JOURNAL OF BACTERIOLOGY, Apr. 1985, p. 92–97
0021-9193/85/040092-06$02.00/0

Vol. 162, No. 1

Transformation and Fusion of *Streptococcus faecalis* Protoplasts

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Received 10 October 1984/Accepted 2 January 1985

Nonconjugative plasmids were transferred by protoplast fusion among *Streptococcus faecalis* strains and from *Streptococcus sanguis* to *S. faecalis*. *S. faecalis* protoplasts were also transformed with several different plasmids, including the Tn*917* delivery vehicle pTV1. Transformation was reproducible, but low in frequency (10^{-6} transformants per viable protoplast). A new shuttle vector (pAM610), able to replicate in *Escherichia coli* and *S. faecalis*, was constructed and transformed into *S. faecalis* protoplasts. pAM610 was mobilized by the conjugative plasmid pAMβ1 in matings among *S. faecalis* strains and from *S. sanguis* to *S. faecalis*. Chimeric derivatives of pAM610 were also transformed into *S. faecalis*.

Genetic studies in *Streptococcus faecalis* have been hampered by the lack of a reliable transformation system, despite efforts in this laboratory and elsewhere (6, 24). Since the first report (1) of the genetic transformation of *Streptomyces* sp. protoplasts in the presence of polyethylene glycol (PEG), similar procedures have been successfully applied to protoplasts of *Bacillus subtilis* (4) and many other species, including *Streptococcus lactis* (16). Transformation of *Streptococcus faecium* autoplasts has been reported (P. A. Carson and L. Daneo-Moore, Abstr. Annu. Meet. Am. Soc. Microbiol. 1983, H60, p. 60). Fusion of bacterial protoplasts in the presence of PEG was described originally for *B. subtilis* (25) and subsequently for many species, including *S. lactis* (9).

The generation and regeneration of protoplasts of *S. faecalis* were studied in detail by several groups (10, 11, 14, 22). The results vary from strain to strain, but up to 90% of the *S. faecalis* protoplasts regenerate on agar containing hypertonic concentrations of salt, sucrose, or a combination of the two (9, 11, 14). Initially, the colonies consist of L-phase cells, which lack a complete cell wall and are osmotically sensitive. Normal cells eventually appear, which have a complete cell wall and grow on media without osmotic support (10).

In this study, the transformation of *S. faecalis* protoplasts with plasmid DNA was low in frequency, but reproducible. Plasmids were also transferred by fusion of *S. faecalis* protoplasts to each other and to *Streptococcus sanguis* protoplasts. A new shuttle vector (pAM610) and three chimeric derivatives able to replicate in *Escherichia coli* and *S. faecalis* were introduced to *S. faecalis* by transformation. pAM610 was transferred by conjugation with the mobilizing plasmid pAMβ1, allowing pAM610 transfer to strains not as easily transformed. Finally, the Tn*917* delivery vehicle pTV1 (29) was introduced to *S. faecalis* by transformation. pTV1 was not stably inherited in *S. faecalis* and so may possibly be useful in *S. faecalis* for the selection of Tn*917* insertions into the chromosome.

MATERIALS AND METHODS

Bacteria and media. Most of the bacterial strains used are shown in Table 1. Several strains are not listed because they were simple derivatives [e.g., OG1-RF(pVA736)]. *S. faecalis* were grown with gentle shaking at 37°C in nutrient broth no. 2 (GIBCO Laboratories, Grand Island, N.Y.) supplemented with 0.2% glucose and buffered with 0.1 M Tris, pH 7.7 (N2GT), and plated on antibiotic medium no. 3 (Difco Laboratories, Detroit, Mich.) containing 1.5% agar (AB3). Blood plates contained 1% defibrinated horse blood in AB3 agar. *S. sanguis* were grown in Difco brain heart infusion and transformed by the method of Lawson and Gooder (17). *E. coli* cells were grown in L broth and transformed by the calcium chloride-heat shock procedure (21). Conjugative plasmids were transferred by 4-h filter matings in the presence of DNase as described previously (26) on brain heart infusion agar. When present in selective plates, antibiotics were used at the following concentrations: tetracycline, 8 μg/ml (*S. faecalis*), 4 μg/ml (*E. coli*), or 2 μg/ml (*S. sanguis*); erythromycin, 10 μg/ml; rifampin, 25 μg/ml; fusidic acid, 25 μg/ml; streptomycin, 1 mg/ml; spectinomycin, 0.5 mg/ml; kanamycin, 0.5 mg/ml (*S. faecalis* and *S. sanguis*) or 30 μg/ml (*E. coli*). PEG 6000 was from Matheson, Coleman & Bell, Northwood, Ohio. PEG 4000 and PEG 400 were from Baker. Spectinomycin was a gift from the Upjohn Co., Kalamazoo, Mich. Other reagents and their sources have been described previously (7, 12).

