JOURNAL OF BACTERIOLOGY, June 1984, p. 777–783
0021-9193/84/060777-07$02.00/0
Copyright © 1984, American Society for Microbiology
Vol. 158, No. 3

Genetic Analysis of the pAD1 Pheromone Response in *Streptococcus faecalis*, Using Transposon Tn*917* as an Insertional Mutagen

YASUYOSHI IKE† AND DON B. CLEWELL*

Dental Research Institute, Department of Oral Biology, School of Dentistry, and Department of Microbiology and Immunology, School of Medicine, The University of Michigan, Ann Arbor, Michigan 48109

Received 30 September 1983/Accepted 27 February 1984

The conjugative plasmid pAD1 (56.7 kilobases) in *Streptococcus faecalis* has been shown to confer a mating response to the sex pheromone cAD1 excreted by recipient strains. The response is characterized by the synthesis of a proteinaceous adhesin which coats the surface of the pAD1-containing donor cell and facilitates the formation of mating aggregates. Donors exposed to cAD1-containing filtrates of recipients undergo self-aggregation (clumping), an event believed to be associated with an interaction between the adhesin and a binding substance always present on the surface of both recipients and donors. To analyze the molecular processes involved in the mating response, mutants were generated by the erythromycin resistance transposon Tn*917*. Transpositions to pAD1 in *S. faecalis* DS16 gave rise to a number of derivatives that exhibited "constitutive clumping" and the ability to transfer at high frequencies in short (10-min) matings. These mutants fell into two subclasses, which exhibited colony morphologies that were "dry" or "normal". The Tn*917* insertions were mapped by restriction enzyme analysis to two separate clusters, designated *traA* and *traB*. The dry colony subclass corresponded to *traA* and represented a span of 1.5 kilobases, whereas the normal subclass corresponded to *traB* and spanned 1.3 kilobases. The two clusters were separated by 1.7 kilobases in which insertions of Tn*917* did not affect the ability to respond normally to cAD1. Neither type of constitutive clumper produced cAD1. Another series of insertions exhibited reduced donor potential. In two cases, the reduction in transfer was three to four orders of magnitude; these mapped in *traA*. In two other cases, the reduction was one to two orders of magnitude. These mapped outside of *traA* and *traB*, and one was associated with an increase in plasmid copy number.

Two types of conjugative plasmids have been observed in streptococci. One type, with a molecular size generally in the range of 22 to 30 kilobases (kb), does not give rise to mating aggregates in liquid broth; mating on solid surfaces (e.g., filter membranes) is required for transfer. These plasmids are usually able to transfer between a variety of streptococcal species and even between different genera. In contrast, the other type of plasmid discussed in this communication (i) usually has a size of greater than 45 kb, (ii) transfers efficiently in broth, (iii) is characterized by its response to small heat-stable substances (sex pheromones) excreted by potential recipients, and (iv) has thus far been reported to occur only in *Streptococcus faecalis* (3, 7, 8, 10, 11). (For a review of plasmid transfer in streptococci, see reference 2.) The response to the sex pheromone is characterized by the synthesis of a proteinaceous adhesin (16, 22) on the donor cell surface; this substance is referred to as aggregation substance (AS) and exhibits antigenic properties only associated with induced cells. Donor cells induced by exposure to filtrates of recipients undergo self-clumping. AS is presumed to adhere to a substance referred to as binding substance, which is located on the surface of both donor and recipient cells. Mating aggregates arise upon random collisions between the nonmotile donors and recipients; once a copy of plasmid DNA has been acquired by the recipient, the production of the related pheromone ceases. Different pheromones, specific for donors harboring different classes of plasmids, continue to be excreted.

Figure 1 illustrates a previously proposed (11) hypothetical model suggesting how pheromones, aggregation, and mating may interrelate. Applied here toward the 56.7-kb hemolysin-bacteriocin plasmid pAD1 (5, 21), a determinant designated *IcAD1* has been proposed to be involved in the shutoff of chromosome-determined pheromone cAD1. Recent evidence (14) indicates that this is accomplished through a chemical addition of ca. 350 daltons to the 1,050-dalton pheromone and involves the formation of a phosphodiester bond. A determinant involved in controlling expression of AS has also been proposed; designated *RcAD1*, it is most simply conceived as encoding a substance which represses the synthesis of AS.

The derivation and characterization of plasmid mutants altered in the mating response would be very useful in revealing the mechanisms by which aggregation and plasmid transfer are controlled. For example, if AS were indeed negatively controlled, it should be possible to obtain mutants defective in *RcAD1* which would give rise to constitutive expression of AS. Such variants would self-aggregate continuously. Mutations in *IcAD1* might also give rise to constitutive expression due to a response to endogenous pheromone.

Using the erythromycin resistance transposon Tn*917* (5, 19, 20), we generated a number of insertions into pAD1, many of which gave rise to abnormal mating behavior. We found that mutations resulting in constitutive clumping can indeed be obtained and map in two regions on the plasmid. In neither case, however, did these variants appear to

* Corresponding author.
† Present address: Department of Microbiology, Gunma University School of Medicine, Maebashi City, Japan.

