (by erythromycin resistance).

Mapping of Tn*917* insertions in pAD1. Plasmid DNA was isolated by a modification of the methods of Ish-Horowicz and Burke (14) and Wirth et al. (28). A 5-ml sample of cells from a fresh overnight culture of *S. faecalis* was pelleted and suspended in 200 μl of a 100 mM glucose–50 mM Tris hydrochloride (pH 8)–10 mM EDTA solution containing 3 mg of lysozyme per ml and incubated for 30 min at 37°C. The samples were then transferred to 1.5-ml Eppendorf tubes, 300 μl of a freshly prepared 1% sodium dodecyl sulfate in 0.2 N NaOH was added to each tube and mixed, and the tubes were placed in an ice bath for 5 min. Then 200 μl of a 30% potassium acetate solution (pH 5) was added, and the mixture was maintained on ice for another 5 min before a 5-min centrifugation in a model 5412 Eppendorf centrifuge. The clear supernatant was transferred to a second tube and extracted once with a 1:1 (vol/vol) phenol-chloroform mixture. The aqueous phase was then precipitated with an equal volume of isopropanol (approximately 750 μl) at room temperature. Nucleic acids were pelleted by a 15-min centrifugation at room temperature, dissolved in 100 μl of 125 mM Tris hydrochloride–300 mM sodium acetate (pH 8), and reprecipitated by the addition of 200 μl of 95% ethanol. The pellet produced after a final 8-min centrifugation at room temperature was dissolved in 15 μl of 50 mM Tris hydro-

chloride (pH 8), and the entire sample was used for a single restriction enzyme digestion. Restriction enzyme digestions were done in the three buffer systems of Maniatis et al. (18), and the specifications of the manufacturers (Bethesda Research Laboratories, Inc., Gaithersburg, Md., and New England BioLabs, Inc., Beverly, Mass.) for temperature and enzyme concentration were followed. RNA present in the samples was removed by using RNase at a concentration of 10 μg/ml during the restriction enzyme digestions.

The location of the Tn*917* insertion point was found by digestion of the plasmid by the restriction enzymes *Eco*RI (eight sites in pAD1 and none in Tn*917*), and *Sal*I (three sites in pAD1 and one at the midpoint of Tn*917*, at base pair 2508 of 5,257 base pairs [21, 23]). This method allowed the determination of the Tn*917* insertion point to within approximately 250 base pairs, but did not identify the orientation of the transposon at that site. Insertions of Tn*917* were mapped to individual loci on the map of pAD1 shown in Fig. 1. pAD1::Tn*917* derivatives which were altered in the mating response are also listed (with their map location) in Table 2. The map of pAD1 shown in Fig. 1 is an updated and slightly revised (corrected) version of those previously published (6, 11). Changes include an increase in apparent total size to 59.6 kb, the addition of a second *Bam*HI site in the *Eco*RI A fragment, and the designation of the *Sal*I site as the origin of the plasmid map. The estimated sizes of *Eco*RI fragments A through H are 19.3, 15.4, 12.0, 4.1, 3.4, 2.7, 1.5, and 1.2 kb, respectively. The positioning of the *traA* and *traB* loci was revised by remapping of the Tn*917* insertions which previously defined their boundaries.

Conjugative transfer experiments. The 1-h broth and overnight filter matings were done as previously described (3). Generally 0.05 ml of an overnight donor culture [OG1X (pAD1::Tn*917*)] was mixed with 0.5 ml of an overnight recipient culture (FA2-2) in 4.5 ml of N2GT. Serial dilutions (1:10) of the mating mixtures were made in 5-ml aliquots of broth before 0.1 ml of the diluted mixture was plated onto selective media. Mating assays of pAD1 derivatives were done in groups of 12, with each group including the pAD1::Tn*917* derivative pAM714 (which has wild-type transfer properties) as a standard for wild-type transfer (11). The transfer frequencies (transconjugants per donor cell) listed for both mating assays were normalized to the transfer rate of pAM714 of each individual experiment (transfer frequency of pAM714 was assigned a value of unity). Filter matings were done twice and normalized to the transfer frequency of pAM714 before being averaged.

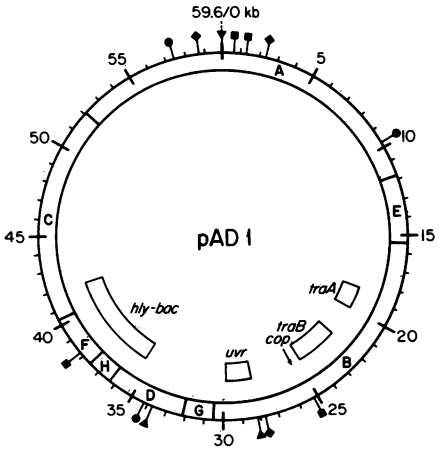

FIG. 1. Physical map of pAD1. Mapping was based on analyses of single and double digestions with the appropriate restriction enzymes. *Eco*RI fragments are labeled in the inner circle; other restriction sites are *Sal*I (▼), *Bam*HI (■), *Kpn*I (◆), and *Pst*I (●). Abbreviations: *hly-bac*, hemolysin-bacteriocin determinant; *cop*, copy number control; *uvr*, resistance to UV light. The boxes indicate the regions within which transposon insertions disrupt function.