Isolation of plasmid DNA and gel electrophoresis. Plasmid DNA was isolated as described previously (27). Plasmid DNA was treated with restriction endonucleases from Bethesda Research Laboratories, Inc. (Rockville, Md.) with conditions suggested by the manufacturer. Electrophoresis was in 0.7% agarose gels as described previously (27). Gels were stained with ethidium bromide (1 μg/ml) and visualized with shortwave UV light. Lambda DNA was *c*I857*Sam*7 (Bethesda Research Laboratories).

Protoplast solutions. DM3 regeneration agar was described by Chang and Cohen (4). 2 × SMM was 1 M sucrose–40 mM maleic acid–40 mM $MgCl_2$–200 μg of bovine serum albumin per ml, pH 6.5 (4). 4 × AB3 was Difco antibiotic medium no. 3 made at 4 times normal strength (4). SMMP was equal volumes of 2 × SMM and 4 × AB3 (4). 2 × SMTB was 20 mM Tris-hydrochloride–40 mM $MgSO_4$–1.5 M sucrose, pH 7.6 (28). PEG solutions were in SMM (4). Donor DNA was prepared by mixing equal volumes of DNA (in 0.15 M NaCl, 0.015 M sodium citrate) and 2 × SMTB.

Generation of protoplasts. Overnight cultures in N2GT were diluted 50-fold into N2GT. Usually, 150 ml of cells was grown. After 60 min, sterile glycine was added to 0.2%, which had no effect on the growth rate. The cultures were grown to the midlog phase (reading of 30 on a Klett-Summerson colorimeter, no. 54 filter). The cells were harvested

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TABLE 1. Bacterial strains

Species	Strain	Description[a] and source
S. sanguis	V288	Transformable (18)
	V736	V288(pVA736) (18)
S. faecalis	JH2-2	JH2 Rif^r Fus^{rb}
	JH2-SS	JH2 Str^r Spc^{rb}
	JH203(pMV163)	JH2 derivative (2)[c]
	FA2-2	JH2 Rif^r Fus^{rb}
	FA1001(pAM602)	JH2 Rif^r Fus^r derivative (8, 27)
	OG1-RF	OG1 Rif^r Fus^r gelatinase (8)
	OG1-X	Str^r gelatinase-negative (12)
	OG1-10	Str^r OG1 gelatinase (12)
	OG1-11	OG1-10(pAMα1)gelatinase
E. coli	DH1	HsdR Rec (21)
	AB2497	pMK20 (15)[d]
	DH1(pVA838)	(19)
	DH1/pAM910	pMK20::pMV163 *PstI-EcoRI*[e]
	DH1/pAM920	pAM910::pAD1 *EcoA*[e]
	DH1/pAM925	pAM910::pAD1 *EcoF*[e]

[a] Drug resistances: Rif, rifampin; Fus, fusidic acid; Str, streptomycin; Spc, spectinomycin.
[b] Strain origins described in reference 26.
[c] Received from V. Burdett.
[d] Received from D. Helinski.
[e] Constructed by E. Ehrenfeld.

(all centrifugations were for 10 min at 4,400 × *g*), suspended in 6% of the original culture volume in 0.25 M EDTA–10% glycerol (pH 8.0), and frozen at −70°C in 2.5-ml samples. There were usually 2 × 10^{10} CFU in each frozen sample. *S. sanguis* cells were grown to the early log phase in brain heart infusion and harvested after 60 min in brain heart infusion containing 1% glycine (20).

Frozen cells were thawed, centrifuged, and suspended in 10 ml containing 1 mg of lysozyme per ml and 10 mg of bovine serum albumin per ml in 25% sucrose–50 mM Tris-hydrochloride–5 mM EDTA–50 mM NaCl (pH 8.0). After 60 min at 37°C, the protoplasts were centrifuged. The efficacy of the lysozyme treatment and protoplast viability were measured by plating dilutions of protoplast suspensions on DM3 regeneration plates and on AB3 plates. Usually, osmotically sensitive CFU (seen on DM3 plates) were in 10,000-fold excess over the CFU observed on AB3 plates. Most of these cells were probably protoplasts, since they gave rise to L-phase colonies on the DM3 plates (see below). Protoplasts were routinely observed in a phase-contrast microscope and usually constituted the vast majority of cells after lysozyme treatment.