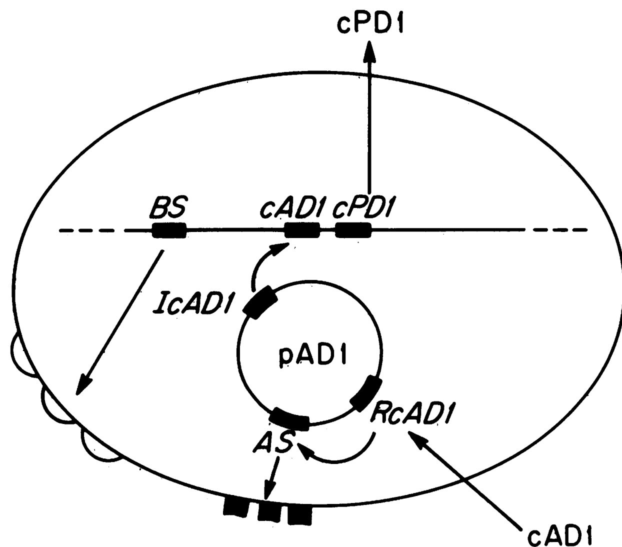

FIG. 1. Model illustrating certain aspects of the pheromone response of pAD1 and the shutoff of endogenous cAD1. *IcAD1* is a determinant for the modification (inactivation) of endogenous cAD1. The cell continues to excrete different pheromones such as cPD1. *RcAD1* determines a regulatory protein involved in controlling the synthesis of AS via a direct, or indirect, interaction with exogenous cAD1. The dark surface structures represent AS and are believed to represent an adhesin that uniformly coats the cell as a result of induction by cAD1. The clear (half-circle) surface structures represent binding substance (BS); it is encoded by the chromosome and is always present on the surface of both recipients and donors. Binding substance interacts with AS in the generation of mating aggregates.

produce endogenous cAD1. These and related results are presented here.

MATERIALS AND METHODS

Bacteria, media, and reagents. The *S. faecalis* strains used in this study are listed in Table 1. Unless otherwise indicated, the medium used throughout this study was N2GT (Oxoid Nutrient Broth no. 2 supplemented with glucose [0.2%] and Tris-hydrochloride [0.1 M], pH 7.7.), and turbidity was monitored with a Klett-Summerson colorimeter with a no. 54 filter. Selective agar plates contained Todd-Hewitt broth (Difco Laboratories, Detroit, Mich.), and the drug concentrations used were as follows: erythromycin, 25 μg/ml; rifampin, 25 μg/ml; fusidic acid, 25 μg/ml; streptomycin, 500 μg/ml; kanamycin, 500 μg/ml; spectinomycin, 500 μg/ml; and tetracycline, 5 μg/ml. Hemolysin detection was on Todd-Hewitt plates containing 4% horse blood (Colorado Serum Co., Denver, Colo.). The sources of the reagents used in the study were as previously described (5, 11–13, 20, 22).

[With regard to bacterial strains, we wish to note that derivatives of strain JH2-2 harboring pAD1 generally do not exhibit a typical clumping response when exposed to pheromone-containing filtrates, despite the fact that isogenic strains such as JH2SS(pAD1) or FA2-2(pAD1) exhibit normal responses. In addition, the transfer frequency in broth from strain JH2-2(pAD1) is very low (less than 10^{-6} per donor); however, transfer occurs at ca. 10^{-5} per donor in filter matings. Indeed, it was for this reason that strain FA2-2 (5), with essentially identical markers as strain JH2-2, was originally constructed. When strain JH2-2 harbored a derivative of pAD1 previously shown to give rise to constitutive clumping, aggregation did occur; thus the host defect is not in the ability to express AS but rather may relate to the ability to recognize and take up exogenous cAD1.]

Isolation of pAD1 derivatives with Tn*917* insertions. Insertions were derived as a result of transposition of Tn*917* (5 kb) from pAD2 to pAD1 in strain DS16. pAD2 is a 25.7-kb nonconjugative plasmid which, in addition to the transposon, bears determinants for resistance to streptomycin and kanamycin (5, 21). Logarithmic cultures of strain DS16 were exposed to erythromycin (0.5 μg/ml) for 4 h to enhance the frequency of transposition (19, 20). The cells were then washed and mated in broth with strain OG1RF or FA2-2 for 4 h or 10 min, respectively. A mating mixture corresponded to 0.05 ml of donors and 0.5 ml of recipients added to 4.5 ml of fresh broth. Selection was on blood agar plates containing rifampin, fusidic acid, and erythromycin. Transconjugants that were sensitive to streptomycin and kanamycin generally contained pAD1::Tn*917* derivatives.

In the cases involving matings between strains DS16 and OR1RF (i.e., 4-h matings), 30 independent experiments were done; and of 1,500 OG1RF(pAD1::Tn*917*) derivatives examined, ca. 2% proved to be "constitutive clumpers." In the matings between strains DS16 and FA2-2 (i.e., 10-min matings), 100 independent experiments were carried out, yielding 4,000 FA2-2(pAD1::Tn*917*) derivatives. Of these, ca. 20% exhibited constitutive clumping. We point out that the 10-min matings would be expected to select for such derivatives, since most other derivatives would still require the 30- to 40-min period necessary to respond to the pheromone of the recipients. The derivatives not exhibiting constitutive aggregation were tested for their ability to respond to pheromone (11) and with only a few exceptions (indicated in the text) were found to be normal. The transfer of pAD1::Tn*917* derivatives from one strain to another was done in broth or in cross-streak matings as previously described (5).