TABLE 2. Map positions of pAD1::Tn*917* derivatives exhibiting altered response to pheromone

Sector	Derivative (map position [kb])
1	pAM930 (0.5), pAM931 (0.75)
2	pAM932 (3.1), pAM933 (3.3), pAM934 (3.3), pAM935 (3.3), pAM936 (3.3), pAM937 (3.8), pAM938 (4.4)
3	pAM939 (4.4), pAM940 (4.8), pAM941 (5.5)
4	pAM942 (8.4), pAM943 (8.9)
5	pAM944 (9.4), pAM945 (9.8), pAM946 (10.7), pAM947 (10.9)
6	pAM948 (12.8)
7	pAM949 (14.1)
8	pAM950 (17.9), pAM951 (17.9), pAM952 (17.9), pAM953 (17.9), pAM954 (17.9), pAM955 (18.4), pAM956 (18.4), pAM957 (18.9), pAM958 (18.9)
9	pAM959 (19.4), pAM960 (19.6), pAM961 (19.6)
10	pAM962 (19.6), pAM963 (19.9), pAM964 (19.9), pAM965 (19.9), pAM966 (19.9), pAM967 (19.9), pAM968 (20.1), pAM969 (20.2), pAM970 (20.2), pAM971 (20.3), pAM972 (20.4), pAM973 (20.4), pAM974 (20.4), pAM975 (20.4), pAM976 (20.6), pAM977 (20.8), pAM978 (20.9), pAM979 (20.9), pAM980 (20.9), pAM981 (20.9), pAM982 (21.1), pAM983 (21.2), pAM984 (21.4), pAM985 (21.5), pAM986 (21.9)
12	pAM987 (53.1), pAM988 (53.3), pAM989 (53.6), pAM990 (54.6), pAM991 (55.4), pAM993 (56.3), pAM994 (56.5), pAM995 (56.5), pAM996 (56.6), pAM997 (56.6), pAM998 (58.1)
13	pAM922 (56.1)

Assays for production of cAD1 and iAD1, and response to exogenous cAD1. The concentration of pheromone (cAD1) and inhibitor (iAD1) present in culture filtrates was assayed by dilution in the wells of a microtiter plate as described previously (8, 12). DS16 (6, 24), the strain in which pAD1 was originally identified, was used as a responder for both the cAD1 and the iAD1 assays. The pheromone titer was defined as the reciprocal of the highest dilution of pheromone which induced aggregate formation of responder cells. iAD1 units quantified the reduction in the dilutions necessary to titrate a given cAD1 when a known amount of the latter was diluted through a filtrate containing inhibitor. By this definition, an inhibitor-caused reduction in pheromone titer from 32 to 8 would indicate the presence of 4 U of inhibitor in the sample tested. For the analysis of pheromone-induced aggregation of the mutant plasmid-containing strains, the response of each pAD1 derivative to cAD1 was measured and the titer was calculated.

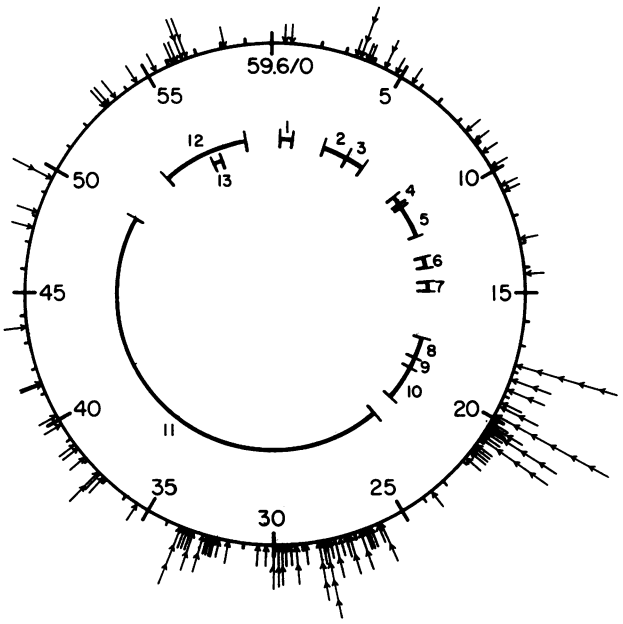

FIG. 2. Physical map of Tn*917* insertions into pAD1. Each arrow represents a single Tn*917* insertion into a copy of pAD1. The numbered segments of the inner circle represent the phenotypically defined sectors in which the Tn*917* insertions are located. Sectors 6, 7, and 13 are sized arbitrarily and correspond to a single insertion in each case.

