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Transmissible Toxin (Hemolysin) Plasmid in *Streptococcus faecalis* and Its Mobilization of a Noninfectious Drug Resistance Plasmid

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Streptococcus faecalis strain DS-5 has been shown previously to contain three plasmids, pAMα1, pAMβ1, and pAMγ1. Mixed incubation of DS-5 with strain JH2-2, a plasmid-free *S. faecalis* recipient, results in the transfer of pAMα1 (which determines resistance to tetracycline) and/or pAMγ1. Analyses of recipients carrying various combinations of these plasmids have revealed the pAMγ1 codes for toxin (hemolysin) production and two bacteriocin activities. JH2-2 strains carrying both pAMα1 and pAMγ1, or pAMγ1 only, can donate their plasmids to other recipients, whereas strains carrying only pAMα1 cannot serve as donors. This indicates that pAMγ1 actually mobilizes the otherwise nontransferable pAMα1.

An important trait determined by certain bacterial plasmids is the ability to promote gene transfer. The gentic material transferred may include the plasmid itself as well as other plasmid or chromosomal deoxyribonucleic acid (DNA). This phenomenon of plasmid-mediated conjugal transfer of genetic information has been extensively studied in gram-negative organisms, especially the *Enterobacteriaceae*. Recent reports of the existence of plasmids in various *Streptococcus* strains (7, 9-11) have been followed by communications dealing with apparent conjugation in *Streptococcus faecalis* (13, 14, 17). Previous studies in our laboratory (7) have shown that *S. faecalis* strain DS-5 carries three plasmids (henceforth officially designated pAMα1, pAMβ1, and pAMγ1 and abbreviated here α, β, and γ) with molecular weights of 6×10^6, 17×10^6, and 34×10^6. The β plasmid was shown to code for erythromycin (Ery) and lincomycin resistance, and more recent experiments indicate that the α plasmid determines resistance to tetracycline (TC) (6a). We now present evidence indicating that strain DS-5 is capable of transferring its α and/or γ plasmid to another *S. faecalis* strain during mixed incubation in broth. We further demonstrate that the γ plasmid codes for toxin (hemolysin) and bacteriocin production and is capable of promoting the transfer of the α plasmid as well as itself.

MATERIALS AND METHODS

Materials. Most of the reagents and sources have been previously described (4, 6, 7). TC, rifampin (Rif), and streptomycin (SM) were from Calbiochem. Ery (Ilotycin Gluceptate) was from Eli Lilly. [*methyl*-^{3}H]thymidine (60 Ci/mmol) and [*methyl*-^{14}C]thymidine (56 mCi/mmol) were from Nuclear Dynamics. Fusidic acid (Fus) was generously provided by F. Neidhardt.

Bacteria and media. *S. faecalis* strain DS-5 has been described previously (7). Strain JH2-2 was kindly provided by A. Jacob (14). Strain DS-3, used as an indicator in bacteriocin assays, is a clinical isolate received from E. Britt of St. Joseph's Mercy Hospital, Ann Arbor. All strains discussed in this paper are listed in Table 1 along with their respective markers.

Oxoid nutrient broth no. 2 was utilized in all mating experiments. For labeling cells an M9-glucose-Casamino Acids medium (8) with 0.3% yeast extract (M9-YE) was used. Bacteriocin assays were done in Oxoid nutrient broth no. 2 agar or Difco antibiotic medium no. 3 (Penassay broth) agar. Hemolysin production was observed on Todd-Hewitt broth agar plates containing 4% blood. Horse and rabbit blood (defibrinated) was from BBL (Division of Bioquest), and human blood was drawn from one of the authors (G.D.) and defibrinated by swirling with glass beads before being added to the agar solutions. Sheep blood plates were purchased from BBL.