Fusion. Protoplasts were suspended in 0.25 ml of SMTB; 50 μl of donors, 50 μl of recipients, and 5 μl of pancreatic DNaseI (5 mg/ml) were mixed. Then 1.5 ml 50% PEG 6000 was added, and the phases were mixed for 20 s. Samples were diluted in SMTB, and three serial 10-fold dilutions were plated on DM3 plates without drug. After 2 days at 37°C, the growth on each plate was suspended in 2 ml of SMTB–10% glycerol, and 0.1-ml samples were plated on AB3 plates containing the appropriate drugs. Colonies having fusion phenotypes were checked for donor chromosomal markers and streaked on bile esculin (Difco) and gelatin (12) plates to verify that they were *S. faecalis* with the correct gelatinase phenotype. No fusion phenotypes were observed when identical procedures were used in each experiment on mixtures containing only donors or only recipients.

Protoplast transformation. Protoplasts were washed with 10 ml of SMMP and suspended in 1 ml of SMMP. DNA (80 μl) was added to 0.1 ml of the protoplast suspension, and then 1.5 ml of 40% PEG 6000 was added quickly. After 2.5 min, 5 ml of SMMP was added, and the mixture was centrifuged for 15 min at 4,400 × *g*. The protoplasts were suspended in 1 ml of SMMP and incubated at 37°C for 4 h with mild shaking. Samples (0.1 and 0.01 ml) of the suspensions were plated on DM3 plates containing erythromycin or tetracycline. Transformants were tested for catalase, streaked on AB3 containing the appropriate drugs and on bile-esculin, gelatin (12), and horse blood plates to verify antibiotic resistance, ability to hydrolyze esculin, gelatinase phenotype, and blood hemolysis phenotype. Transformants often took 7 or more days to grow and often were L-phase colonies that could be subcultured only on DM3 plates. Upon subculture on DM3 plates, or on prolonged incubation on selective plates, L-phase colonies gave rise to normal cells, which were streaked onto an AB3 plate containing appropriate antibiotics. Every transformation experiment had a control in which no donor DNA was added to the recipients.

RESULTS

Generation of *S. faecalis* protoplasts. OG1-RF and FA2-2 cultures were grown in N2GT to the early log phase, and the cells were washed and incubated with various concentrations of lysozyme for various times. The results (data not shown) showed that for the generation of protoplasts the optimal concentration of lysozyme was 1 mg/ml and that the optimal time of incubation was 60 min. Generation of OG1 protoplasts was easier than the generation of FA2-2 or JH2-2 protoplasts (data not shown). The total CFU on DM3 after lysozyme treatment varied from 10 to 90% of that before treatment, in accord with previously published data (9, 14).

Growth from *S. faecalis* protoplasts on DM3 (regeneration) medium. When *S. faecalis* protoplast suspensions were plated on DM3 and AB3 plates, colonies were seen on both types of plates after 18 h of incubation at 37°C. These CFU evidently represented cells that retained some cell wall and were thus able to multiply quickly. After 2 days of incubation, small colonies arising from protoplasts became easily visible on the DM3 plates. Many of these colonies consisted

TABLE 2. Transfer of pAMα1 by protoplast fusion

Experiment	Mixture[a]	Rif^r Fus^r Tc^r CFU/plate[b]	
		Replica	Resuspension
IA	36% PEG 6000	20	1,500
IB	45% PEG 6000	15	15,000
IC	54% PEG 6000	24	3,000
IIA	36% PEG 4000	10	1,600
IIB	45% PEG 4000	5	9,100
IIC	54% PEG 4000	5	764
IIIA	36% PEG 400	0	3,400
IIIB	45% PEG 400	0	130,000
IIIC	54% PEG 400	6	4,200

[a] Donor (OG1-11) CFU before the addition of PEG were 1 × 10^{10}/ml on DM3 and 2 × 10^5/ml on AB3. Recipient (OG1-RF) CFU were 1 × 10^{10}/ml on DM3 and 4 × 10^5/ml on AB3. The average molecular weight and final percentage of PEG (weight/volume) in the fusion mixture are indicated.
[b] A 0.1-ml sample of a 100-fold dilution of the fusion mixture was plated on DM3 plates. The growth on the DM3 plates was replicated on agar containing rifampin, fusidic acid, and tetracycline or was suspended in 2 ml buffer, and 0.1 ml was plated on plates containing the drugs. Drug resistances: Rif, rifampin; Fus, fusidic acid; Tc, tetracycline.

