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Streptococcus faecalis Sex Pheromone (cAM373) Also Produced by *Staphylococcus aureus* and Identification of a Conjugative Transposon (Tn*918*)

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Streptococcus faecalis **RC73 was found to harbor a conjugative plasmid (pAM373) which confers a mating response to a sex pheromone (cAM373) excreted by plasmid-free members of the same species. The pheromone was also detected in culture filtrates of all of 23 *Staphylococcus aureus* strains but in only 2 of 22 coagulase negative staphylococcus strains. *Streptococcus sanguis* Challis and G9B also produced the activity, but 10 other *Streptococcus sanguis* strains did not. The activity was also produced by *Streptococcus faecium* 9790. A tetracycline resistance (Tc) determinant present in *S. faecalis* RC73 was not associated with pAM373 but served as a useful marker in efforts to identify pAM373 among other plasmids present in the strain. Analyses of the Tc determinant showed that it was located on a conjugative transposon very similar to Tn*916*. Designated Tn*918*, the transposon could insert into pAM373 as well as into two other hemolysin plasmids. Whereas pAM373 derivatives transferred very well between strains of *Streptococcus faecalis*, the plasmid would not establish in *Staphylococcus aureus* or *Streptococcus sanguis*. However, a derivative of pAM373 carrying Tn*918* proved to be a useful delivery vehicle for generating transposon insertions into multiple sites on the staphylococcal chromosome.**

Recipient strains of *Streptococcus faecalis* excrete small peptide sex pheromones that induce donors harboring certain conjugative plasmids to synthesize a proteinaceous adhesin that facilitates the formation of mating aggregates (see reference 6 for a recent review of this topic). Referred to as "aggregation substance," the adhesin has been revealed as a unique antigen that uniformly coats the donor cell surface (18, 32). When exposed to a recipient culture filtrate, donor cells undergo a self aggregation or clumping; for this reason, sex pheromone is also referred to as clumping inducing agent (CIA).

When a recipient strain acquires a copy of the plasmid, the strain ceases to excrete the related pheromone; however, pheromones specific for donors harboring unrelated conjugative plasmids continue to be excreted (10). The "shut-off" of pheromone production in donor cells is accomplished by a plasmid-encoded inactivation of the peptide via a chemical addition (16). A phosphodiester bond is generated in the process, and the modified pheromone is capable of inhibiting exogenous pheromone activity, probably by competition for a pheromone receptor site.

Since recipient cells excrete multiple sex pheromones, a specific pheromone is defined by the plasmid system being used to resolve it. The activity designated cAD1 represents the pheromone to which strains harboring pAD1 respond; similarly, donors harboring pPD1, pAMγ2, etc., respond to cPD1, cAMγ2, etc., respectively. Two pheromones cAD1 and cPD1 have been purified and sequenced (20, 26). Both are very hydrophobic octapeptides, and each has a single serine residue that is probably involved in the plasmid-determined modification that generates a phosphodiester bond.

In the bacterial world, sex pheromones that induce conjugation and plasmid transfer have so far been reported only in *Streptococcus faecalis*. In an effort to determine whether other species of bacteria might produce similar peptides, we screened filtrates of a variety of species for CIA activities resembling the *Streptococcus faecalis* pheromones. In this report we show that numerous *Staphylococcus aureus* strains and at least some *Streptococcus faecium* and *Streptococcus sanguis* strains excrete a CIA activity responded to by *Streptococcus faecalis* strains harboring a plasmid designated pAM373. In the course of the study a tetracycline resistance determinant that served as a convenient marker in the characterization of pAM373 was found to be located on a conjugative transposon which was designated Tn*918*.

MATERIALS AND METHODS

Bacteria, media, and reagents. The bacterial strains and plasmids used in this study are listed in Tables 1 and 2, respectively, or are described in the text. *Streptococcus faecalis* RC73 and the other isolates used as responders in the initial screening of culture filtrates of the different bacterial species were part of a collection generated by R. Carron and used in a previously published study (10) on the correlation of drug resistance and the ability to respond to or produce, or both, CIA activities. Clinical isolates were obtained from University Hospital, Ann Arbor, Mich. *Streptococcus faecalis* was grown in N2GT (Oxoid nutrient broth no. 2 supplemented with Tris buffer [pH 7.0] and glucose [0.2%]) for the preparation of culture filtrates. All other species were grown in Todd-Hewitt broth (THB; Difco Laboratories). In general, broth cultures were grown at 37°C and monitored with a Klett-Summerson colorimeter (no. 54 filter). In connection with the cloning and transformation experiments, *Escherichia coli* was grown in LB medium (8). In mating experiments between *Streptococcus faecalis* and different species, both donors and recipients were pregrown and mated in THB (in broth or on filter membranes), and the mating mixtures were plated on THB agar. Unless otherwise

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TABLE 1. Bacterial strains used in this study