Analysis of surface proteins. Zwittergent 3-12 (Calbiochem-Behring, La Jolla, Calif.) extracts of surface proteins from uninduced and induced cells were prepared as previously described (10). Samples were separated on sodium dodecyl sulfate-polyacrylamide (10%) gels (17) and then electrophoretically transferred to nitrocellulose filters and visualized by a modification of the Western blotting (immunoblotting) method of Towbin et al. (26), as previously reported (10).

RESULTS

Generation of pAD1::Tn*917* derivatives. Analysis of conjugative functions of pAD1 was initiated by the generation of transfer-deficient plasmids by using the transposon Tn*917*. Insertions into pAD1 were derived in *S. faecalis* OG1X by using the temperature-sensitive delivery vector pTV1-ts, as described in Materials and Methods. After induction of transposition and elimination of pTV1-ts, chloramphenicol-sensitive, erythromycin-resistant colonies were exmained to ascertain whether Tn*917* was present on pAD1 or on the cell chromosome. This was accomplished by using the cells as donors in individual mating experiments to determine whether the phenotypic traits associated with pAD1 and Tn*917* (hemolysin production and erythromycin resistance, respectively) were transferred together, independently, or not at all. Cross-streak matings were performed on agar plates between 378 independently isolated chloramphenicol-sensitive, erythromycin-resistant colonies and the plasmid-free strain FA2-2. Recipient cells were selected from mating mixtures and screened for hemolysin production and erythromycin resistance. Of the 378 matings, 69% yielded transconjugants that were hemolysin positive and erythromycin resistant, indicating the transfer of Tn*917* on pAD1. Approximately 2% gave transconjugants that were erythromycin resistant but did not produce hemolysin, implying the presence of Tn*917* in the hemolysin gene of pAD1. About 4% of the matings produced transconjugants that were hemolysin positive but erythromycin sensitive, indicating that Tn*917* was on the OG1X chromosome; and 25% of the matings yielded no hemolysin-positive or erythromycin-resistant transconjugants, indicating that pAD1 did not transfer from the donor cell and that the transposon may be inserted in regions of pAD1 required for conjugation. Thus approximately 96% of the Tn*917* transposon events resulted in the insertion of Tn*917* into pAD1, and up to 25% of the insertions occurred into genes required for transfer. A strong preference for insertion into pAD1 relative to insertion into the chromosome was clearly evident.

Subsequently a more sensitive method was developed for the detection of transfer-deficient mutants of pAD1 among the insertion derivatives. This involved broth matings in the wells of a microtiter plate. Ten independent mutagenesis experiments resulted in 140 potential transfer-deficient pAD1::Tn*917* derivatives on the basis of these two screening methods. The positions of the Tn*917* insertions were mapped (Fig. 2; Table 2) by restriction enzyme analyses of purified plasmid DNA.

Transfer frequency of pAD1::Tn*917* mutants. After initial screening by the qualitative assays described above, we quantitated the transfer potential of the 140 pAD1::Tn*917* derivatives as follows. We performed 1-h broth matings of donor [OG1X(pAD1::Tn*917*)] and recipient (FA2-2) cells and determined the transfer frequency in each case. On the basis of their location and frequency of plasmid transfer, the Tn*917* insertions were then grouped into sectors, with each sector having gaps no greater than 2 kb between insertions. Three pAD1 insertions did not fit into any sector defined by these criteria and were treated as individual sectors (insertions at 12.8, 14.1, and 56.1 kb). Table 3 lists the transfer frequencies for all 140 strains analyzed. pAM714 (11), a pAD1::Tn*917* derivative with a Tn*917* insertion mapping at 26.9 kb, was shown to be unaffected in transfer functions and was used to determine the wild-type frequency of transfer in each mating experiment. The transfer frequencies listed have been normalized to the value of pAM714 in each individual experiment (the transfer frequency of pAM714 was generally about 5×10^{-3} per donor). The majority of the pAD1 derivatives fell into two sectors (sectors 10 and 11) showing little or no difference in transfer frequency from the wild type. The single strain containing an insertion in sector 7 of pAD1 also transferred at wild-type levels. pAD1 derivatives with insertions in sectors 3, 4, and 5 and the single insertion in sector 13 showed levels of transfer reduced to 4 to 5% of the wild type. Insertions of Tn*917* in sectors 1, 2, 8, 9, and 12 of pAD1, as well as the individual insertion at 12.8 kb, all had transfer levels reduced below 0.4% of the frequency of the wild-type plasmid. (These strains showed no detectable transfer after 1 h, and the numbers listed represent the lower limit of detectable transfer by the assay.) Therefore the region running clockwise from 53.1 to 19.6 kb appears to encode functions necessary for normal transfer frequencies.