Preparation of Sarkosyl lysates and density gradient analysis of DNA. Cultures (15 ml of M9-YE) were grown to late log phase with shaking at 37 C in the presence of 0.15 ml of isotope. The cells were then chilled, harvested, and lysed by the lysozyme-Pronase-Sarkosyl procedure, which has been described in detail previously (7). Dye-buoyant density and sucrose gradient centrifugation was performed as previously described (4, 6). ^{14}C-labeled marker DNA consisting of the α and γ plasmids was

TABLE 1. *S. faecalis strains and relevant properties*

Strain	Chromosomal resistances[a]			Plasmid markers[b]				Remarks[c]
	Rif[d]	Fus[d]	SM	TC resistance	Hemolysis	Bac I	Bac II	
DS-5	−	−	−	+	+	+	+	Carries α, β, and γ plasmids
JH2-2	+	+	−	−	−	−	−	Plasmid-free strain obtained from A. Jacob (14)
JH2-2S	+	+	+	−	−	−	−	SM-resistant mutant of JH2-2 (resistant to >1 mg/ml)
DT-4	+	+	−	+	+	+	+	JH2-2 exconjugant containing α and γ
DT-34	+	+	−	+	+	+	+	Properties identical to DT-4
DT-9	+	+	−	+	−	−	+	JH2-2 exconjugant containing α and an "altered" γ
DT-10	+	+	−	+	−	−	+	Properties similar to DT-9
DT-11	+	+	−	+	−	−	−	JH2-2 exconjugant containing α
DT-30	+	+	−	+	−	−	−	Properties identical to DT-11
HD-1	+	+	−	−	+	+	+	JH2-2 exconjugant containing γ
DS-3	−	−	−	−	−	−	−	Indicator strain used for bacteriocin assays

[a] +, Resistant; −, not resistant.
[b] Association of the α plasmid with TC resistance and the γ plasmid with the bacteriocin and hemolysin markers is described in the text and elsewhere (6a).
[c] All exconjugants listed in this table were obtained in matings between JH2-2 and DS-5.
[d] These resistances were described by Jacob and Hobbs (14).

isolated from an Ery-sensitive derivative of DS-5 called DS-5C1 as described previously (7).

Mating procedures. We used a mating procedure similar to the one employed by Jacob and Hobbs (14). In typical mating experiments, overnight cultures (5 ml) of donor and recipient cells were grown in Oxoid nutrient broth no. 2. To 4.5 ml of fresh broth, 0.5 ml of the recipient culture and 0.05 ml of the donor culture were added. The mixture was incubated at 37 C with gentle agitation for 2 h (unless otherwise indicated) and was then vortexed and plated out on nutrient broth plates with appropriate selective antibiotics. Colonies were counted after 48 h of incubation. Controls consisting of various dilutions of donors and recipients alone were always plated separately. The concentrations of antibiotics in the plates were as follows: TC, 10 μg/ml; Rif and Fus, 25 μg/ml; SM, 1,000 μg/ml; and Ery, 50 μg/ml. It should be noted that the minimum inhibitory concentration for TC is about 25 μg/ml for a JH2-2 host carrying the α plasmid, as compared with 250 μg/ml in the case of strain DS-5 (7). This presumably reflects differences in the intrinsic resistances of the two hosts.

Bacteriocin assays. An overnight culture of the indicator strain was grown in Difco antibiotic medium no. 3 or Oxoid nutrient broth no. 2, and 0.05 ml of this culture was added to 4 ml of liquefied soft agar (0.75% agar) and poured over an agar plate. When this had solidified, samples from appropriate colonies or liquid cultures of producer strains were stabbed into the plates and after incubated overnight at 37 C; clear zones of inhibition were visible around the "stabs" of bacteriocinogenic strains.

RESULTS

Hemolysin production by strain DS-5. Strain DS-5 was originally obtained from the American Type Culture Collection and had not previously been designated as being hemolytic. When tested on sheep blood agar, the strain is in fact not hemolytic; however, when grown on plates containing rabbit, horse, or human blood, this organism produces a clear zone of hemolysis. This strain is, therefore, beta-hemolytic and thus a *zymogenes* variant (16). (Interestingly, when we examined 50 clinical *S. faecalis* isolates from St. Joseph's Mercy Hospital, Ann Arbor, which were nonhemolytic on sheep blood [which is the type of blood commonly used for diagnostic purposes], about 25% of these isolates were found to be hemolytic on horse blood.)