Strain	Relevant chromosome markers	Plasmid content	Source or reference
Streptococcus faecalis			
JH2-2	*rif fus*	None	17
FA2-2	*rif fus*	None	5
JH2SS	*str spc*	None	28
JH2SS (pAMα1::Tn*917*)	*str spc*	pAMα1::Tn*917*	This study
FA1001	*rif fus*	None	This study (derivative of JH2-2)
Y11	*str spc tet* (Tn*916*)	None	16
39-5		pPD1, pPD2, pPD3, pPD4, pPD5, pPD6	32
DS16	*tet* (Tn*916*)	pAD1, pAD2	5, 29
DS5	*tet*	pAMγ1, pAMγ2, pAMγ3, pAMα1, pAMβ1	2, 7
RC73	*tet* (Tn*918*) (probable chromosomal location)	pAM373, at least four additional plasmids	This study; University Hospital, Ann Arbor, Mich.
FA373	*rif fus tet* (Tn*918*)	pAM373	This study
FA381	*str spc tet* (TN*918*)	None	This study
CG110	*rif fus tet* (Tn*916*)	None	13
Streptococcus faecium			
9790 (ATCC)		30-kb cryptic plasmid	R. Kessler
9790RF	*rif fus* (derivative of 9790)	30-kb cryptic plasmid	This study
Streptococcus sanguis			
Challis (NCTC 7868) (V288)		None	F. Macrina
Challis RF	*rif fus* (derivative of Challis)	None	This study
G9B		None	22, G. Jones
Staphylococcus aureus			
879R4	Restriction negative	12.5-kb cryptic plasmid	25, P. Pattee
879R4S	*str* (derivative of 879R4)	12.5-kb cryptic plasmid	23
879R4RF	*rif fus* (derivative of 879R4)	12.5-kb cryptic plasmid	23
RN450		None	21, R. Novick
E. coli			
DH1	F⁻ *recA1 endA1 gyrA96 thi-1 hsdR17 supE44*	None	14
CG170	DH1 background	pAM170	14

noted, matings between *Streptococcus faecalis* strains were in AB3 (broth matings) or on THB-blood (see below) agar (filter matings), and the final platings were on AB3 agar containing appropriate selective drugs. When hemolysin production was being monitored, THB agar containing 4% horse blood (Colorado Serum Co.) was used. Antibiotics were present in selective plates at the following concentrations (micrograms per milliliter): tetracycline, 10 (in all cases except for *E. coli*, where a concentration of 5 was used); erythromycin, 50; rifampin, 25; fusidic acid, 25; streptomycin, 1,000; spectinomycin, 500. The sources of the reagents used in this study were as previously described (7, 9, 12, 16).

Clumping assays. Culture filtrates were prepared as previously described (10). CIA (pheromone) assays were performed by the microtiter, serial twofold dilution assay (10). The modified pheromone (CIA inhibitor) assay was described elsewhere (16). *Streptococcus faecalis* FA373 was used as a responder when specifically determining cAM373 or mcAM373.

Mating procedures. Broth matings were performed as follows. Fresh overnight cultures (5 ml) of donor and recipient cells were prepared in each case. To 4.5 ml of fresh broth, 0.5 ml of the recipient culture and 0.05 ml of the donor culture were added. This mixture (representing a donor-recipient ratio of 1:10) was incubated at 37°C with gentle agitation for 4 h, then vortexed, and plated on media with appropriate selective antibiotics. Colonies were counted after 48 h of incubation. Separate platings where donors alone were selected provided a basis for estimating the transfer frequency (per donor). In the case of filter matings, similarly prepared 1:10 mixtures of donors and recipients were collected on a membrane filter (type HA; pore size, 0.45 μm; diameter, 13 mm; Millipore Corp.). A Swinnex-13 Millipore disk filter holder attached to a Luer-Lok tip plastic syringe (5 cm^3; Becton Dickinson and Co.) was used in the filtration step. The filters were placed on horse blood agar and allowed to incubate at 37°C for 18 to 20 h. The cells were then suspended in 1.0 ml of broth and spread on plates containing the appropriate selective drugs. In some cases, cross-streak matings were performed to determine whether specific resistance determinants transferred at high frequency. Donor cells to be tested were streaked across a perpendicular streak of recipient cells. After growth overnight on drug-free agar, cells in the intersection were streaked onto selective plates. If the determinant being monitored

was linked to the conjugative plasmid (e.g., pAM373 or pAD1), many colonies were readily apparent, whereas few or no colonies implied an absence of linkage.

DNA isolation, characterization, and hybridization. Isolation of plasmid DNA and its characterization by alkaline sucrose density gradients, agarose gel electrophoresis, and restriction enzymes were essentially as described elsewhere (5, 12, 14, 15). Lambda DNA was used as a standard for molecular size estimates of fragments generated by treatment with restriction enzymes. Filter-blot hybridization was as described by Southern (24), as modified by Wahl et al. (30). The isolation of chromosomal DNA, the preparation of ^{32}P-labeled probe, and related autoradiography procedures were as described by Gawron-Burke and Clewell (14).

Genetic techniques. The generation of Tn*916* and Tn*918* inserts into plasmid DNA was as previously described for Tn*916* (12, 13). Briefly, the appropriate plasmid was introduced into a plasmid-free strain harboring Tn*916* (or Tn*918*) on the chromosome. The resulting strain was then used as a donor in a filter mating with a plasmid-free recipient. Transconjugants were then tested for plasmid-transposon linkage by testing donor potential in cross-streak matings.

For mating purposes, it was necessary to introduce selectable chromosomal resistance markers in *Streptococcus sanguis* Challis and *Streptococcus faecium* 9790. To accomplish this, unmutagenized cells were plated on THB plates containing rifampin (50 μg/ml), fusidic acid (50 μg/ml), or streptomycin (1,000 μg/ml) to select randomly occurring mutants.

Strain JH2SS(pAMα1::Tn*917*) is a derivative of PT450 (a JH2SS derivative harboring both pAMγ1 and pAMα1::Tn*917* [29]) which was cured of the hemolysin plasmid pAMγ1 by growing the cells overnight in novobiocin (5 μg/ml in AB3 broth) and plating the cells on blood agar. Nonhemolytic colonies appeared at a frequency of about 5%.