Table 3 also lists the normalized transfer frequencies obtained in overnight filter matings. These were determined for plasmids which had transfer frequencies undetectable in broth matings. Filter matings bring cells into close contact and are less dependent on the expression of inducible adherence properties (2). The transfer frequencies observed in filter matings for sectors 1, 2, 8, and 12 were still greatly

TABLE 3. Transfer frequencies of pAD1::Tn*917* derivatives

Sector	Map locus (kb)	1-h broth mating		Overnight filter mating	
		No. measured	Normalized value[a] (mean ± SD)	No. measured	Normalized value[b] (mean ± SD)
1	0.5–0.8	2	<0.0008[c]	2	<0.00001
2	3.1–4.4	7	<0.0005[c]	7	0.00761 ± 0.020
3	4.4–5.5	3	0.0512 ± 0.060	0	
4	8.4–8.9	2	0.0475 ± 0.029	0	
5	9.4–10.9	4	0.0395 ± 0.016	0	
6	12.8	1	<0.0002[c]	1	1.15
7	14.1	1	0.62	0	
8	17.9–18.9	9	<0.0004[c]	9	0.00063 ± 0.002
9	19.4–19.6	3	<0.0004[c]	3	0.27 ± 0.272
10	19.6–21.9	25	0.60 ± 0.89	0	
11	23.6–49.8	71	1.24 ± 0.82	0	
12	53.1–58.1	11	<0.0037[c]	11	0.00068 ± 0.001
13	56.1	1	0.0140	0	

[a] Normalized to transfer frequency of pAM714 (approximately 0.005 transconjugants per donor).
[b] Normalized to transfer frequency of pAM714 (approximately 1.5 transconjugants per donor).
[c] Below detectable level of assay.

reduced below those of the wild type. (All insertions had transfer frequencies below 0.8% of that seen for pAM714.) However, in contrast to the situation with broth matings, the single insertion in sector 6 and the three insertions in sector 9 exhibited transfer frequencies close to wild-type levels in filter matings.

Analysis of pheromone-induced aggregation. The amount of pheromone necessary to promote aggregate formation was assayed to determine the sensitivity of cells containing mutant plasmids. The lowest concentrations of exogenous pheromone necessary for the induction of clumping in the mutant strains are shown in Table 4 (response to cAD1). The "titers" (reflecting relative sensitivities) were determined by serial dilution of pheromone in the wells of a microtiter plate, to which cells containing the pAD1::Tn*917* derivatives were added. All of the Tn*917* insertions in sectors 5 and 8 of pAD1 prevented visible aggregate formation by the host cells, as did two of the three insertions in sector 9 (pAM959 and pAM960 mapping at 19.4 and 19.6 kb, respectively). Cells containing these plasmid derivatives failed to respond to even the lowest (twofold) dilution of culture filtrate from the plasmid-free strain JH2-2. In comparison, cells harboring the pAM714 control were induced to aggregate by filtrate diluted 32-fold. Cells containing the third insertion in sector 9 (pAM961, which mapped at 19.6 kb) were able to aggregate in pheromone diluted 16-fold (titer of 16). Insertions in sector 10 decreased the sensitivity of the cells to the exogenous pheromone, so that the cells responded at titers of 2 to 8. pAD1 derivatives with insertions outside of these four sectors were sensitive to eightfold or greater dilutions.

Production of cAD1 and iAD1 by pAD1::Tn*917*-containing cells. The level of production of the pAD1-specific pheromone (cAD1) and the pAD1-encoded inhibitor (iAD1) was analyzed for a number of strains carrying mutant plasmids. Culture filtrate from each of the specified pAD1 derivatives was prepared and used to promote (cAD1) or inhibit (iAD1) aggregation of an indicator strain containing pAD1 (DS16; see Materials and Methods for details). None of the strains examined produced detectable levels of cAD1 (titers of <2) and thus resembled the wild type in this regard. Levels of inhibitor produced by a number of strains are listed in Table 4. Insertions of Tn*917* in pAD1, except for those in sector 10, exhibited mean inhibitor levels of approximately 2 U, ranging from <2 to 4 U. Insertions in sector 10 exhibited a mean inhibitor level of 6.5 U. It is noteworthy that this is the same sector of pAD1 in which insertions of Tn*917* caused de-

TABLE 4. Response to cAD1, and production of iAD1 by pAD1::Tn*917* derivatives including pAM714