Clumping assays. Detection of aggregation was as previously described (10, 11). Pheromone preparations (cAD1) were culture filtrates of strain JH2-2. Generally, 0.5 ml of culture filtrate from late logarithmically growing cells was mixed with 0.5 ml of fresh N2GT broth and 20 μl of overnight-cultured cells to be tested for their ability to respond. The

TABLE 1. *S. faecalis* strains used in this study

Strains	Chromosomal genotype	Plasmid content	Comments or references
DS16	*tet*	pAD1 (Hem-Bac); pAD1 [Sm^r Km^r Tn*917*(Em^r)]	Original isolate (21)
OG1RF	*rif fus*	None	Derivative of OG1 (18)
OG1-10	*str*	None	Derivative of OG1 (11)
OG1-10(pAM700)	*str*	pAM700 (pAD1::Tn*917*)	(14)
OG1X	*str*	None	Protease negative mutant of OG1-10 (14)
JH2-2	*rif fus*	None	Mutant of JH2 (15)
FA2-2	*rif fus*	None	Mutant of JH2 (5)
JH2SS	*str spc*	None	Mutant of JH2 (20)

mixtures were cultured for 4 h at 37°C with shaking and were examined for clumping. This method was also used when testing strains harboring pAD1::Tn*917* derivatives for their ability to excrete pheromone; culture filtrates of the latter strains were tested by using strain OG1-10(pAM700) as a responder. When pheromone titers or the sensitivity of a particular responder was desired, a microtiter dilution assay (11) was used. Examination for constitutive clumping involved inoculating fresh N2GT with an overnight inoculum (5%) for 4 h and visually monitoring. The assay for modified pheromone (inhibitor) was as previously described (14).

Isolation of plasmid DNA and mapping. The isolation of plasmid DNA made use of Sarkosyl lysis procedure, followed by separation of satellite DNA by ethidium bromide-cesium chloride equilibrium centrifugation (1, 6). Satellite DNA, located by exposure to an ultraviolet lamp, was removed by puncturing the side of the centrifuge tube with an 18.5-gauge syringe needle. In the case in which the relative copy number of plasmid DNA was estimated, the cells were previously grown in radioactive thymidine, and the resulting gradient was collected in its entirity and counted as previously described (1, 4). The amount of plasmid DNA was estimated relative to chromosomal DNA. Further preparation of DNA and materials for subsequent restriction enzyme mapping and the details of the mapping procedure were done as described elsewhere (5, 12).

Immunofluorescence analysis. Immunofluorescence analysis was performed as previously described (22). The antiserum had been prepared against the "aggregation substance" of pPD1 but has been shown (22) to strongly cross-react with the "AS" of pAD1.

RESULTS

Generation of insertion mutants altered in mating response. Insertions of Tn*917* into pAD1 were obtained as a result of erythromycin-induced transposition (19, 20) from the non-conjugative pAD2 in *S. faecalis* DS16, using the procedure described above. A list of these derivatives along with their phenotypic properties is shown in Table 2.

A number of insertions, called classes B and C, gave rise to constitutive clumping accompanied by increased mating potential, whereas other variants exhibited a reduction in transfer frequency (classes D through G). Transferability in broth matings was examined in a "JH2" background (from strain FA2-2 to strain JH2SS) as well as an "OG1" background (from strain OG1-10 to strain OG1RF). The phenotypes (relative to the wild types represented by members of class A) were similar in both backgrounds, although the transfer frequencies in the OG1 background were usually ca. one to two orders of magnitude higher than in the JH2 background. (This difference in the two backgrounds is also seen for the normal phenotype.) The mating results shown in Table 2 involved the JH2 background.

The strains characterized by constitutive clumping (classes B and C) aggregated without exposure to pheromone in both the JH2 and OG1 backgrounds. The corresponding plasmids transferred at relatively high frequencies in 10-min matings in contrast to the wild type, which usually requires 30 to 40 min of exposure to pheromone before significant transfer occurs. Transfer during 10 min was almost as high as after 4 h. The constitutive clumping strains fell into two subclasses, which exhibited different colony morphologies. In one case, class C, the colonies appeared normal; whereas the other, class B, had a "drier" texture. The differences were easily seen when the colonies were scraped with the end of a toothpick. We also note that the constitutive aggregation exhibited by class B is much more extensive than that of class C. Class B strains form much larger clumps; indeed the aggregation is even more extensive than that observed in the response of normal strains (e.g., class A) to exogenous pheromone. Class C clumping had a more