Streptococcus faecalis FA1001, defective in the excretion of cAD1 and cPD1, was generated by using nitrosoguanidine (31) and a CIA-detecting colony halo assay that was used in the previous isolation of the Tn*916*-generated mutant Y11 (16).

The cloning of Tn*918* in *E. coli* was essentially as described by Gawron-Burke and Clewell (14) for similar studies involving Tn*916*.

Characterization of cAM373 and mcAM373. Treatments with trypsin and chymotrypsin, as well as gel filtration chromatography and related size estimates by using a Bio-Rad P2 polyacrylamide column, were performed as described by Ike et al. (16). A unit of inhibitor (i.e., mcAM373) is defined as the amount required to reduce the related CIA activity by 50% or essentially a one-well reduction in the microtiter dilution assay.

TABLE 2. Relevant plasmids used in this study

Plasmid	Marker	Comment
pAD1	Hem-Bac	57-kb, conjugative plasmid from *Streptococcus faecalis* DS16
pAM373	None	36-kb, conjugative plasmid from *Streptococcus faecalis* RC73
pAM377	Em (Tn*917*)	pAM373 with Tn*917* insertion
pAM378	Tc (Tn*918*)	pAM373 with Tn*918* insertion
pAM380	Em (Tn*917*) Tc (Tn*916*)	pAM377 with Tn*916* insertion
pAMα1::Tn*917*	Tc Em (Tn*917*)	Nonconjugative; mobilizable (1, 28)
pAM180	Em Tc (Tn*916*)	Derivative of the 26-kb, conjugative, Em plasmid pAM81 with Tn*916* insertion (13)
pAM360	Tc (Tn*918*)	pAD1 with Tn*918* inserted in *Eco*RI fragment D
pAM361	Tc (Tn*918*)	pAD1 with Tn*918* inserted in *Eco*RI fragment B
pAM210	Tc (Tn*916*)	pAD1 with Tn*916* inserted in *Eco*RI fragment D (12)
pAM170	Ap Tc (Tn*916*)	*E. coli* plasmid vector pGL101 with a cloned fragment carrying Tn*916* (14)

TABLE 3. CIA titers of culture filtrate from different species[a]

Expt	Strain	*Streptococcus faecalis* responder strain			
		39-5	DS16	DS5	RC73
A	*Streptococcus faecalis* JH2-2	64	32	64	128
	Streptococcus faecium 9790	<2	<2	<2	8
	Streptococcus sanguis				
	Challis	<2	<2	<2	16
	G9B	<2	<2	<2	8
	Staphylococcus aureus				
	879R4S	<2	<2	<2	32
	RN450	<2	<2	<2	32
B	*Streptococcus faecalis*				
	JH2-2	64	32	64	128
	FA1001	2	<2	<2	64
	Y11	2	<2	2	128

[a] The values represent the reciprocal of the highest serial (twofold) dilution which still exhibited a detectable clumping response with the indicated responder strain.

RESULTS

CIA activities produced by species other than *Streptococcus faecalis*. Ten *Streptococcus faecalis* strains exhibiting high sensitivity to induced clumping by filtrates of plasmid-free cells (*Streptococcus faecalis*) were used to screen filtrates from a variety of other bacterial sources. Table 3A shows results for four of these responder strains, three of which (39-5, DS16, and DS5) previously were characterized with respect to their plasmid content (2, 5, 7, 29, 32). Strains 39-5, DS16, and DS5, as well as the six *Streptococcus faecalis* strains not shown (DU9, RC11, RC26, RC48, RC56, and RC87), failed to respond to any of the filtrates, except that derived from the JH2-2 control. Interestingly, strain RC73 responded to filtrates from *Streptococcus faecium* 9790, *Streptococcus sanguis* Challis and G9B, and *Staphylococcus aureus* 879R4S and RN450. Filtrates from the following bacteria (not shown in Table 3A) exhibited no CIA activity for any of the 10 responders: *Streptococcus sanguis* A1, *Streptococcus pyogenes* AC1, *Streptococcus agalactiae* T-2, *Streptococcus lactis* 4027, *Streptococcus cremoris* 4158, *Streptococcus milleri* CRS8507, *Staphylococcus epidermidis* UM899, *Bacillus subtilis* BR151, and *E. coli* DH1. It was also of interest that two mutants of *Streptococcus faecalis* (FA1001 and Y11) that were defective in cPD1 and cAD1 production and whose filtrates generated little or no response by strain 39-5, DS16, or DS5 produced essentially

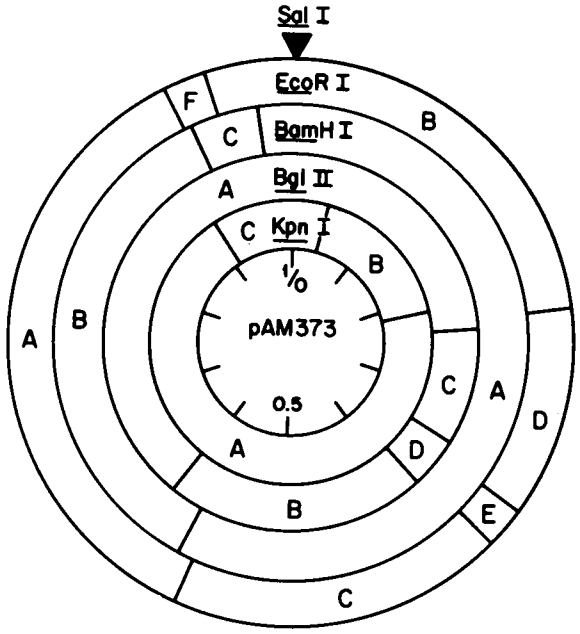

FIG. 1. Restriction map of pAM373. Mapping was based on analyses of double and partial digestions with appropriate enzymes.

unaltered levels of CIA activity to which strain RC73 responded (Table 3B).