Sector	Map locus (kb)	Response to cAD1			Production of iAD1		
		No. measured	Titer[a]		No. measured	Units of activity[a]	
			Mean	Range		Mean	Range
1	0.5–0.8	2	32.0	32	1	2.0	
2	3.1–4.4	7	22.9	16–32	5	<2.4	<2–4
3	4.4–5.5	3	18.7	8–32	3	2.0	2
4	8.4–8.9	2	32.0	32	1	2.0	
5	9.4–10.9	4	<2.0	<2	4	2.5	2–4
6	12.8	1	32.0		1	2.0	
7	14.1	1	32.0		0		
8	17.9–19.4	7	<2.0	<2	3	2.0	2
9	19.4–19.6	3	<6.7	<2–16	2	3.0	2–4
10	19.9–21.9	25	3.3	2–8	13	6.5	4–8
11	22.5–49.8	27	48.9	8–128	2	<2.0	<2–2
12	53.1–58.1	11	39.3	16–128	6	2.3	2–4
13	56.1	1	32.0	32	0		
pAM714	26.9		32.0			2.0	

[a] Values of <2 are calculated into the mean as a value of 2.

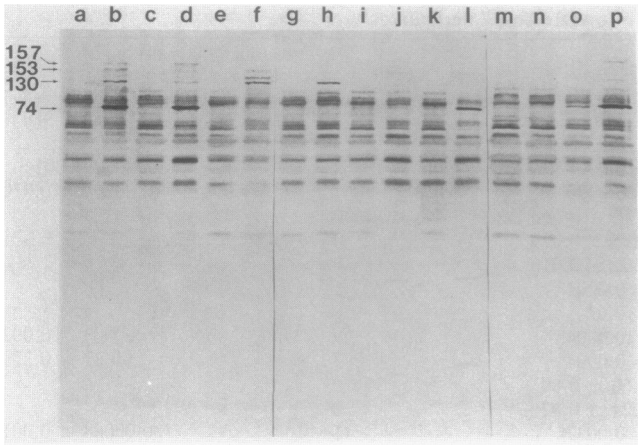

FIG. 3. Western blot of cell surface proteins extracted from uninduced and induced cells carrying derivatives of pAD1. Primary antisera [raised against induced FA2-2(pAM714)] were diluted 1:100, and the secondary antisera (goat anti-rabbit) were diluted 1:2,000. Lanes: a (uninduced) and b (induced), pAM714-carrying cells; c (uninduced) and d (induced), a sector 12 mutant (pAM990); e (uninduced) and f (induced), a sector 5 mutant mapped to 9.8 kb (pAM945); g (uninduced) and h (induced), a sector 5 mutant mapped to 10.9 kb (pAM947); i (uninduced) and j (induced), sector 6 mutant (pAM948); k (uninduced) and l (induced), sector 7 mutant (pAM949); m (uninduced) and n (induced), a sector 9 mutant mapped to 19.6 kb (pAM960); and o (uninduced) and p (induced), a sector 9 mutant mapped to 19.6 kb (pAM961). Sizes of induced bands (in kilodaltons) are listed on the left.

creased sensitivity to exogenous pheromone; the two phenomena are presumably related.

Comparison of surface proteins. Previous work (10) identified four novel surface proteins produced by pAD1-containing cells following pheromone induction. These proteins were identified by Western blot analysis; specific functions could not be assigned to any of them. The use of antibodies in identification of proteins has been indispensable, because most of the pheromone-associated changes on the cell surface are not detectable on Coomassie brilliant blue-stained gels. However, it should be noted that Western blot analysis does not give quantitative measurements of the proteins, because band intensity is dependent not only on protein concentration, but also on antibody concentration and affinity.

Proteins were extracted from induced and uninduced cells carrying derivatives of pAD1 by agitation in 0.2% Zwittergent 3-12. The proteins were separated by polyacrylamide gel electrophoresis and transferred to nitrocellulose filters for immunoblotting. Figure 3 shows extracts of representative strains containing insertions in the various sectors of pAD1, incubated with antisera against induced pAD1-containing cells. The extracts were paired by strain: the first lane of each pair represents uninduced cells, and the second lane corresponds to cells induced with cAD1. Lane b shows an extract from pAM714, which produces the four inducible protein bands AD74, AD130, AD153, and AD157 (10) (named according to their molecular weights of 74,000, 130,000, 153,000, and 157,000, respectively). Lane d shows an extract from a sector 12 strain with an insertion at 54.6 kb on the pAD1 map (pAM990). This strain was able to form mating aggregates, but showed greatly reduced transfer frequencies in both 1-h broth and overnight filter matings. The four induced proteins visible in lane d appear identical to the wild-type proteins seen in lane b. This pattern of inducible proteins was observed with all 8 strains carrying sector 12 insertions, as well as strains with insertions in sectors 1 (1 strain examined), 2 (3 examined), 3 (1 examined), 4 (3 examined), 10 (24 examined), 11 (5 examined), and 13 (1 examined). Lanes e and f show extracts from a sector 5 insertion mapping at 9.8 kb (pAM945). Induced proteins are visible at 130 kDa (AD130) and 153 kDa (AD153), and a novel protein is visible at 140 kDa (lane f). Two other strains containing pAD1 with insertions in sectors 5 (pAM944 and pAM946) exhibited the same three proteins. However, the strain with the fourth sector 5 insertion (pAM947), mapping at 10.9 kb, showed a different protein profile, with induced bands at 130 and 110 kDa (lane h). Lanes i and j show protein extracts from the strain containing the single insertion of sector 6. This mutant (pAM948), which formed aggregates in response to exogenous pheromone, exhibited only AD153 (lane j). The strain carrying a sector 7 insertion (pAM949) produced AD74 and AD153 after pheromone induction (lanes k and l). Lanes m and n show extracts from a strain containing a sector 9 insertion located at 19.6 kb (pAM960). No induction of any surface proteins was seen after exposure to pheromone (lane n). The same result was also observed with one of the other insertions in sector 9 (pAM959, mapping at 19.4 kb), as well as the sector 8 insertions (five strains examined). However, cells containing the third insertion in sector 9 (pAM961), which mapped at 19.6 kb) exhibited low-level production of AD74 when uninduced, and postinduction produced the protein profile seen for wild-type induced cells: AD74, AD130, AD153, and AD157 (lane p).