Transfer of plasmids from DS-5 to JH2-2 in mixed culture. When 18-h broth cultures of these strains were mixed for 2 h as described in Materials and Methods, TC resistance was transferred at a frequency of 4.3 per 10^5 donors or 6.5 per 10^6 recipients. (In other experiments [such as shown in Fig. 2 and discussed below] higher frequencies of transfer have been observed.) Twenty-eight TC-resistant exconjugant colonies were rechecked for proper markers and further analyzed. When these strains were grown on horse blood plates, 5 of

the 28 were beta-hemolytic. Two of these hemolytic strains and 10 of the nonhemolytic exconjugants were grown in 15 ml of M9-YE medium containing [^{3}H]thymidine and lysates from these cultures were centrifuged in CsCl-ethidium bromide density gradients. The fractions containing the satellite peak were pooled, dialyzed, and sedimented through 5 to 20% neutral sucrose gradients along with ^{14}C-labeled DS-5Cl plasmid DNA (marker). Data from four representative strains are shown in Fig. 1. All of the 12 strains had the 28S α plasmid peak associated with TC resistance (6a), and all strains except DT-11 also had a faster-sedimenting peak corresponding to the approximate position (about 58S) of the γ plasmid (7). All TC-resistant exconjugants tested were sensitive to Ery and showed no signs of having acquired the β plasmid, which determines resistance to this antibiotic (7). Moreover, we have repeatedly failed to observe transfer of Ery resistance by adding Ery to the selective medium (without TC).