The response of strain RC73 to the two *Staphylococcus aureus* filtrates was of particular interest because it involved not only a different genus but an important human pathogen. This prompted an examination of additional clinical isolates of *Staphylococcus aureus*, as well as some of its less pathogenic, coagulase-negative counterparts (e.g., *Staphylococcus epidermidis*). All 21 additional strains of *Staphylococcus aureus* examined excreted activities (titers of 16 to 64) to which strain RC73 responded (data not shown). In contrast, among 22 isolates of coagulase-negative staphylococci, only 2 excreted such an activity. in the two cases in which activity was observed, the titer in both cases was only 2.

In contrast to *Staphylococcus aureus*, *Streptococcus sanguis* strains were not as prevalent in producing the RC73-related activity. Whereas production was observed by strains Challis and G9B, no activity was detected in the case of 10 additional strains (data not shown).

With regard to *S. faecium*, no additional strains were tested.

Identification of the plasmid in *Streptococcus faecalis* RC73 encoding the related CIA response. *Streptococcus faecalis* RC73 is hemolytic, bacteriocinogenic, and resistant to tetracycline (MIC of tetracycline was 60 μg/ml in AB3 broth). Sedimentation (alkaline sucrose density gradients) and electrophoretic analyses revealed at least five plasmids, the largest one being about 57 kilobases (kb) (data not shown). In an effort to generate strains harboring only one or a few of these plasmids, matings were performed between strain RC73 and the plasmid-free *Streptococcus faecalis* FA2-2. Although the nature and location of the Tc determinant were initially unknown, selection for tetracycline-resistant transconjugants from filter matings yielded transconjugants at a frequency of about 10^{-6} per donor (about 10% were hemolytic), and these contained a variety of plasmid combinations. Analyses of plasmid DNA from four derivatives (two hemolytic and two nonhemolytic) by alkaline sucrose density gradient centrifugation revealed one strain, designated FA373 (nonhemolytic and nonbacteriocinogenic), which exhibited a CIA response to filtrates of *Streptococcus faecalis* JH2-2, *Staphylococcus aureus* 879R4S, *Streptococcus sanguis* Challis, and *Streptococcus faecium* 9790 and harbored a single plasmid. The plasmid was designated pAM373, and its size was estimated at 36 kb. A restriction map of pAM373 is shown in Fig. 1. The plasmid did not bear the Tc determinant from strain RC73, as could be judged initially by the fact that not all tetracycline-resistant transconjugants harbored pAM373. (The nature of the Tc determinant is addressed below.) As expected, filtrates of *Streptococcus faecalis* FA1001 and Y11, which were greatly reduced in cPD1 and cAD1 production, had titers similar to that of strain JH2-2 with respect to the pAM373-related CIA.

Properties of the pheromone cAM373. As in the cases for cAD1 and cPD1, the pheromone that induces pAM373 transfer (now referred to as cAM373) was resistant to heat (100°C for 5 min), resistant to trypsin, and sensitive to chymotrypsin. The results of gel filtration chromatography are shown in Fig. 2A, in which cAM373 is observed to elute between cAD1 and cPD1. Although the molecular weights of cPD1 and cAD1 are now known from their amino acid content to be 912 (26) and 818 (20), respectively, their elution positions are reversed from what one would normally expect. This is probably due to differences in hydrophobicity between the two compounds, both of which are octapeptides. It is likely that the size of cAM373 is in this range.

It was also found that strains harboring pAM373 or related derivatives (e.g., pAM378 [see below]) excreted low levels of an inhibitor activity presumed to represent a modified form of the pheromone. Designated mcAM373, this activity was observed to have a mass about 300 to 400 daltons larger than that of cAM373 (Fig. 2B).

Construction of pAM373 derivatives harboring Tn916, Tn917, or both. The Em transposon Tn*917* (27, 28) was introduced onto pAM373 by the following approach. Strain FA373, which harbors only pAM373, was mated (overnight on a filter membrane at a donor-to-recipient ratio of 1:1) with

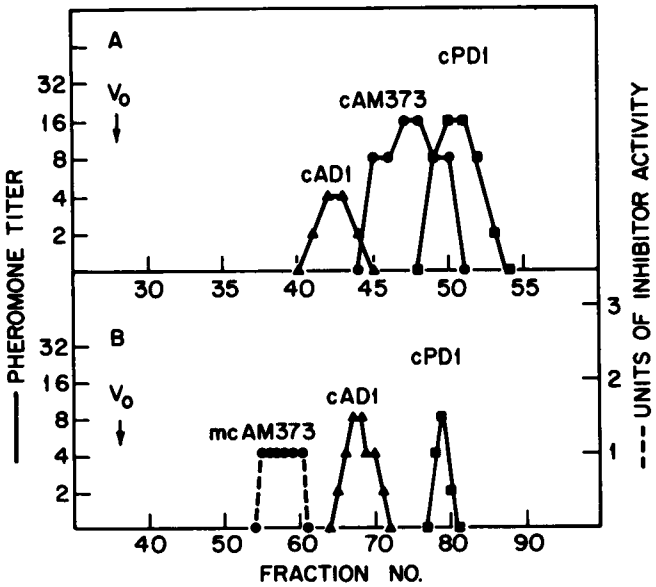

FIG. 2. Gel filtration chromatography (Bio-Rad P2 polyacrylamide) of culture filtrates of *Streptococcus faecalis* strains. V_o, Void volume. A, Filtrate of JH2-2; B, filtrate of FA2-2(pAM378).