TABLE 3. Viability of protoplasts and fusion results with cell cultures grown to different densities

Klettunits[a]	Cells	DM3 CFU/AB3 CFU		CFU/ml in suspensions from DM3 plates[d]	OG1-RF(pAMα1) CFU/ml in suspensions
		Before PEG[b]	After PEG[c]		
30	OG1-11	$3 \times 10^{10}/3 \times 10^{3}$	$2 \times 10^{8}/5 \times 10^{3}$	1×10^{10}	
30	OG1-RF	$2 \times 10^{10}/4 \times 10^{4}$	$1 \times 10^{9}/5 \times 10^{3}$	2×10^{10}	3×10^{6}
60	OG1-11	$4 \times 10^{11}/2 \times 10^{6}$	$5 \times 10^{9}/1 \times 10^{5}$	3×10^{9}	
60	OG1-RF	$1 \times 10^{11}/5 \times 10^{5}$	$4 \times 10^{8}/1 \times 10^{5}$	5×10^{9}	6×10^{4}
90	OG1-11	$2 \times 10^{10}/3 \times 10^{6}$	$7 \times 10^{8}/5 \times 10^{5}$	3×10^{10}	
90	OG1-RF	$3 \times 10^{10}/4 \times 10^{5}$	$5 \times 10^{8}/5 \times 10^{5}$	3×10^{9}	2×10^{4}

[a] Samples (100 ml) were removed from cell cultures and treated with lysozyme when the cell density reached the indicated Klett value.
[b] Samples were removed from the protoplast suspensions just before mixing of donors and recipients, diluted in SMTB, and spotted on AB3 and DM3 plates. Values were calculated to total CFU.
[c] Titers were determined as described in footnote *b* after the addition of PEG to mixtures containing only donors or only recipients.
[d] OG1-11 and OG1-RF protoplasts were mixed, and PEG was added. A 0.1-ml sample of a 100-fold dilution of each of the fusion mixtures was plated on DM3. After 2 days, the growth was suspended in SMTB and plated on AB3 plates containing streptomycin (counts of OG1-11 CFU); rifampin and fusidic acid (counts of OG1-RF CFU); or rifampin, fusidic acid, and tetracycline [counts of OG1-RF(pAMα1) CFU].

of L-phase bacteria that had not regenerated complete cell walls, as evidenced by cell morphology (phase-contrast microscopy), susceptibility to lysis upon suspension in AB3, and ability to be subcultured on DM3, but not AB3, plates. After prolonged incubation (3 to 5 days), most L-phase colonies were overtaken by cells that had regenerated their cell wall and were growing quickly. At this point, the colonies were normal looking (i.e., white), but contained a small, squarish, translucent central core representing the original L-phase colony. These results are in accord with published data on *S. faecalis* L-phase cells (10). The regeneration of normal cells from L-phase cells appeared to be slower in OG1-X backgrounds than in OG1-RF backgrounds. As has been reported (22), colonies arising from cells that retain some cell wall or whole cells of *S. faecalis* inhibited the formation of L-phase colonies nearby, placing an upper limit (dependent on the fraction of protoplasts in the culture) on the amount of a protoplast suspension that could be plated on a drug-free DM3 plate.

Fusion of *S. faecalis* protoplasts. Several protoplast fusion experiments were performed with OG1-11 [OG-10(pAMα1)] donors and OG1-RF recipients, in which the transfer of pAMα1 from donor to recipient was followed. Donors and recipients were treated with lysozyme, mixed, exposed to PEG, and plated on DM3 regeneration plates in the absence of drug. After 2 days of growth, the DM3 plates were covered with a lawn of colonies. OG1-RF(pAMα1) offspring were detected either by replica plating the DM3 lawn to selective plates or by suspending the lawn in medium and plating the suspension on selective plates (Table 2). Gel electrophoresis of plasmid-enriched lysates of several offspring showed that they contained pAMα1 (data not shown). The fusion results were not strongly dependent on the concentration or molecular weight of the PEG used (Table 2). More colonies were counted with the resuspension method, perhaps because a single offspring L-phase colony that regenerated cell walls early might yield many CFU upon resuspension (experiment III-B in Table 2). Recombination of chromosomal markers was below the level of detection (data not shown).

Transfer of pAMα1 by fusion was more efficient when the parental cells were harvested at the early log phase, rather than the middle or late log phase (Table 3). This experiment also demonstrates typical protoplast yields and viability after PEG treatment. The ratio of protoplasts to osmotically stable CFU was greater for the early log cells than for middle or late log cells (Table 3). The survival of CFU in the PEG ranged from 1 to 10% for protoplasts, but was better for those CFU able to grow on AB3 (Table 3).

Transfer of pAMα1 by fusion to JH2-2 and FA2-2 was undetectable with our standard conditions (data not shown), but when the fused protoplasts were plated on DM3 plates in 0.5% agar, the results were nearly equal for all recipients (Table 4). As demonstrated above (Table 3), these results may not accurately reflect fusion efficiency.