TABLE 2. pAD1::Tn*197* derivatives and related phenotypes

Classification	Plasmid derivatives	Colony morphology	Clumping[a]		Frequency of transfer from FA2-2 to JH2SS	
			No exposure to pheromone	Exposure to pheromone	10-min mating	4-h mating
A (Normal clumping)	pAM709, pAM713, pAM714, pAM738, pAM739, pAM740, pAM741, pAM742, pAM743, pAM744, pAM745, pAM750, pAM752, pAM753, pAM754.	Normal	−	+	$<10^{-7}$	1×10^{-4} to 1×10^{-3}
B (Constitutive clumping)	pAM721, pAM722, pAM723, pAM724, pAM725, pAM726, pAM729, pAM730, pAM731, pAM733, pAM734, pAM735, pAM736, pAM737.	Dry	+	+	1×10^{-3} to 1×10^{-3}	2×10^{-3} to 5×10^{-3}
C (Constitutive clumping)	pAM701, pAM702, pAM703, pAM705, pAM746, pAM747, pAM748, pAM749.	Normal	+	+	1×10^{-4} to 1×10^{-3}	1×10^{-3} to 2×10^{-3}
D (No clumping)[b]	pAM727	Dry	−	−	$<10^{-7}$	1×10^{-5}
	pAM728		−	−	$<10^{-7}$	7×10^{-6}
E (Reduced transfer)	pAM720	Normal	−	+	$<10^{-7}$	6×10^{-5}
F (*cop*)[c]	pAM710	Normal	−	+	$<10^{-7}$	5×10^{-5}
G (Deleted plasmid)	pAM717	Normal	−	−	$<10^{-9}$	$<10^{-9}$

[a] +, Clumping observed; −, no clumping observed.
[b] Constitutive clumping in OG1 background but not in FA2-2.
[c] Location of the insertion of pAM710.

grainy appearance and was more typical to that corresponding to a normal pheromone response. Immunofluorescence analysis of representative strains of classes B and C (pAM721 and pAM702), using antiserum specific for AS (22), showed positive staining (data not shown).

Filtrates from FA2-2 cells containing the constitutive clumping plasmids shown in Table 2, as well as additionally derived similar strains (a total of 800 strains were examined), were devoid of cAD1 activity. In addition, filtrates of representatives of the two subclasses (class B [pAM721, pAM724, pAM734, pAM735] and class C [pAM701, pAM702, pAM705]) contained inhibitor activity (4 U in all cases) corresponding to the modified form of cAD1 (14).

The plasmids pAM727 and pAM728 (class D) promoted constitutive clumping in an OG1 background (strain OG1-10 or OG1X); however, in a JH2 background (strain FA2-2 or JH2SS) no clumping was visible, even upon exposure to exogenous pheromone at a titer of 32. The two exhibited a drier than normal colony morphology in both backgrounds. In the OG1 background, pAM727 and pAM728 both transferred at frequencies of 1×10^{-4} to 3×10^{-4} (10 min) and 5×10^{-4} to 9×10^{-4} (4 h). (These values are two orders of magnitude lower than for pAM701 [class C] and pAM721 [class B] in an OG1 background.) This is in contrast to the much lower values observed for the JH2 background (Table 2). It appears that mating aggregates arise, but to a lesser (visibly undetectable) extent, in the JH2 background. The constitutive aggregation observed in the OG1 background is not as extensive as that of the constitutively clumping class B types; rather, the clumping has a more grainy appearance resembling that of class C types (see above). Interestingly, the strains harboring pAM727 and pAM728 consistently exhibited a higher than normal level of modified endogenous pheromone (6 and 5 U, respectively) in comparison to members of the two constitutive clumping, high-transfer subgroups (classes B and C) and normal phenotypes (class A; e.g., pAM714) in which 4 U was the usual level. (We note here that units are logarithmically related to concentration.)

Strains harboring pAM720 (class E) or pAM710 (class F) gave rise to a typical clumping response when exposed to two-fold-diluted filtrates of strain JH2-2. Both, however, were reduced in transferability in 4-h matings. pAM710 was found to occur at an elevated copy number, a phenomenon first suspected from its ability to give rise to larger than normal zones of hemolysis on blood agar. Interestingly, the degree of increase in copy number depended on the host. Relative to the normal case represented by pAM714 (one to two copies per chromosome in both the OG1 and JH2 backgrounds), pAM710 was increased by 4.8 and 2.5 times in OG1 and JH2 backgrounds, respectively. (These are based on the averages of two independent estimates of the amount of covalently closed circular DNA present in each case.)

Strain OG1RF(pAM717) failed to respond at all to pheromone and was completely incapable of conjugative transfer (even in filter matings). Analyses by agarose gel electrophoresis indicated that this plasmid had sustained a deletion and had a mass of only 33 kb (data not shown). Its copy number appeared normal. Interestingly, cAD1 activity was present in filtrates of these cells at levels corresponding to that produced by plasmid-free strains; thus, the deletion removed the ability to modify endogenous pheromone.

Transfer kinetics of mutant plasmids. Representative variants were examined for their transferability to recipients as a function of time, and the results are shown in Fig. 2. (The donor host was strain FA2-2; the recipient was strain JH2SS.) pAM714 represents normal behavior and is shown to give rise to incresing numbers of transconjugants for ca. 3 h, at which point the frequency levels off. pAM702 and pAM721 represent constitutive clumpers of the normal (class C) and dry (class B) colony morphology, respectively; their behaviors were similar, showing a high degree of transfer during the early moments of mating. In these cases, the increase observed over the first 1 to 2 h may simply reflect growth of the cells during this time (i.e., increasing numbers of donor-recipient collisions arising due to increasing cell density). pAM710 and pAM720 were observed to transfer at increased rates as time progressed but did so more slowly than the wild type (i.e., pAM714). After 4 h, the frequency leveled off at 1 to 1.5 orders of magnitude lower than the case for pAM714. pAM727 and pAM728 reached a maximum very quickly (at ca. 1.5 h) and then leveled off. Three h later, the transfer level remained unchanged at ca. three orders of magnitude lower than the case for pAM714.