Streptococcus faecalis JH2SS(pAMα1::Tn*917*). (pAMα1 is a 9-kb, nonconjugative Tc plasmid [1, 3], known to be readily mobilized by several different conjugative plasmids.) The mating mixture was plated on medium containing rifampin, fusidic acid, and erythromycin; this selected for donors which had transferred pAM373 to recipients and then received back pAM373-mobilized pAMα1::Tn*917*. Transconjugants arose at approximately 10^{-8} per donor; of these, one of three tested transferred Em at high frequency without cotransfer of Tc. pAM373 acquired an insertion (5 kb) into its *Eco*RI A fragment (Fig. 3A, lane 2). The plasmid derivative was designated pAM377 and found to transfer at a frequency of 10^{-3} per donor in 4-h broth matings.

An additional derivative of pAM373 having both Em and Tc determinants was generated by inserting the conjugative transposon Tn*916* (Tc) onto pAM377 (Em) by an approach previously described (12, 13). pAM377 was introduced into plasmid-free *Streptococcus faecalis* CG110 (harbors Tn*916* on the chromosome), and the resulting strain was then used as a donor in subsequent matings (on filters) that selected for transfer of Tc. These matings gave rise to a pAM377 derivative carrying Tn*916*. Designated pAM380, the plasmid acquired the insertion in its *Eco*RI A fragment, which already contained Tn*917* (Fig. 3A, lane 4). Further analyses (data not shown) revealed that Tn*916* had actually inserted into a *Bgl*II fragment within Tn*917*.

The Tc determinant of *Streptococcus faecalis* RC73 is on a transposon similar to Tn*916*. The Tc determinant selected for in the initial RC73 × FA2-2 mating appeared to be chromosome borne; indeed, subsequent filter matings yielded some tetracycline-resistant transconjugants devoid of plasmid DNA. Furthermore, a segment of DNA containing the resistance determinant was found to transpose to plasmid DNA. In filter matings with strain FA373 as a donor and strain JH2SS as a recipient, tetracycline-resistant transconjugants arose at a frequency of 10^{-5} per donor; of 14 isolates tested for donor potential in cross-streak matings, 8 were found to transfer Tc at high frequency. Plasmid DNA from three of these strains was examined and found to contain a discrete segment of DNA inserted into *Eco*RI fragment C of pAM373. A restriction digest of one of these, designated pAM378, is shown in Fig. 3A (lane 3). (The inserted DNA did not contain an *Eco*RI site.)

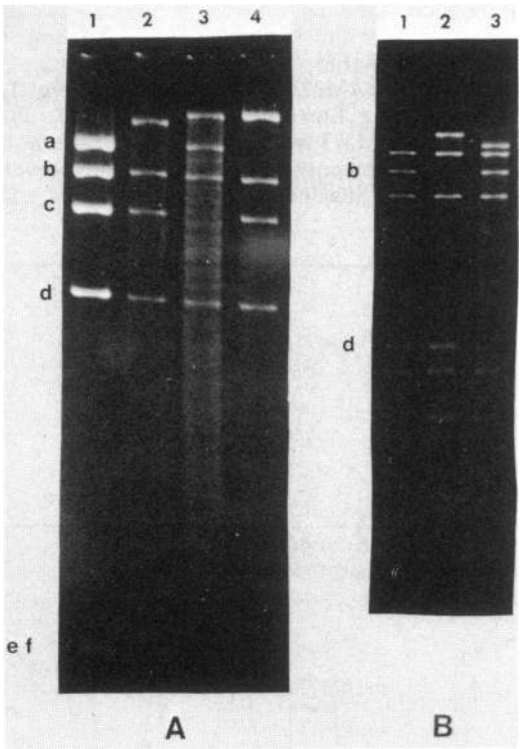

FIG. 3. Agarose gel electrophoresis of plasmid DNA digested with *Eco*RI. A. Lanes: 1, pAM373; 2, pAM377; 3, pAM378; 4, pAM380. The lowercase letter on the left corresponds to the *Eco*RI fragment of pAM373 (fragments E and F migrate together). B. Lanes: 1, pAD1; 2, pAM361 (pAD1::Tn*918*; insertion in the B fragment); 3, pAM360 (pAD1::Tn*918*; insertion in the D fragment). The B and D fragments of pAD1 are indicated on the left.

The Tc determinant was also able to insert into the hemolysin plasmid pAD1. When the latter was introduced into a plasmid-free tetracycline-resistant derivative (FA381) and subsequent matings were performed (on filter membranes) with strain FA2-2 as a recipient, tetracycline-resistant transconjugants arose at 10^{-7} per donor. Of these, about 60% were hemolytic, and 10 to 20% of these had the resistance marker linked to pAD1. Figure 3B (lanes 2 and 3) shows examples of insertions (pAM360 and pAM361) into two different *Eco*RI restriction fragments (B and D), one of which (D, pAM360) gave rise to a hyperhemolytic phenotype. The enhanced hemolysin expression resembled a phenomenon known to occur in the case of some Tn*916* insertions into the D fragment of pAD1 (12).

Finally, we note that among the transconjugants of the original mating between RC73 and FA2-2, a hemolytic derivative subsequently used as a donor in filter matings with strain JH2SS gave rise to tetracycline-resistant, hemolytic strains of which about 10% were hyperhemolytic. In these cases, the Tc determinant was found to be linked to a 57-kb hemolysin plasmid originally present in strain RC73 (data not shown).