Fusion of *S. faecalis* and *S. sanguis* protoplasts. *S. sanguis* V288(pVA736) protoplasts did not regenerate well on DM3 (data not shown). However, in protoplast fusions,

TABLE 4. Protoplast fusion to JH2-2 and FA2-2 recipients

Recipient	CFU on DM3-AB3[a]	Rif^r Fus^r Tc^r CFU/ml[b]
JH2-2	$1 \times 10^{10}/9 \times 10^{9}$	10,000
FA2-2	$2 \times 10^{8}/2 \times 10^{7}$	10,000
OG1-RF	$1 \times 10^{10}/2 \times 10^{5}$	50,000

[a] Viable counts of recipients before adding donors, calculated to total CFU.
[b] Donor (OG1-11) and recipient protoplasts were fused with PEG 6000, and 0.1 ml of a 100-fold dilution of the protoplast mixture was plated in a 5-ml soft (0.5%) agar DM3 layer on DM3 plates. After 2 days of incubation, the plating layer was resuspended in 2 ml of medium and plated on plates containing rifampin, fusidic acid, and tetracycline. Drug resistances are as in footnote *b* of Table 2.

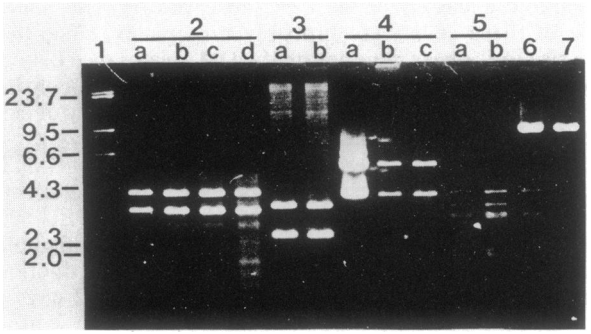

FIG. 1. Agarose gel electrophoresis of endonuclease-treated plasmid DNA. Sections: 1, lambda DNA cleaved with *Hin*dIII; 2, *Eco*RI-*Hin*dIII-cleaved pVA736 DNA from V736 (a), JH2-2(pVA736) (b), OG1-10(pVA736) (c), and OG1-RF(pVA736) (d); 3, *Ava*I-cleaved DNA from FA1001(pAM602) (a) and an OG1-RF(pAM602) transformant (b); 4, *Eco*RI-cleaved pAM610 from DH1(pVA610) (a), an OG1-RF(pAM610) transformant (b), and a V288(pAM610) transformant (c); 5, *Hin*dIII-cleaved pAMβ1 from JH2-2(pAMB1) (a) and an OG1-RF(pAMB1) transformant (b); 6, *Hin*dIII-cleaved DNA from transconjugant JH2-SS(pAMβ1, pAM610); 7, *Hin*dIII-cleaved DNA from DH1(pAM610).

TABLE 5. Transformation of *S. faecalis* protoplasts

Donor plasmid[a]	Size (megadaltons)	Reference	Selection	Transformants/ml[b]
pAM602	3.5	27	Erythromycin	45; 546
pAM603	3.5	27	Erythromycin	300
pAM604	4.4	27	Erythromycin	19; 65
pAM605	4.4	27	Erythromycin	1,200
pVA838	6.2	19	Erythromycin	12
pTV1	8.3	29	Erythromycin	140
pAMα1	6.6	7	Tetracycline	15; 127
pAM610	6.0		Tetracycline	13
pAM910	5.5		Tetracycline	20
pAM920	18		Tetracycline	10
pAM925	7.1		Tetracycline	25
pAMβ1, pAM610	17, 6.0	7	Erythromycin	74; 46; 118
			Tetracycline	110; 186; 30
no DNA			Erythromycin	<1
			Tetracycline	<1

[a] Donor preparations contained about 1 μg DNA by estimation from gel electrophoresis. pAM610 and pAM910 were from DH1 backgrounds. pTV1 was from a *B. subtilis* strain (29). All other plasmids were from *S. faecalis* backgrounds. One donor preparation was from JH2-SS(pAMβ1, pAM610).

[b] Transformation mixtures were suspended in 1 ml, which represented about 10^8 viable protoplasts. The results from different trials are separated by semicolons.

V288(pVA736) transferred pVA736 to JH2-2 (1 CFU/0.1-ml resuspension sample), OG1-10 (200 CFU/0.1-ml resuspension sample), and OG1-RF (10,000 CFU/0.1-ml resuspension sample) recipients. Plasmid DNA from offspring contained pVA736 DNA (Fig. 1, part 2).