The fact that hemolytic exconjugants (e.g.,

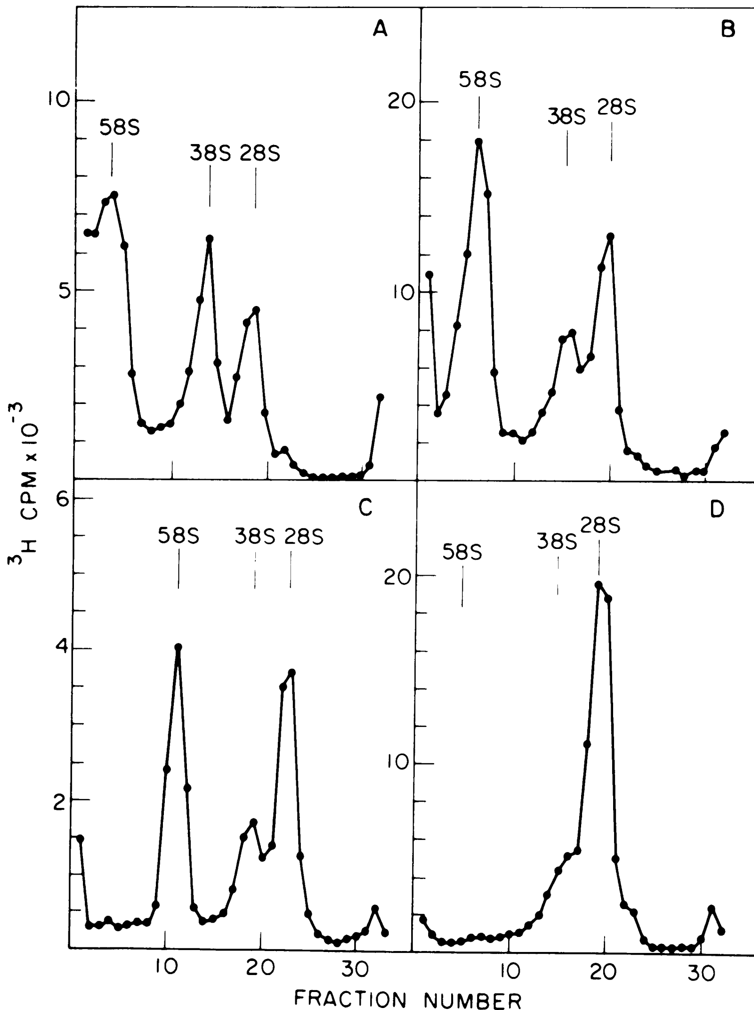

FIG. 1. *Sucrose density gradient analysis of plasmid DNA from TC-resistant exconjugants. Fractions containing the satellite peak from CsCl-ethidium bromide buoyant density gradients were pooled and dialyzed before sedimenting on 5 to 20% neutral sucrose gradients for 60 min at 48,000 rpm in a Beckman SW50.1 rotor. Sedimentation is from right to left. ^{14}C-labeled marker DNA (α and γ DNA as discussed in the text) was also included in the gradients, and the positions of the 58S supercoiled γ plasmid, 38S nicked circular γ, and 28S supercoiled α plasmid are indicated. (A) DT-4, a TC-resistant strain which is also hemolytic. (B) DT-9, a nonhemolytic, TC-resistant strain. (C) DT-10, a nonhemolytic, TC-resistant strain. (D) DT-11, a nonhemolytic, TC-resistant strain.*

strain DT-4 [Fig. 1A]) were found to contain the 58S γ plasmid and DT-11 (Fig. 1D) was nonhemolytic, completely lacking the γ plasmid, suggested to us that the γ plasmid may determine hemolysin production. Jacob et al. (13) have reported hemolysin in *S. faecalis* determined by a plasmid similar in size to the γ plasmid. Inasmuch as strains such as DT-9 and DT-10 (Fig. 1B and C) were nonhemolytic but still carried a γ-like plasmid, we suspected that they may, in fact, carry the γ plasmid in an altered state not distinguishable from normal γ DNA on sucrose gradients. (Evidence supporting this interpretation is presented below.)

Figure 2 shows the results of an experiment to determine the kinetics of plasmid transfer between DS-5 and JH2-2. In this experiment, overnight cultures of donors and recipients were mixed and, at various time intervals, aliquots were removed and plated out to select for donors (on Ery plates, since Ery resistance is nontransferable), recipients (on plates containing Rif and Fus), or TC-resistant recipients (on plates with Rif, Fus, and TC). Colonies of the latter two classes were also checked for hemolysin production.

TC-resistant recipients could be detected very early, and an increasing percentage of these organisms were hemolytic after a longer period of mixed incubation (Fig. 2). The number of TC-resistant recipients reached a maximum level after 1 h and then appeared to drop slightly to a level which then remained constant.

An interesting aspect of this experiment is the dramatic killing of recipients after several hours of mixed incubation. This killing was probably due, at least in part, to the bacteriocinogenic nature of the donor, as will be discussed below. (It is likely that the bacteriocin effect was enhanced by significant growth of the donor cells during the first 4 h). JH2-2 strains which had become hemolytic were resistant to the bacteriocin. Repeated efforts had shown that overnight matings selected for bacteriocin-resistant strains, and therefore a significant percentage (about 3 to 50% in different experiments) of recipient organisms surviving long matings, were found to be hemolytic. Hemolytic exconjugants not resistant to TC could be easily obtained by this method. Figure 3 shows a neutral sucrose gradient of plasmid DNA from a strain (HD-1) obtained in this way, and depicts a profile typical of several hemolytic, TC-sensitive exconjugants we have obtained. Only the γ plasmid was evident in such strains.