The transposable Tc element will henceforth be referred to as Tn*918*. In all of the cases in which it was measured (e.g., those shown in Fig. 3), the size of Tn*918* was estimated at 15 kb (similar to that of Tn*916*). Mating experiments (on filter membranes) with plasmid-free derivatives harboring Tn*918* as the donor showed that the transposon had conjugative properties closely resembling those of Tn*916* (12, 13) (data not shown).

Transferability of pAM373. The pAM373 derivatives pAM377 (Em) and pAM378 (Tc) transferred efficiently in 4-h broth matings between strains of *Streptococcus faecalis* (Table 4). Under the same conditions, however, no transfer could be observed from *Streptococcus faecalis* to *Staphylococcus aureus* 879R4RF, *Streptococcus sanguis* Challis RF, or *Streptococcus faecium* 9790RF. In further efforts to transfer plasmid DNA to these species, overnight filter matings were performed (Table 4). In the case of pAM377 (Em), transfer of Em to *Staphylococcus aureus* 879R4RF or *Streptococcus sanguis* Challis RF again could not be detected. However, in the case of pAM378 (Tc), tetracycline-resistant transconjugants appeared at a significant frequency among *Staphylococcus aureus* (3.0×10^{-5}) and at a very low frequency among *Streptococcus sanguis* (4×10^{-9}). When examined for plasmid content, pAM378 was not detected in the transconjugants of these two species. (Only a 13.5-kb cryptic plasmid already present in the staphylococcal recipient was evident.) Both pAM377 and pAM378 transferred to *Streptococcus faecium* at about 2.5×10^{-6}; transconjugants harbored the corresponding plasmid along with a 30-kb cryptic plasmid already present in the recipient.

TABLE 4. Mating experiments

Donor	Recipient	Selection[a]	Frequency transconjugants per donor
Broth matings (4 h)			
JH2SS(pAM377)	FA2-2	Rif, Fa, Em	5.9×10^{-3}
	879R4RF	Rif, Fa, Em	$<3 \times 10^{-9}$
	Challis RF	Rif, Fa, Em	$<5 \times 10^{-9}$
	9790RF	Rif, Fa, Em	$<1 \times 10^{-9}$
JH2SS(pAM378)	FA2-2	Rif, Fa, Tc	3.0×10^{-2}
	879R4RF	Rif, Fa, Tc	$<1 \times 10^{-9}$
	Challis RF	Rif, Fa, Tc	$<1 \times 10^{-9}$
	9790RF	Rif, Fa, Tc	$<1 \times 10^{-9}$
Filter matings (overnight)			
JH2SS(pAM377)	FA2-2	Rif, Fa, Em	4.6×10^{-2}
	879R4RF	Rif, Fa, Em	$<2 \times 10^{-9}$
	Challis RF	Rif, Fa, Em	$<2 \times 10^{-9}$
	9790RF	Rif, Fa, Em	2.5×10^{-6}
JH2SS(pAM378)	FA2-2	Rif, Fa, Tc	3.3×10^{-2}
	879R4RF	Rif, Fa, Tc	3.0×10^{-5}
	Challis RF	Rif, Fa, Tc	4×10^{-9}
	9790RF	Rif, Fa, Tc	2.5×10^{-6}
FA2-2(pAM380)	879R4S	Sm, Em	$<1 \times 10^{-9}$
	879R4S	Sm, Tc	1×10^{-8}
JH2-2(pAM180)	879R4S	Sm, Em	1×10^{-7} (6% Tc)
	879R4S	Sm, Tc	1×10^{-7} (83% Em)

[a] Rif, Rifampin; Fa, fusidic acid; Em, erythromycin; Tc, tetracycline; Sm, streptomycin.

Efforts to transfer pAM380 (pAM373::Tn*917*::Tn*916*) from *Streptococcus faecalis* into *Staphylococcus aureus* gave rise to tetracycline-resistant transconjugants at 10^{-8}, whereas erythromycin-resistant transconjugants could not be obtained. Those tetracycline-resistant transconjugants that were obtained were sensitive to erythromycin, and no new plasmid could be detected.

Establishment of the Tn*918* and Tn*916* resistance markers in *Staphylococcus aureus* without detection of plasmid DNA may be interpreted in terms of phenomenon previously described for Tn*916* in *Streptococcus faecalis* (13). When located on a conjugative plasmid such as pAM81 (24 kb; Em), Tn*916* was found to undergo a zygotic induction when introduced into a new strain. That is, after transfer of pAM180 (pAM81::Tn*916*), Tn*916* excises from the plasmid and is either lost or inserts into the recipient chromosome. Intact plasmid DNA devoid of the transposon can be recovered in the recipient (13). A similar behavior was observed when pAM180 was transferred into *Staphylococcus aureus* (Table 4). Tetracycline-resistant, as well as erythromycin-resistant, transconjugants could be obtained; however, not all erythromycin-resistant transconjugants were tetracycline resistant and vice versa. In view of the similarities between Tn*918* and Tn*916*, it is likely that transfer of these elements into *Staphylococcus aureus* from their pAM373-borne locations (i.e., pAM378 and pAM380) involved transpositional insertions from plasmid DNA incapable of autonomous existence in this host. The fact that pAM377 (Em) (bearing the more conventional transposon Tn*917*, which is not known to undergo a zygotically induced transposition) yielded no erythromycin-resistant transconjugants implies that the plasmid cannot integrate into the staphylococcal chromosome. Since in the case of pAM378 the frequency of tetracycline-resistant transconjugants was relatively high (3.0×10^{-5}), it is conceivable that the plasmid entered the recipient and was subsequently lost (segregated). (We note that when a plasmid-free *Streptococcus faecalis* donor able to transfer Tn*918* between *Streptococcus faecalis* strains at frequencies of 10^{-7} per donor in overnight filter matings was mated with *Staphylococcus aureus*, no tetracycline-resistant transconjugants could be detected [$<10^{-9}$].) It is not clear why pAM378 (bearing Tn*918*) gave rise to tetracycline-resistant transconjugants at a much higher frequency than did pAM380 (bearing Tn916) (Table 4); however, it may relate to the fact that inserts of this type of element at different sites on a given replicon are known to subsequently transpose at frequencies that can differ greatly (13).