Transformation of *S. faecalis* protoplasts. A reliable protocol for the transformation of *S. faecalis* protoplasts was established after several preliminary experiments (data not shown). Transformants were selected by plating on regeneration plates containing selective drug concentrations. Transformation frequency varied from $<10^{-8}$ to 10^{-5} per viable protoplast (from <1 to 2,000 transformants per ml of suspension). No transformants were detected in about 20% of the experiments (data not shown). Sample data taken from several experiments show results obtained when transforming with different plasmids (Table 5). Transformants contained the plasmids appropriate to their phenotype as determined by electrophoresis of endonuclease-treated plasmid DNA (Fig. 1 and 2). OG1-RF protoplasts were transformed by plasmid DNA isolated from JH2-SS(pAMβ1, pAM610). Roughly equal numbers of erythromycin-resistant (pAMβ1) and tetracycline-resistant (pAM610) transformants were observed in three separate trials (Table 5). No transformants to both erythromycin and tetracycline resistance were observed. Plasmid DNA from the OG1-X(pTV1) transformant contained the four *Ava*I fragments of pTV1 described by Youngman et al. (30) (Fig. 2). The shuttle plasmid pVA838, able to replicate in *E. coli* and streptococci (19), was transformed into OG1-RF protoplasts (Table 5), and transformants contained authentic pVA838 DNA (data not shown). Transformation of JH2-2 and FA2-2 protoplasts was below the level of detection (data not shown). Transformation was dependent on the concentration of donor DNA used (Fig. 3).

Construction of pAM610. *Streptococcus agalactiae* plasmid pMV163 confers resistance to tetracycline and is mobilized by broad-host-range plasmids between strains of *S. faecalis* (2). *E. coli* cloning vehicle pMK20 (15) is made up of two *Hae*II fragments from ColE1 and the *Hae*II fragment conferring kanamycin resistance from Tn*903*, originally found on plasmid R6 (23). pMV163 and pMK20 were linearized with *Eco*RI and ligated with T4 DNA ligase, and the mixture was used to transform *E. coli* strain DH1. Sixty tetracycline-resistant colonies were observed (13), and each was kanamycin resistant. Plasmid DNA (pAM610) from one such isolate was analyzed by gel electrophoresis (Fig. 2). The restriction map of pAM610 (Fig. 4) was deduced from published data (3, 15), and the *Pst*I digestions of pAM610 (not shown). pAM910 was derived from pAM610 by an *Eco*RI-*Pst*I digestion, followed by ligation of the two largest

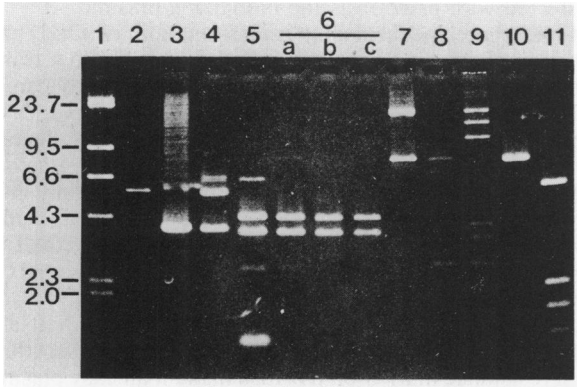

FIG. 2. Agarose gel electrophoresis of endonuclease-treated plasmid DNA. Lanes: 1, *Hin*dIII-cleaved lambda DNA; 2, *Eco*RI-cleaved pMV163; 3, *Eco*RI-cleaved pMK20; 4, *Eco*RI-cleaved pAM610; 5, *Eco*RI-*Pst*I-cleaved pAM610; 6, *Eco*RI-*Pst*I-cleaved pAM910 from DH1(pAM910) (a), an OG1-RF(pAM910) transformant (b), and a V288(pAM910) transformant (c); 7, *Eco*RI-cleaved pAM920 DNA from an OG1-X transformant; 8, *Eco*RI-cleaved pAM925 from an OG1-X transformant; 9, *Eco*RI-cleaved pAD1 from OG1-RF(pAD1); 10, *Eco*RI-cleaved pAM910 from DH1(pAM910); 11, *Ava*I-digested pTV1 from an OG1-X(pTV1) transformant.