Figure 3 shows the extent of transfer in 10-min matings, after prior exposure of donors to filtrates containing cAD1 for increasing lengths of time. pAM714 was found to require

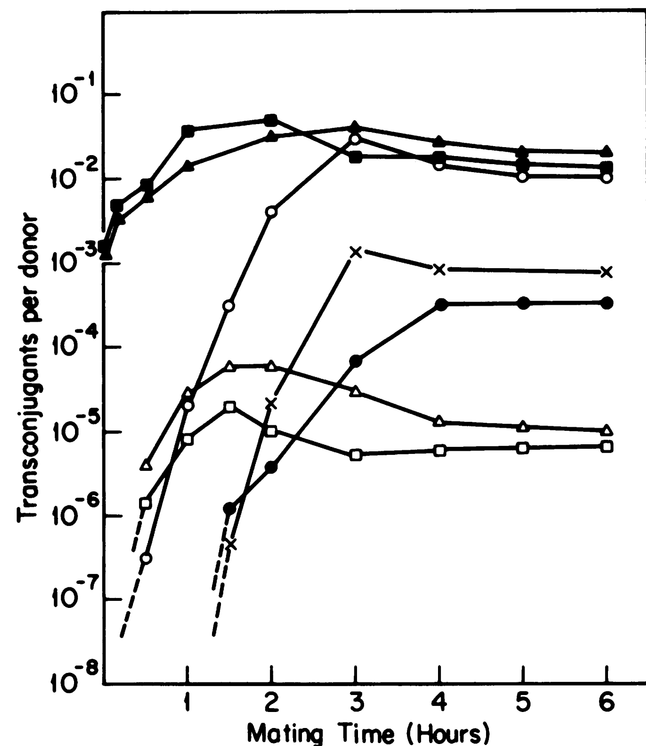

FIG. 2. Kinetics of plasmid transfer of pAD1::Tn*917* derivatives. The recipient was strain JH2SS in all cases but one; the donors were strains FA2-2(pAM721), FA2-2(pAM702), FA2-2(pAM727), FA2-2(pAM728), FA2-2(pAM720), and FA2-2(pAM714). In one case, the recipient was strain FA2-2 and the donor was strain JH2SS(pAM710). Matings were carried out with an initial volume of a mixture of 0.05 ml of donors, 0.45 ml of recipients, and 4.5 ml of fresh N2GT broth. The mixtures were incubated at 37°C with gentle shaking. At the indicated times, 0.1-ml samples were removed, diluted appropriately, and plated for donors (erythromycin, rifampin, and fusidic acid) and transconjugants (erythromycin, streptomycin, and spectinomycin). The reverse was the case for the mating involving pAM710. The number of transconjugants per donor was calculated and plotted as a function of time. Symbols: ○, pAM714; □, pAM727; △, pAM728; ●, pAM720; ■, pAM721; ▲, pAM702; and X, pAM710.

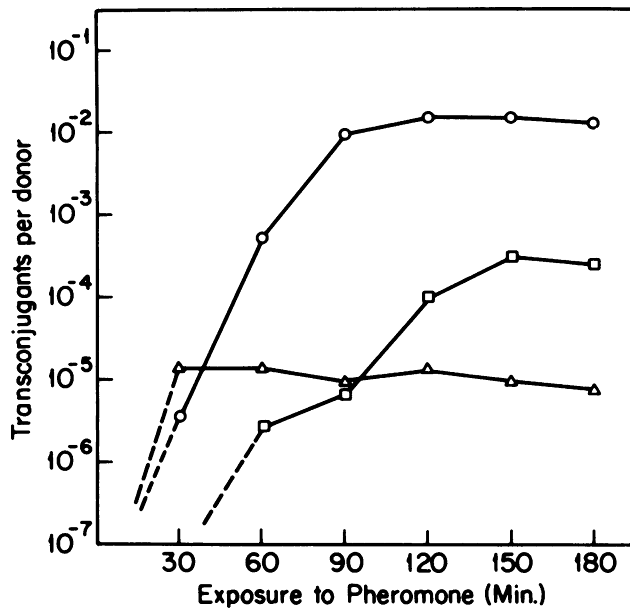

FIG. 3. Transferability of pAD1::Tn917 derivatives in short matings as a function of length of prior exposure to pheromone. Overnight cultures of strains FA2-2(pAM727), FA2-2(pAM720), and FA2-2(pAM714) were diluted (1:10) into a 1:1 mixture of cAD1 (culture filtrate of strain JH2-2) and fresh N2GT broth (total volume, 5 ml). At the indicated times, 0.1-ml samples were removed and mixed with recipients (1 volume of donors to 9 volumes of recipients [JH2SS cells that were similarly diluted and incubated in N2GT broth during the donor incubation]) for 10 min at 37°C. The mixtures were then plated on selective agar. Symbols: ○, transconjugants receiving pAM714; △, transconjugants receiving pAM727; □, transconjugants receiving pAM720.