Further comparison of Tn*918* with Tn*916*. The chimeric plasmid pAM170, harboring the entire Tn*916* element as previously described (14), was used as a probe in filter-blot hybridizations. The hybridizations were with *Hin*cII-cleaved preparations of pAM210 (pAD1 derivative with Tn*916* inserted into *Eco*RI fragment D), pAM360 (pAD1 derivative with Tn*918* inserted into *Eco*RI fragment D), pAM380 (pAM373 with both Tn*918* and Tn*917* on *Eco*RI fragment A), and pAM378 (pAM373 with Tn*918* inserted in *Eco*RI fragment C). The probe (pAM170) actually contains the *Eco*RI D′ fragment (D::Tn*916*) of pAM210 and, thus, contains pAD1 D-fragment sequences. The autoradiogram obtained from the Southern blot is shown in Fig. 4, and homology between Tn*918* and Tn*916* is readily evident. Tn*916* is known to have five internal *Hin*cII fragments (C. Gawron-Burke, unpublished data); these are indicated by arrows adjacent to the corresponding bands of pAM210 (Fig. 4).

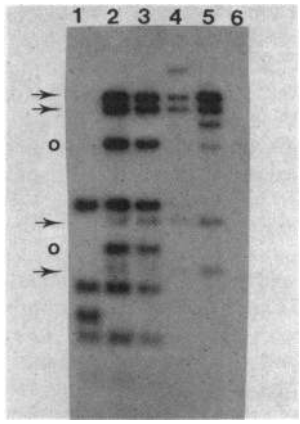

FIG. 4. Autoradiogram obtained from filter blot of *Hin*cII-digested plasmid DNA and hybridization to ^{32}P-labeled pAM170 (contains Tn*916*). Lanes: 1, pAD1; 2, pAM210; 3, pAM360; 4, pAM380; 5, pAM378; 6, pAM373. The arrows mark four of the five *Hin*cII fragments located within Tn*916*. (The fifth could not be resolved here due to its very small size.) Two of the transposon-plasmid junction fragments in pAM210 and pAM360 (marked with an o), as well as those *Hin*cII fragments derived from the pAD1 *Eco*RI D fragment, are more intense than some of the internal fragments. This is because the probe used contained a substantial amount of DNA from which Tn*916* had excised, regenerating a segment of DNA corresponding to that DNA flanking the transposon in pAM210 and pAM360.

(One of the fragments was too small to resolve.) The four visible internal *Hinc*II fragments are also present in the cases of pAM360, pAM380, and pAM378. In the case of pAM360 even the junction fragments appear the same as for pAM210, suggesting that both transposons inserted in the same site (or very close) in pAD1; this is not surprising considering the similar hyperhemolytic phenotype exhibited by both derivatives. This site (or region) may represent a hot spot for insertion. Other data (not shown) revealed that Tn*918*, like Tn*916*, had single recognition sites for *Hin*dIII and *Kpn*I, and these were in locations similar to those of Tn*916*.

It was possible to clone the pAD1 *Eco*RI D::Tn*918* fragment (i.e., of pAM360) in *E. coli* DH1 in a manner essentially identical to the method used to clone the equivalent fragment of pAM210 containing Tn*916* (14). The *Eco*RI D' fragment of pAM360 was ligated to *Eco*RI-digested plasmid vector pGL101 (2.4 kb, Ap) (19). After transformation of *E. coli* DH1, derivatives resistant to both ampicillin and tetracycline could be obtained; however, Tc was lost at high frequency (e.g., over 90% of the cells became sensitive to tetracycline during unselected overnight growth) without loss of Ap. Analysis (data not shown) of the sensitive derivatives showed that Tn*918* had excised and that there was a regeneration of *Eco*RI fragment D (i.e., the pAD1 fragment into which Tn*918* had been inserted).

Analysis of pAM373-delivered Tn*918* and Tn*916* inserts in *Staphylococcus aureus*. Tn*916* has been reported (13) to insert into a number of different sites in the *Streptococcus faecalis* chromosome; this was demonstrated by hybridizing a ^{32}P-labeled probe containing Tn*916* to chromosomal DNA cleaved with *Hin*dIII. In the case of each insert, two fragments hybridized; these represented the two transposon-host DNA junction fragments extending from the single *Hin*dIII site within Tn*916*. Because the size of these fragments differed in the various isolates, insertions into different sites must have occurred (13). Figure 5 shows the results of similar experiments in which ^{32}P-labeled pAM170 was hybridized to *Hin*dIII-digested chromosomal DNA isolated from several staphylococcal strains containing Tn*918* or Tn*916* inserts. The transposons had been introduced from their positions on plasmid DNA (i.e., from pAM378 or pAM380 [see above]). It is readily apparent that insertions of both Tn*918* and Tn*916* occurred into different sites on the recipient chromosome, and in a few cases more than one transposon insertion was evident.