Bacteriocin production associated with the γ plasmid. We have determined that strain

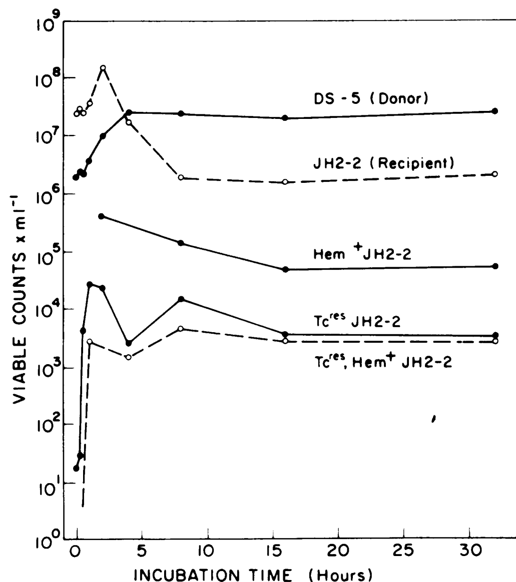

FIG. 2. *Kinetics of gene transfer between DS-5 and JH2-2. Overnight cultures of donors and recipients were mixed as described in the text and aliquots were removed and plated out to select for donors, recipients, or TC-resistant recipients at 0 min, 30 min, 1 h, 2 h, 4 h, 8 h, 16 h, and 32 h after mixing. The recipients were also checked for hemolysis by replating onto horse blood plates.*

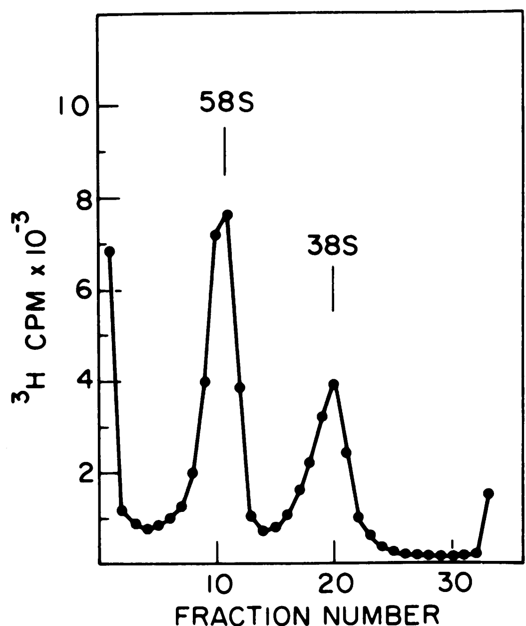

FIG. 3. *Neutral sucrose gradient of plasmid DNA from strain HD-1. Plasmid DNA from a CsCl-ethidium bromide gradient was pooled, dialyzed, and sedimented for 60 min on a 5 to 20% gradient along with ^{14}C marker DNA as described in the legend to Fig. 1.*

DS-5 is bacteriocinogenic to JH2-2 and DS-3 by the assay described in Materials and Methods. We have also tested many JH2-2 exconjugants carrying α and/or γ plasmids. Results of some strains tested, shown in Table 2, indicated that there were actually two bacteriocin activities associated with the γ plasmid. One bacteriocin (Bac I) was produced by all hemolytic strains, acted against all nonhemolytic strains (including exconjugants of JH2-2 containing "γ-like" DNA), and was closely linked and probably identical to the hemolysin, as demonstrated previously in other *S. faecalis* systems by Brock and Davie (3) and Granato and Jackson (12). The other bacteriocin (Bac II), which was present but in a sense "masked" in hemolytic strains, was also produced by nonhemolytic strains such as DT-9 and DT-10 and acted against DS-3 but not against any JH2-2 strains. Because of differences in the size of the zones of inhibition produced by different derivatives, we believe that strain DS-3 is sensitive to both bacteriocins. We reasoned that if the two bacteriocin genes were independent of each other, they might not be closely linked on the γ plasmid, and therefore TC-resistant exconjugants determining neither bacteriocin (i.e., non-bacteriocinogenic to DS-3) could be missing the γ plasmid entirely. Fifty TC-resistant exconjugant colonies from a 2-h mating experiment were tested for bacteriocin production against DS-3. Four of the 50 were negative on the DS-3 indicator, and three of these four strains had only an α plasmid as revealed by sucrose gradient analysis (data not shown). The fourth strain appeared to have a second peak sedimenting at 34S, possibly representing a dimer of α. (We are continuing to investigate the properties of this strain.) These data demonstrate that, in general, TC-resistant exconjugants lacking both bacteriocin activities tend to contain only α plasmids, supporting the notion that both activities are determined by, but are not closely linked on the γ plasmid.