90 to 120 min to fully respond to pheromone, whereas pAM720 responded more slowly and plateaued at a lower level. (In these two cases, the onset of a stationary phase may have prevented plateauing at even high levels.) Interestingly, pAM727 gave rise to a constant level of transfer throughout, suggesting little or no response at all to the pheromone.

Mapping of Tn*917* inserts of pAD1. A physical map of pAD1 showing the locations of *Eco*RI, *Sal*I, and *Bam*HI restriction sites has been previously published (5). A map of pAD2 was also generated (5) and revealed that Tn*917* has a single *Sal*I restriction site located nearly exactly in its center. This restriction site was useful in determining the location of the inserts relative to other restriction sites; the results of such analyses are summarized in Fig. 4 and 5.

The constitutive clumpers were observed to map in two clusters, designated *traA* and *traB*, located at 14.5 to 16.0 and 17.7 to 19.0 kb, respectively. (The coordinates are measured clockwise from a *Bam*HI site located at 12 o'clock on the pAD1 map.) The dry colony morphology derivatives (classes B and D) are all located in *traA*, whereas those with normal colony morphology (class C) correspond to *traB*. A number of insertions were found to map between *traA* and *traB*; these derivatives all appeared to be normal (class A) with respect to their mating response. Interestingly, the transfer-defective plasmids pAM727 and pAM728 (class D) map near the center of *traA*. pAM720 (class E) mapped far away at a coordinate of 40.7 or 45.4 kb. (We did not distinguish between these two positions.) The plasmid of the copy mutant pAM710 (class F) mapped at 20.8 kb.

Figure 4 also indicates the region lost in the deletion of pAM717; the missing portion involves about half of pAD1 and includes *traA* and *traB*. As mentioned above, this plasmid is incapable of transfer and allows the production of normal pheromone.

DISCUSSION

Tn*917* insertions into pAD1 have given rise to several different classes of mutations altered in the normal mating response. Two classes synthesize AS constitutively and transfer plasmid DNA at relatively high frequencies in short matings. In this regard, they resemble a mutant recently reported in the pCF-10 system (9). The ability to obtain such mutants suggests a form of negative control and also suggests that repression of AS synthesis may involve the products of two genes. Alternatively, one class could be due to a promoter on Tn*917* which facilitates reading of the AS structural gene. Although we had considered the possibility that one type of mutation giving rise to constitutive AS synthesis could involve the putative *IcAD1* gene, neither of the observed classes was of this type. The failure to obtain such mutants argues that Tn*917* insertions into the putative *IcAD1* are quite rare or that such strains are still incapable of responding to endogenous pheromone.

It is possible that products from the two segments, designated *traA* and *traB*, interact in some way in the repression of AS. The fact that *traA* mutants have a distinguishable colony texture (dry) and give rise to unusually large clumps suggests differences in surface properties of the cell and also suggests that the AS in this case may be altered. Two plasmids mapping in *traA* (pAM727 and pAM728) were greatly reduced in transferability in a JH2 background. Neither plasmid was a constitutive clumper (in this background) and neither responded to pheromone. Both behaved

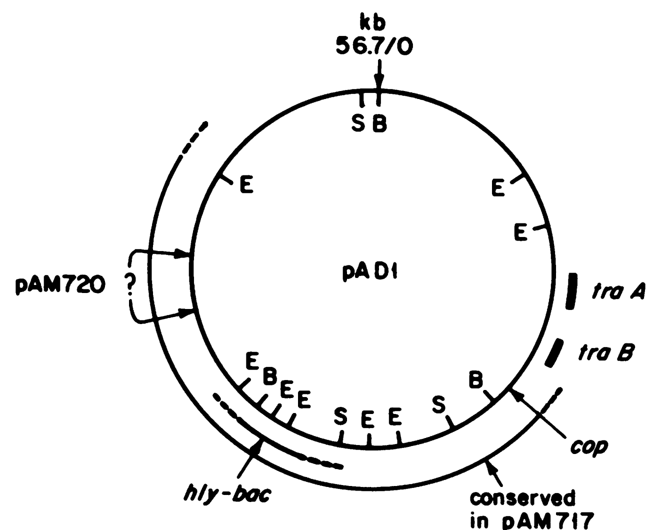

FIG. 4. Map of pAD1 based on analyses of Tn*917* insertions. *Hly-bac*, hemolysin-bacteriocin determinant; *cop*, location of the insertion of pAM710. *traA* and *traB* are segments of DNA into which insertions give rise to constitutive clumping. A more detailed description of the region containing *traA*, *traB*, and *cop* is shown in Fig. 5. The restriction sites were as follows: E, *Eco*RI; S, *Sal*I; and B, *Bam*HI.