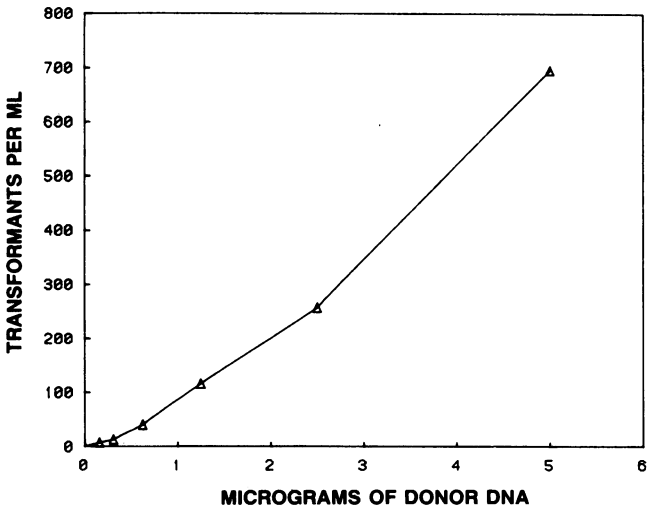

FIG. 3. Dependence of transformation on DNA concentration. Donor pAM910 DNA was isolated from DH1(pAM910), and concentration was estimated by absorbtion at 258 nm. Total transformants in the 1-ml resuspension (calculated from 0.3 ml plated) is plotted against total DNA added to the transformation mix (0.5 ml of cells plus 0.4 ml of DNA plus 1.5 ml of PEG). The addition of DNase to 5 μg of donor DNA before transformation resulted in <2 transformants per ml.

fragments and transformation of *E. coli* to tetracycline and kanamycin resistance (E. Ehrenfeld, personal communication).

The copy number of pAM910 in DH1(pAM910) cells growing in M9CY broth was determined from dye buoyant density gradients with tritiated thymidine (5). There were 18 and 95 copies per chromosome before and after 16 h of incubation with chloramphenicol, respectively.

Deleted forms of pAM610 and pAM910 were often observed in plasmid preparations isolated from *E. coli* cells (data not shown). These forms usually constituted a small fraction of the plasmid DNA.

Transformation of *S. sanguis* and *S. faecalis* with pAM610 and derivatives. *S. sanguis* strain V288 and *S. faecalis* strain OG1-RF were transformed with DNA from DH1(pAM610) and DH1(pAM910). In each case, tetracycline-resistant transformants were obtained which contained pAM910 or pAM610 DNA (Fig. 2). pAM610 was stable in both *S. sanguis* and *S. faecalis* since the tetracycline resistance marker was present in all 50 colonies examined even after four passes of the strains on drug-free agar. In contrast, after one passage of DH1(pAM610) in the absence of drug both kanamycin and tetracycline resistance markers were missing from about 10% of the CFU. To show that *S. faecalis* could be transformed with useful chimeras, *S. faecalis* strain OG1-X was transformed with pAM910 derivatives pAM920 and pAM925. The plasmids in the transformants were identical to those in the original *E. coli* host (Fig. 2).

Mobilization of pAM610. Conjugative plasmids pAMβ1 and pAD1 were transferred by filter mating to V288 and OG1-RF strains that contained pAM610 or pAM910. The resulting strains were used as donor to JH2-SS recipients in filter mating experiments. pAM610 was mobilized by pAMβ1, but not pAD1 (Table 6). Gel electrophoresis of plasmid-enriched lysates of transconjugants showed that they contained pAMβ1 and pAM610, not a cointegrate of the two (data not shown). pAM910 was not mobilized by either pAMβ1 or pAD1 (Table 6). pAM610 is transferable by filter mating to *S. lactis* strain 4125 from JH-2-22 or V288 strains that contain pAM610 and pAMβ1 (L. Pearce, personal communication).

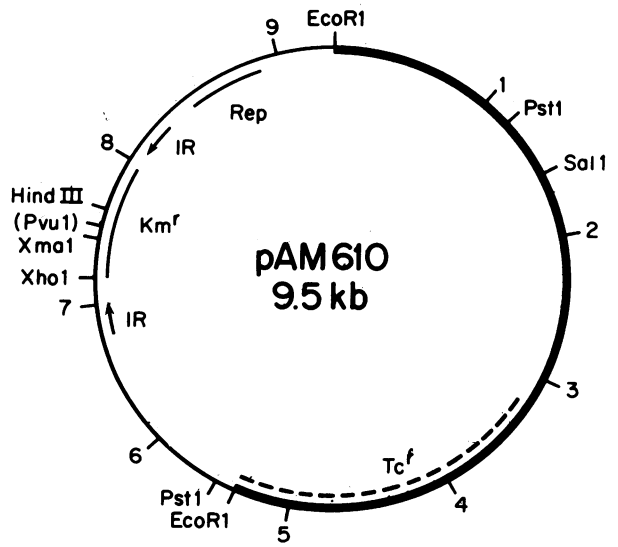

FIG. 4. Restriction map of pAM610 based on the published maps for pMK20 (15) and pMV163 (3). The streptococcal part of the plasmid is drawn with a heavy line. The replication region of ColE1 (Rep), kanamycin resistance gene (Km^r), and part of the IS*903* inverted repeat (IR) are indicated. The tetracycline resistance gene is within the area indicated (3). There is a second, unmapped *Pvu*I site in pAM610.