Demonstration that transferability is determined by the γ plasmid. We have carried out experiments using strain HD-1 as a donor and JH2-2S as a recipient to test for hemolysin transfer in the absence of the α plasmid. After 26 h of mating, approximately 50% of the viable recipients (which represent only 0.1% of the original count, presumably due to killing by bacteriocin activity) had become hemolytic. Thus the γ plasmid is able to promote its own transfer.

Other experiments have shown that the transfer of the α plasmid is actually the result of mobilization by the γ plasmid. Two hemolytic, TC-resistant donors (DT-4 and DT-34) were compared with 2 nonhemolytic donors (DT-11 and DT-30) that contained only α plasmids for the ability to transfer TC resistance to JH2-2S (all four donor strains are exconjugants from matings between JH2-2 and DS-5 as noted in Table 1). The results are shown in Table 3. It can be seen that transfer of the α-coded TC resistance was possible only in strains that also carried a γ plasmid. Strains DT-11 and DT-30 were not capable of transferring their TC resistance. From these data we conclude that the γ plasmid is capable of mobilizing the α plasmid as well as promoting its own transfer.

Number of copies of plasmid DNA in the new host. We have calculated the number of plasmid copies per chromosome for the α plasmid in DT-11 and the γ plasmid in HD-1 based on the amount of the plasmid DNA in CsCl-ethidium bromide, taken as a percentage of the chromosomal DNA, and assuming the molecular weight of the *S. faecalis* genome to be 1.47×10^9 (2). The results indicate that, per chromosomal genome equivalent, there are about 10

TABLE 2. *Bacteriocin activities of S. faecalis strains*[a]

Producer strain	Indicator strain						
	JH2-2	DT-11	DS-3	DT-10	DT-9	DS-5	HD-1
Nonhemolytic							
JH2-2	−	−	−	−	−	−	−
DS-3	−	−	−	−	−	−	−
DT-11	−	−	−	−	−	−	−
Hemolytic							
DS-5	+	+	+	+	+	−	−
HD-1	+	+	+	+	+	−	−
DT-4	+	+	+	+	+	−	−
Nonhemolytic							
DT-9	−	−	+	−	−	−	−
DT-10	−	−	+	−	−	−	−

[a] "+" indicates that the producer strain is bacteriocinogenic to the indicator strain by the assay described in the text.

copies of α in DT-11 and about 3 copies of γ in HD-1. This is in relatively close agreement with the level of the α plasmid in the DS-5 host (7), but represents a lower level in the case of the γ plasmid (γ was calculated to be 5.3 copies in DS-5 [7]).

On the mechanism of transfer. Previous studies by others have shown that the most probable mechanism of the genetic transfer observed in *S. faecalis* is by conjugation (14, 17). We have carried out several experiments (data not shown) which indicate that culture filtrates of the donor are ineffective in promoting plasmid transfer. In addition, transfer in mixed cultures occurs normally in the presence of deoxyribonuclease. These data support the notion that transduction or transformation is not the mechanism of transfer.