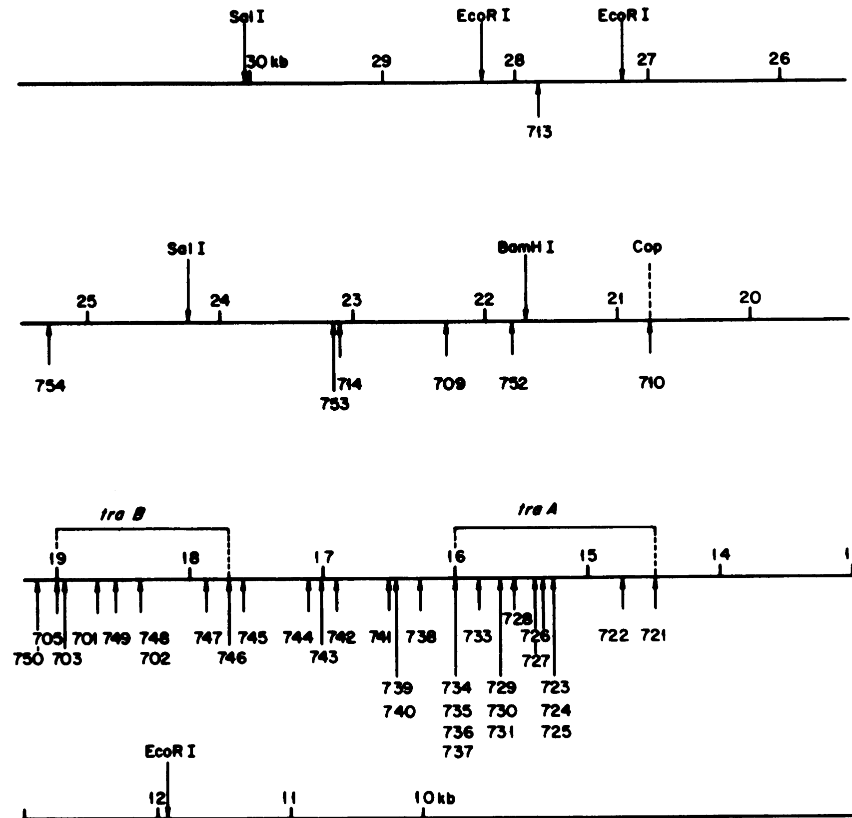

FIG. 5. Map of region of pAD1 containing *traA*, *traB*, and *cop*. The arrows indicate the point of each Tn*917* insertion and the identification number of the related pAM plasmid.

as if the low level of transferability was of a constitutive nature. That is, the transfer kinetics paralleled that of other *traA* (or *traB*) mutants, except that the frequency was several orders of magnitude lower. It is conceivable that the structure of AS itself is altered. (Possibly due to an absence of appropriate processing?) The formation of aggregates may simply be too inefficient to be noticable.

The two other plasmids reduced in transfer (pAM720 and pAM710) mapped elsewhere on pAD1; whereas transfer was reduced, the ability to give rise to a normal clumping response was not affected. With respect to donor potential, both were somewhat sluggish in responding to pheromone. One of the mutants, pAM710, exhibited an increased plasmid copy number. Another plasmid, pAM717, was incapable of transfer and sustained a large deletion which included *traA* and *traB*.

It should be kept in mind that the procedure for generating the Tn*917* insertion mutations tends to select for variants with increased ability to transfer; thus, a great majority of the derivatives were of the *traA* or *traB* type. The few mutants reduced in transfer may have arisen from insertions acquired just before mating, and unaltered proteins may still have been available for use in transfer. The possibility of generating such derivatives is afforded by the inducibility of Tn*917* transposition. That is, induced insertions occuring in the donor may be followed by plasmid transfer before phenotypic expression.

The present data suggesting a negative control are supportive of, but not proof of, a plasmid-borne locus for the AS gene. (The uncertain location of this determinant has been noted previously [11]). If the negative control were exerted on a chromosomal AS locus, plasmid-free strains would be expected to express AS constitutively. This, of course, does not happen.

These studies represent the beginning of a genetic analysis of the mating response conferred by pAD1, a plasmid recently shown to be closely related to several different *S. faecalis* hemolysin plasmids of diverse geographical origin (17). In addition, the utility of Tn*917* in conducting genetic analyses in streptococci has been demonstrated; it is noteworthy that this transposon has also recently been used in mutational studies in *Bacillus subtilis* (23).

ACKNOWLEDGMENTS

We thank E. Ehrenfeld, C. Gawron-Burke, M. Smith, B. White, Y. Yagi, and J. Shaw for helpful discussions. We are also grateful for technical assistance from F. An and E. Ehrenfeld.

This work was supported by Public Health Service grants DE02731 and AI10318 from the National Institutes of Health.

LITERATURE CITED

1. **Clewell, D. B.** 1972. Nature of Col E1 plasmid replication in *Escherichia coli* in the presence of chloramphenicol. J. Bacteriol. **110**:667–676.
2. **Clewell, D. B.** 1981. Plasmids, drug resistance, and gene transfer in the genus *Streptococcus*. Microbiol. Rev. **45**:409–436.
3. **Clewell, D. B., and B. Brown.** 1980. Sex pheromone cAD1 in *Streptococcus faecalis*: induction of a function related to plasmid transfer. J. Bacteriol. **143**:1063–1065.
4. **Clewell, D. B., and D. R. Helinski.** 1970. Properties of a

deoxyribonucleic acid-protein relaxation complex and strand specificity of the relaxation event. Biochemistry 9:4428–4440.