The protein extracts were also analyzed by Western blotting techniques with antisera raised against cells containing a different conjugative plasmid (pPD1) that highly cross-reacted with AD130, AD153, and AD157, but not AD74 (10). The extracts (Fig. 4) are paired, with the first and second

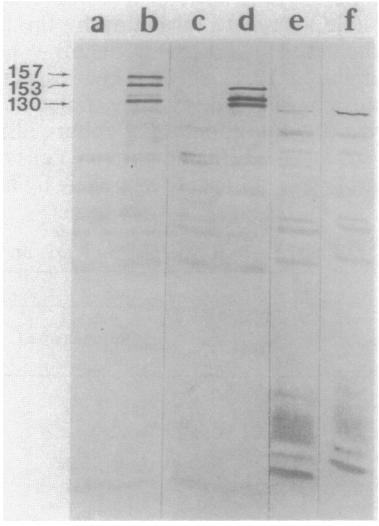

FIG. 4. Western blot of cell surface proteins extracted from uninduced and induced cells carrying derivatives of pAD1. Primary antisera (raised against induced *S. faecalis* 39-5) were diluted 1:500, and secondary antisera (goat anti-rabbit) were diluted 1:2,000. Lanes: a (uninduced) and b (induced), pAM714-carrying cells; c (uninduced) and d (induced), a sector 5 mutant mapped to 9.8 kb (pAM945); e (uninduced) and f (induced), a sector 5 mutant mapped to 10.9 kb (pAM947). Sizes of induced bands (in kilodaltons) are listed on the left.

lanes representing uninduced and induced cells, respectively. Lanes a and b show extracts of cells containing pAM714, while AD130, AD153, and AD157 are visible in the induced lane (lane b). Lane d shows the sector 5 insertion at 9.8 kb (pAM945), and the three proteins seen in the Western blots with antisera raised against cells containing pAD1 (Fig. 3, lane f; 130-, 140-, and 153-kDa bands) are visible. This suggests that the 140-kDa protein is related to AD157 rather than AD74 (both of which are absent in this mutant). Lanes e and f show protein extracts from the strain containing the insertion in sector 5 that mapped at 10.9 kb (pAM947). In this case AD130 is seen, but the 110-kDa band did not cross-react with antisera raised against pPD1-containing cells (lane f). Protein extracts from the other strains shown in Fig. 3 were also screened by using antisera against pPD1-containing cells, and only AD157, AD153, and AD130 (when present in Fig. 3) were observed (data not shown).

DISCUSSION

A 31.2-kb region of the conjugative hemolysin plasmid pAD1, stretching clockwise from 53.1 to 24.7 kb on the plasmid map, encodes functions necessary for conjugal transfer. We isolated 69 pAD1 derivatives containing Tn*917* insertions in this region, and all exhibited changes in at least one transfer-related property. The results are summarized in Fig. 5 and Table 5. In addition to the two previously identified regions (*traA* and *traB*), six new regions important for conjugation are shown and are labeled C through H. These regions are characterized by different phenotypic changes caused by the insertion of the transposon. Usage of *tra* terminology is being withheld at present, since there are probably multiple determinants within at least some of these regions.

Region H, 53.1 to 4.4 kb on the pAD1 map, is composed of sectors 1, 2, 12, and 13, all of which are required for normal plasmid transfer but not for aggregate formation. A total of 21 insertions were isolated in this region, all of which exhibited greatly reduced transfer frequencies. In all cases except one, the frequency of transfer was below detectable limits during 1-h broth matings and was greatly reduced in overnight filter matings. One insertion derivative internal to this region, designated sector 13 (shown by the arrow at map position 56.1 on Fig. 5), exhibited transfer in broth, but at only 1.4% of the wild-type level. Even though insertions in region H rendered it essentially transfer negative, cells exhibited apparently normal aggregation and synthesized the four surface proteins AD74, AD130, AD153, and AD157.