DISCUSSION

The data presented here demonstrate the ability of the α and γ plasmids of *S. faecalis* strain DS-5 to transfer and become established in a new streptococcal host. As a result of mating experiments between DS-5 and JH2-2 where selection was carried out for the α-determined TC resistance, a relatively high percentage (90%) of the exconjugants were found to have also acquired the γ (or a "γ-like") plasmid, and a significant number of these strains had become hemolytic and bacteriocinogenic. Subsequent experiments confirmed that the hemolytic activity as well as two bacteriocin activities (Bac I and Bac II) were determined by the γ plasmid. Bac I and Bac II were resolved by using two indicator strains, one of which (JH2-2) was sensitive to only one of the activities (Bac I), whereas the other strain (DS-3) was sensitive to both activities. Nonhemolytic, Bac I-negative TC-resistant exconjugants of JH2-2 that carried "γ-like" plasmid produced Bac II, whereas TC-resistant exconjugants lacking both Bac I and Bac II were free of γ plasmid DNA. Hemolysin and Bac I production were always associated and possibly represent the same protein as has been shown by others (3, 12) in the case of *zymogenes* variants of *S. faecalis*.

Strains harboring only the α plasmid were incapable of acting as donors in the transfer of TC resistance. The additional presence of the γ plasmid, however, allowed transfer of drug resistance. In addition the γ plasmid was capable of transferring separately, and strains harboring only this plasmid could transfer the ability to produce hemolysin.

The β plasmid (which determines resistance to Ery, lincomycin, and vernamycin B_α) of strain DS-5 did not transfer during mating. The reason for his is not clear. It may in fact reflect only an inability to be maintained in the JH2-2 host. It is interesting that a plasmid resembling the β plasmid and conferring a similar resistance pattern (i.e., co-resistance to Ery, lincomycin, and vernamycin B_α) has been identified in a clinical isolate of *S. pyogenes* (5). This plasmid has essentially the same molecular weight (about 17 million) as, and is 95% homologous to (19), the β plasmid, indicating the likelihood of a common origin. The plasmid has possibly been passed around between strains (and across species barriers) with perhaps minor modifications arising.

TABLE 3. *Frequency of transfer between JH2-2 donors and JH2-2S*[a]

Donor	Frequency of TC resistance transferred per donor
DT-4	4.7×10^{-5}
DT-34	2.4×10^{-5}
DT-11	$<7.4 \times 10^{-8}$
DT-30	$<7.7 \times 10^{-8}$

[a] Strains listed in the table were mixed with JH2-2S cells as described in the text. After 2 h of mating, the mixture was plated on plates containing 10 μg of TC and 1,000 μg of SM per ml.

Some features of the DS-5 system are similar to certain plasmid systems in the gram-negative enteric bacilli. For example, a mobilizing factor, Δ, has been shown by Anderson and Lewis (1) to promote the transfer of small resistance plasmids in *Salmonella*. Biochemical analyses of these plasmids by Milliken and Clowes (15) have shown that the resistance plasmids are physically separate from the Δ factor. Another system with even more similarity to DS-5 has been described by van Embden and Cohen (18). A mobilizing factor was shown by these workers to promote the transfer of an independent TC plasmid in *Salmonella* and *Escherichia coli*. The fact that isogenic strains can be constructed which carry various combinations of α and/or γ plasmids allows for more detailed studies on the functions of these plasmids. One application has related to the study of the phenomenon of gene amplification of the TC determinant on the α plasmid (6a). Strains such as DT-11 (Fig. 1) are being utilized to study this process in detail, yielding interesting insights into its mechanism (6a; Yagi and Clewell, submitted for publication). We are also studying the γ plasmid more extensively, making use of strains having different combinations of hemolysin and bacteriocin markers. Preliminary studies indicate that nonhemolytic strains containing an "altered" γ plasmid, such as DT-9 and

DT-10 (Fig. 1), are also capable of mobilizing the α plasmid, indicating that the transfer function of these plasmids is still intact. Experiments are also in progress to test other *S. faecalis* strains as recipients, and these experiments may help to answer several intriguing questions such as: (i) will the γ plasmid mobilize plasmids other than α? (ii) Will the γ plasmid mobilize chromosomal genes? (iii) Can the β plasmid be transferred if a different recipient is used? Exploitation of the experimental potential offered by this system should shed some light on these questions.

In general, we feel that the newly revealed phenomenon of plasmid transfer in streptococci will be extremely useful in relation to studies of the nature of plasmids in this genus.

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