5. **Clewell, D. B., P. K. Tomich, M. C. Gawron-Burke, A. E. Franke, Y. Yagi, and F. Y. An.** 1982. Mapping of *Streptococcus faecalis* plasmids pAD1 and pAD2 and studies relating to transposition of Tn*917*. J. Bacteriol. **152**:1220–1230.
6. **Clewell, D. B., Y. Yagi, G. M. Dunny, and S. K. Schultz.** 1974. Characterization of three plasmid deoxyribonucleic acid molecules in a strain of *Streptococcus faecalis*: identification of a plasmid determining erythromycin resistance. J. Bacteriol. **117**:283–289.
7. **Clewell, D. B., Y. Yagi, Y. Ike, R. A. Craig, B. L. Brown, and F. An.** 1982. Sex pheromones in *Streptococcus faecalis*: multiple pheromone systems in strain DS5, similarities of pAD1 and pAMγ1, and mutants of pAD1 altered in conjugative properties, p. 97–100. *In* D. Schlessinger (ed.), Microbiology-1982. American Society for Microbiology, Washington D.C.
8. **Dunny, G., C. Funk, and J. Adist.** 1981. Direct stimulation of the transfer of antibiotic resistance by sex pheromones in *Streptococcus faecalis*. Plasmid 6:270–278.
9. **Dunny, G., M. Yuhasz, and E. Ehrenfeld.** 1982. Genetic and physiological analysis of conjugation in *Streptococcus faecalis*. J. Bacteriol. **151**:855–859.
10. **Dunny, G. M., B. Brown, and D. B. Clewell.** 1978. Induced cell aggregation and mating in *Streptococcus faecalis*. Evidence for a bacterial sex pheromone. Proc. Natl. Acad. Sci. U.S.A. **75**:3479–3483.
11. **Dunny, G. M., R. A. Craig, R. Carron, and D. B. Clewell.** 1979. Plasmid transfer in *Streptococcus faecalis*. Production of multiple sex pheromones by recipients. Plasmid 2:454–465.
12. **Franke, A. E., and D. B. Clewell.** 1981. Evidence for a chromosome-borne resistance transposon (Tn*916*) in *Streptococcus faecalis* that is capable of "conjugal" transfer in the absence of a conjugative plasmid. J. Bacteriol. **145**:494–502.
13. **Gawron-Burke, C., and D. B. Clewell.** 1982. A transposon in *Streptococcus faecalis* with fertility properties. Nature (London) **300**:281–284.
14. **Ike, Y., R. A. Craig, B. A. White, Y. Yagi, and D. B. Clewell.** 1983. Modification of *Streptococcus faecalis* sex pheromones after acquisition of plasmid DNA. Proc. Natl. Acad. Sci. U.S.A. **80**:5369–5373.
15. **Jacob, A. E., and S. J. Hobbs.** 1974. Conjugal transfer of plasmid-borne multiple antibiotic resistance in *Streptococcus faecalis* var. *zymogenes*. J. Bacteriol. **117**:360–372.
16. **Kessler, R. E., and Y. Yagi.** 1983. Identification and partial characterization of a pheromone-induced adhesive surface antigen of *Streptococcus faecalis*. J. Bacteriol. **155**:714–721.
17. **LeBlanc, D. J., L. N. Lee, D. B. Clewell, and D. Behnke.** 1983. Broad geographical distribution of a cytotoxin gene mediating beta-hemolysis and bacteriocin activity among *Streptococcus faecalis* strains. Infect. Immun. **40**:1015–1022.
18. **Oliver, D. R., B. L. Brown, and D. B. Clewell.** 1977. Analysis of plasmid deoxyribonucleic acid in a cariogenic strain of *Streptococcus faecalis*: an approach to identifying genetic determinants on cryptic plasmids. J. Bacteriol. **130**:759–765.
19. **Tomich, P., F. An, and D. B. Clewell.** 1978. A transposon (Tn*917*) in *Streptococcus faecalis* which exhibits enhanced transposition during induction of drug resistance. Cold Spring Harbor Symp. Quant. Biol. **43**:1217–1221.
20. **Tomich, P. K., F. Y. An, and D. B. Clewell.** 1980. Properties of erythromycin-inducible transposon Tn*917* in *Streptococcus faecalis*. J. Bacteriol. **141**:1366–1374.
21. **Tomich, P. K., F. Y. An, S. P. Damle, and D. B. Clewell.** 1979. Plasmid-related transmissibility and multiple drug resistance in *Streptococcus faecalis* subsp. *zymogenes* strain DS16. Antimicrob. Agents Chemother. **15**:828–830.
22. **Yagi, Y., R. E. Kessler, J. H. Shaw, D. E. Lopatin, F. An, and D. B. Clewell.** 1983. Plasmid content of *Streptococcus faecalis* strain 39-5 and identification of a pheromone (cPD1)-induced surface antigen. J. Gen. Microbiol. **129**:1207–1215.
23. **Youngman, P. J., J. B. Perkins, and R. Losick.** 1983. Genetic transposition and insertional mutagenesis in *Bacillus subtilis* with *Streptococcus faecalis* transposon Tn*917*. Proc. Natl. Acad. Sci. U.S.A. **80**:2305–2309.