Region G is juxtaposed to region H, at 4.4 to 8.9 kb on the map, as both regions contain individual insertions at 4.4 kb. The insertions in region G (defined as sectors 3 and 4) exhibited reduced transfer (mean frequency reduced to 5% of wild-type levels). As in the case of region H, insertions in this region did not affect aggregate formation, induction of surface proteins, or the level of production of iAD1. The proteins encoded in both regions G and H are likely to include those involved in DNA transfer.

Region F, mapping at 9.4 to 10.9 kb, is composed of sector 5. Insertions here caused a decrease in transfer frequency to an average of 4% of the wild-type. Unlike those in region G, these derivatives do not exhibit a visible clumping response. Moreover, the insertions resulted in an altered protein profile upon exposure to cAD1. Cells containing insertions at 9.4, 9.9, and 10.7 kb all produced three proteins visible in Western blot analyses; their molecular weights were 130,000, 140,000, and 153,000 (AD74 and AD157 were absent). The 140-kDa band may be related to AD157, because antisera reactive against AD157 but not against AD74 reacted with the 140-kDa band in a Western blot analysis. It is unlikely that the 140-kDa band is simply a truncated version of AD157 that was generated by insertion of Tn*917*, because insertions in three different loci (9.4, 9.8, and 10.7 kb) all gave rise to the 140-kDa band. It is more likely that this region codes for a processing, modifying, or transport enzyme(s) necessary for generation of the normal surface proteins. The fourth insertion in region F, mapping at 10.9 kb, produced only two bands: a 110-kDa protein plus AD130. The new band, in this case, does not share antigenic determinants with AD130, AD153, or AD157, and it is tempting to speculate that it is an unprocessed form of AD74.

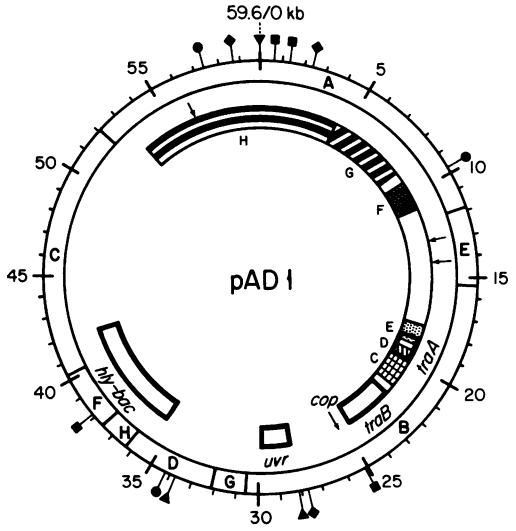

FIG. 5. Physical map of pAD1 showing the eight regions which have been identified as necessary for normal transfer (*traA*, *traB*, and regions C through H). Single insertions with unique phenotypes are represented by arrows (at 12.8, 14.1, and 56.1 kb). *Eco*RI fragments are labeled in the inner circle; other restriction sites are *Sal*I (▼), *Bam*HI (■), *Kpn*I (◆), and *Pst*I (●). The phenotypes of cells carrying plasmids mutant in these regions are listed in Table 5.

The derivatives with insertions at 12.8 and 14.1 kb (indicated by the arrows at those locations in Fig. 5) could both be induced to aggregate, but represented different phenotypes with respect to transfer. The 12.8-kb insertion resulted in undetectable levels of transfer in broth, but transfer occurred at wild-type levels in overnight filter matings. In contrast, the derivative containing the insertion at 14.1 kb transferred at wild-type levels in broth. Interestingly, the insertion at 12.8 kb produced only AD153, whereas the insertion at 14.1 kb produced AD74 and AD153 after induction. Although it would appear that cells producing only AD153 are able to form aggregates in broth, the role (if any) of this protein in aggregation is more complex; region F insertions, which produced AD153 (or a similar-sized protein) along with AD130 and a band at 140 kDa, did not give rise to visible aggregates.

Regions E (17.9 to 18.9 kb) and D (19.4 to 19.6 kb) appear to overlap the previously defined *traA* region. Region D is entirely a subset of *traA*, while region E also encompasses DNA adjacent to the area defined as *traA*. (Previously reported [11] individual insertions in *traA* were mapped [E. Ehrenfeld, unpublished data] to 18.6 [pAM721], 19.2 [pAM727], 19.7 [pAM728], and 19.9 [pAM737] kb.) It is