TABLE 6. Mobilization of pAM610 by pAMβ1

Donor	Transconjugants/donor[a]		
	Hem$^+$	Em^r	Tc^r
V288(pAMβ1, pAM610)		1×10^{-3}	2×10^{-7}
V288(pAM610)		$<10^{-9}$	$<10^{-9}$
OG1-RF(pAMβ1, pAM610)		5×10^{-4}	5×10^{-7}
OG1-RF(pAD1, pAM610)	0.2		$<10^{-9}$
OG1-RF(pAM610)		$<10^{-9}$	$<10^{-9}$
OG1-RF(pAMβ1, pAM910)		4×10^{-4}	$<10^{-9}$
OG1-RF(pAD1, pAM910)	0.04		$<10^{-9}$

[a] Filter matings between the indicated donor and JH2-SS recipients were plated on agar containing streptomycin plus spectinomycin and either erythromycin or tetracycline. All tetracycline-resistant (Tc^r) transconjugants were also erythromycin resistant (Em^r) when further tested. Hem$^+$ transconjugants (pAD1 transfer) were detected by plating mating mixtures on plates containing streptomycin plus spectinomycin and overlaying with 5 ml of agar containing 1% horse blood. No transconjugant phenotypes were observed when donors and recipients were plated separately, and transconjugants lacked resistance to rifampin and fusidic acid.

DISCUSSION

There are now three pathways to transfer chimeric DNA to *S. faecalis* backgrounds. All three may use shuttle plasmids that are able to replicate in *E. coli* and *S. faecalis*. (i) Shuttle plasmids pVA838 and pAM610 from *E. coli* were transformed into OG1 protoplasts (this study). (ii) Cloning vector pVA736 (not a shuttle plasmid) was transferred from *S. sanguis* to *S. faecalis* by protoplast fusion in this study. This mechanism may be extended to the transfer of shuttle plasmids that cannot be mobilized (such as pAM910). Fusion of *E. coli* and *S. faecalis* protoplasts was attempted, but without success (data not shown). (iii) Shuttle plasmids may be transformed into *S. sanguis* and then mobilized from *S. sanguis* to *S. faecalis* with a conjugative plasmid. This strategy was used first with pVA838 and derivatives with the mobilizing plasmid pVA797 (27). In this study pAM610 was transferred with pAMβ1 as the mobilizing plasmid.

Of the three mechanisms, transformation of OG1 protoplasts is the most direct, but is limited by the low transformation frequency. The methods used in this study can be used to transform OG1 derivatives with a plasmid DNA preparation, but may not be suitable for using ligation mixtures as donor DNA unless the transformation frequency is improved. Transformation is also limited in that only OG1 derivatives have been transformed successfully in this study, but protoplast fusion or conjugative mobilization could be employed to transfer the plasmids from OG1 backgrounds to other *S. faecalis* strains.

Shuttle vectors pVA838 and pAM610 are both useful plasmids with several complementary features. The erythromycin resistance marker on pVA838 make it an easy plasmid to select for in most backgrounds (19), whereas the tetracycline resistance conferred by pAM610 can sometimes be difficult to select because of the low level of resistance conferred. On the other hand, transfer of pVA838 by conjugative mobilization requires cointegrate formation with pVA797 (27), whereas pAM610 can be mobilized by pAMβ1 and probably by other similar plasmids (2). The cloning sites available on pVA838 (*Eco*RI, *Sph*I, *Bam*HI, *Sal*I, *Nru*I, and *Xba*I [19]) are complemented by the cloning sites on pAM610 (*Hin*dIII, *Xma*I, *Xho*I).

ACKNOWLEDGMENTS

This work was supported by Public Health Service grants DE2731 and AI10318 from the National Institutes of Health to Don Clewell, in whose laboratory these experiments were carried out. M.D.S. was a recipient of Postdoctoral Fellowship PF-2065 from the American Cancer Society.

Protoplast fusion work was done in association with E. Ehrenfeld. The studies were aided considerably by P. Patee, who provided a detailed protocol for protoplast transformation and fusion in *S. aureus*. I thank Y. Yagi for unpublished results, F. Macrina for the *S. sanguis* transformation protocol, E. Ehrenfeld, D. Clewell, V. Burdett, and L. Pearce for many helpful suggestions and for sharing unpublished data and strains, D. Helinski for pMK20, P. Youngman for pTV1, and B. White, J. Shaw, and C. Gawron-Burke for helpful discussions. I also thank A. Vidaver and D. Clewell for critical reading of the manuscript and L. Burdette for typing it.

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