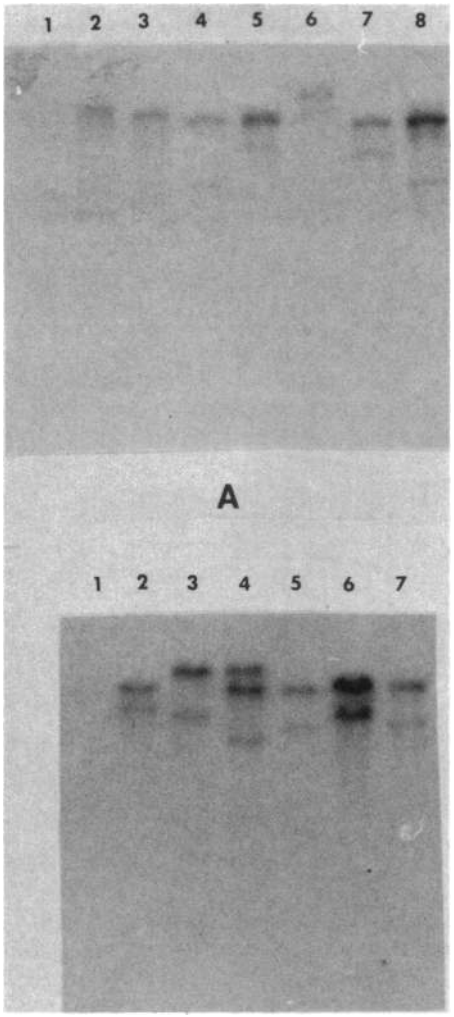

FIG. 5. Autoradiograms obtained from filter blot of *Hin*dIII-digested chromosomal DNA of tetracycline-resistant transconjugants of *Staphylococcus aureus* and hybridization to ^{32}P-labeled pAM170 (contains Tn*916*). A. Lanes 2 through 8 represent chromosomal DNA from seven different transconjugants acquiring Tn*918* from matings between *Streptococcus faecalis* JH2SS(pAM378) and *Staphylococcus aureus* 879R4RF. Lane 1 represents *Staphylococcus aureus* 879R4RF. B. Lanes 2 through 7 represent chromosomal DNA of transconjugants acquiring Tn*916* as a result of matings between *Streptococcus faecalis* FA2-2(pAM380) and *Staphylococcus aureus* 879R4S. Lane 1 is *Staphylococcus aureus* 879R4RF.

DISCUSSION

A conjugative plasmid pAM373 originating in *Streptococcus faecalis* RC73 has been shown to confer a CIA response to a small peptide (cAM373) present in culture filtrates of *Streptococcus faecalis* and certain strains of *Streptococcus faecium*, *Streptococcus sanguis*, and *Staphylococcus aureus*. A number of other species did not produce the cAM373 activity. At 36 kb, pAM373 is somewhat smaller than other known pheromone-related plasmids which are all larger than 45 kb (3), and its restriction map showed no resemblance to the previously mapped pAD1 (5) and pPD1 (32). The unaltered production of cAM373 by two mutants defective in the excretion of both cAD1 and cPD1 further distinguishes this system.

The fact that some other bacterial species excrete a substance resembling a specific sex pheromone in *Streptococcus faecalis* does not necessarily imply a pheromonal role in those species. Indeed, since pAM373 derivatives could not be established in *Staphylococcus aureus* or *Streptococcus sanguis*, the possible absence of a direct relationship between cAM373 and mating in these organisms is suggested. As previously noted (3), it is conceivable that bacterial production of the substances which donor strains recognize as sex pheromones could have preceded the evolution of the related conjugative plasmid systems, with the latter simply taking advantage of these available, extracellular compounds as mating signals. If this is true, the question arises as to the nature of the original, perhaps continuing, function of these peptides. The production of cAM373 activity by all *Staphylococcus aureus* strains tested, and a general absence of the activity in most coagulase-negative staphylococci, raises the possibility of a role in virulence. The derivation of mutants defective in cAM373 production and their examination in virulence assays could shed light on this interesting question.

The element Tn*918*, revealed in connection with efforts to identify and characterize pAM373, was essentially indistinguishable from a previously characterized conjugative transposon Tn*916* (12–14). Although it is possible that in its original host *Streptococcus faecalis* RC73 Tn*918* may be located on one of the several plasmids present, it is a reasonable (if not likely) possibility that it is located on the chromosome, as was the case for Tn*916* in its original host DS16 (12). It is noteworthy that a similar element (Tn*919*) has recently been identified (4, 11) in a strain of *Streptococcus sanguis* (FC-1). A common origin for these elements is implied, and the fact that Tn*916* has been shown able to transfer into a number of gram-positive species (4) suggests that these elements are probably widespread.

Tn*916* and Tn*918* could be introduced onto pAM373, and although the plasmid derivatives could not establish in *Staphylococcus aureus*, the fact that they could facilitate insertions of the transposons at multiple sites in the staphylococcal chromosome suggests a potential for these plasmids as suicide delivery vehicles for the transposons. As recently reported (14), Tn*916* may serve as a useful targeting element for the cloning of gram-positive genes in *E. coli*. That is, restriction fragments containing Tn*916* could be cloned on an *E. coli* plasmid vector by selecting for Tn*916*-encoded Tc. Growth of this clone in the absence of tetracycline would then give rise to a regenerated segment of DNA containing the specific locus of interest. A similar potential for Tn*918* is evident, and the relatively high delivery frequency observed with a derivative such as pAM378 may offer a special advantage for use of this element in staphylococci.

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