TABLE 5. Summary of regions of pAD1 required for normal transfer

Region	Map locus (kb)	Sectors[a]	Phenotype of Tn*917* insertions[b]
traA	18.6–19.9		Constitutive aggregation, increased tf, dry colonies
traB	22.0–24.7		Constitutive aggregation, increased tf
C	19.9–21.9	10	Decreased sensitivity to cAD1, increased production of iAD1, isp$^+$, normal tf
D	19.4–19.6	9	2/3 insertions isp$^-$ aggregation$^-$, 1/3 insertions isp$^+$ aggregation$^+$, tf below detection in broth and wild type on filters
E	17.9–19.4	8	Aggregation$^-$ isp$^-$, tf below detection in broth and reduced below 0.7% of wild type on filters
Single[c]	12.8	6	Aggregation$^+$ isp-altered, tf below detection in broth and wild type on filters
Single	14.1	7	Aggregation$^+$ isp-altered, tf wild type in broth and on filters
F	9.4–10.9	5	Aggregation$^-$ isp-altered, tf reduced in broth
G	4.4–8.9	3, 4	Aggregation$^+$ isp$^+$, tf reduced in broth
H	53.1–4.4	1, 2, 12	Aggregation$^+$ isp$^+$, tf below detection in broth and reduced below 0.7% of wild type on filters
Single	56.1	3	Aggregation$^+$ isp$^+$, tf reduced in broth

[a] See Fig. 2.
[b] tf, Transfer frequency; isp, induced surface proteins.
[c] "Region" corresponds to a single insert.

important that the *traA* overlap of region D and most of region E is within the margin of error of the gel analyses, and more detailed analysis is required to determine the exact locations of insertions in this region (work in progress). Regions E and D are composed of sectors 8 and 9 (respectively), and insertions in both regions cause a reduction in plasmid transfer to below 0.04% of wild-type levels during a 1-h broth mating. In region E a decrease in transfer potential (decreased to about 0.06% of wild-type levels) also occurred in overnight filter matings; however, insertions in region D allow transfer in overnight filter matings to occur at levels approaching those of the wild type (transfer occurred at 27% of the wild-type level).

In addition to decreasing mating potential, insertions in region E interfered with the ability of cells to form mating aggregates and blocked their ability to produce the four inducible surface proteins. Two of the insertions in region D (at 19.4 and 19.6 kb) also failed to form aggregates or produce surface proteins. The third insertion, which maps at 19.6 kb (the end of region D distal to region E), differed in that it was able to form aggregates and, following pheromone stimulation, also produced AD74, AD130, AD153, and AD157.

The salient feature of the insertions in regions D and E is that they disrupt multiple functions associated with pheromone induction (i.e., transfer, aggregate formation, and induction of surface proteins). Preliminary data (Ehrenfeld, unpublished data) also indicate that the mutations in this region shut off the expression of cell surface exclusion. Insensitivitiy to induction suggests a defect in positive control functions. One such function could correspond to a pheromone receptor protein on the bacterial surface. The overlap, or close proximity, of regions D and E with the *traA* determinant, whose product appears to act negatively (11), points out the overall importance of this 2-kb region in the regulation of the pheromone response.

Region C lies between the previously identified *traA* and *traB* regions, mapping from 19.6 to 21.9 kb on pAD1 (Fig. 5). Insertions in this region maintain wild-type mating frequencies and produce the four normal surface proteins. However, they exhibit an apparent increase in the production of iAD1 by the cell; this is accompanied by an expected decrease in sensitivity to exogenous pheromone. A decrease in endogenous cAD1 levels or an increase in actual iAD1 production would have the same effect, and the data presented exclude neither interpretation. It is interesting that two of the previously reported *tra* mutants (pAM727 and pAM728, which mapped at 19.2 and 19.7 kb, respectively) also produced increased levels of iAD1 (19).

Analysis of the conjugative functions of pAD1 by Tn*917* mutagenesis has elucidated the basic organization of the transfer genes of pAD1. Because all of the transfer functions mapped to a contiguous stretch of DNA, it is possible that the pheromone response genes are arranged in a large transfer operon, as is seen for the *Escherichia coli* plasmid F (27). However, the observation that the loss of transfer functions as a result of transposon insertion was not cumulative throughout the 31.2-kb contiguous region suggests that more than one promoter is present. There is a similarity with the *S. faecalis* plasmid pCF-10 in that Tn*917* insertions in a contiguous region of pCF-10 cause either constitutive expression of the pheromone response or an almost complete shutdown of the inducible functions (1). This resembles the pAD1 *traA* and regions D and E. For pCF-10, however, insertions which reduced or blocked plasmid transfer in broth matings were limited to a ca. 1.5-kb contiguous region; this is in contrast to pAD1, for which insertions over a ca. 27-kb region (covering regions E, F, G, and H) affected transfer. In addition, pCF-10 contains ca. 10 kb of noncontiguous DNA, into which insertions give rise to constitutive clumping; in pAD1 such regions (*traA* and *traB*) total only about 3 kb of DNA. It would appear, therefore, that pAD1 and pCF-10 differ significantly in their overall organization.

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