Highly Efficient Protoplast Transformation System for *Streptococcus faecalis* and a New *Escherichia coli*-*S. faecalis* Shuttle Vector

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A highly efficient protoplast transformation system for *Streptococcus faecalis* has been developed by systematically optimizing different parameters. Up to 10^6 transformants per μg of DNA were consistently obtained within 3 days, and cell wall regeneration of protoplasts was virtually 100%. A systematic search for useful vectors showed that the broad-host-range plasmid pIP501 could transform *S. faecalis* at a high frequency (6.3×10^4 transformants per μg). By combining a high-copy-number derivative of pIP501, designated pGB354, with the *Escherichia coli* vector pACYC184, we constructed a new *E. coli*-*S. faecalis* shuttle vector (pAM401) having nine unique restriction sites. In a shotgun cloning experiment, we ligated a tetracycline resistance determinant from *Streptococcus sanguis* chromosomal DNA into pAM401 by direct transformation of *S. faecalis*, establishing the utility of the protoplast transformation system and of the new shuttle vector.

Genetic analyses of *Streptococcus faecalis* are largely impaired by the lack of an efficient transformation system. While conjugation of plasmids is common in *S. faecalis* (5, 7), neither transduction nor naturally occurring transformation has been previously described. Since protoplasts of *S. faecalis* could be reversibly generated by various groups (15, 18, 23), attempts were undertaken in this laboratory to develop a protoplast transformation system in this species. A recent report by Smith (29) showed that protoplast transformation could be achieved; however, that system worked only at a low frequency and, in our hands, was often difficult to reproduce. The primary objective of the work presented here was to optimize a protoplast transformation system for *S. faecalis* to a point where its efficiency was high enough that genetic manipulations such as shotgun cloning of chromosomal DNA would be possible.

By optimizing various parameters (e.g., growth medium, transformation medium, types of plasmids used, etc.), we have developed a system which reproducibly results in up to 10^6 regenerated transformants per μg of DNA within 48 h.

In addition we constructed a new *Escherichia coli*-*S. faecalis* shuttle vector, pAM401, which has nine unique restriction sites. By direct transformation of *S. faecalis* protoplasts, a chromosomal tetracycline resistance determinant from *Streptococcus sanguis* was cloned into pAM401, thus demonstrating the general utility of this system.

MATERIALS AND METHODS

Bacterial strains and plasmids. The bacterial strains and plasmids used in this study are listed in Table 1.

Media. Unless otherwise noted, the growth medium for *E. coli* was L broth (26); for *S. sanguis*, *S. faecalis*, and *Bacillus subtilis*, we used brain heart infusion, Todd-Hewitt broth (THB), and Pennassay broth (all from Difco Laboratories), respectively. Other media tested were DM3 (4), nutrient broth no. 2 (Oxoid Ltd.), and methionine assay medium (Difco). When present, antibiotics were added at the following final concentrations (in micrograms per milliliter):

tetracycline, 10; chloramphenicol, 20; erythromycin, 20; and kanamycin was used at 50 $\mu\text{g}/\text{ml}$ for *E. coli* and 500 $\mu\text{g}/\text{ml}$ for *S. faecalis*. The concentrations of antibiotics used for selection in protoplast transformation experiments are given in Table 2.

DNA procedures. Plasmid DNA was isolated with two isopycnic ethidium bromide-CsCl gradient centrifugations by published methods for *E. coli* (26), *S. sanguis*, and *S. faecalis* (12). DNA restriction cleavages, ligations, and filling in of sticky ends were performed as suggested by the manufacturers (New England BioLabs, Inc., Bethesda Research Laboratories, Inc., and Boehringer Mannheim Biochemicals) of the corresponding enzymes.

For the rapid preparation of small amounts of plasmid DNA (susceptible to restriction enzymes), the method of Ish-Horowicz and Burke (20) was modified as follows. A 1.5-ml sample of cells from a fresh overnight culture of *E. coli* was pelleted and suspended in 100 μl of a 2% glucose-50 mM Tris hydrochloride (pH 8)-25 mM EDTA solution and incubated for 5 min at room temperature. After the addition and mixing of 200 μl of freshly prepared 1% sodium dodecyl sulfate in 0.2 N NaOH, the 1.5-ml centrifuge vial was incubated for 5 min in an ice bath, and 150 μl of a 30% potassium acetate solution (pH 5) was added. The contents were mixed (by inverting the vial five times) and incubated again for 5 min at 0°C. After a 5-min centrifugation, the clear supernatant was transferred to another vial. One milliliter of 95% ethanol was then added, and the precipitation of nucleic acids was facilitated by a 30-min incubation at -70°C. Nucleic acids were pelleted by a 5-min centrifugation at room temperature, dissolved in 100 μl of 125 mM Tris-300 mM sodium acetate (pH 8), and reprecipitated by the addition of 200 μl of 95% ethanol. The pellet of a final 5-min centrifugation at room temperature was dissolved in 25 μl of water; 5- μl samples were used for restriction analysis. For *S. faecalis*, the procedure was altered in that 5-ml (rather than 1.5-ml) samples from fresh overnight cultures were used and incubated for 30 min at 37°C in 100 μl of 2% glucose-50 mM Tris hydrochloride-25 mM EDTA (pH 8) containing 0.3 mg of lysozyme. Further treatment was as for *E. coli*.

Optimization of protoplasting conditions. To obtain 10 samples of cells showing identical behavior in transforma-

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TABLE 1. Strains and plasmids used in this study

Species, strain, and plasmid	Host genotype	Plasmid markers	Reference
<i>S. faecalis</i>			
OG1RF	<i>rif fus</i>		9
OG1X	<i>str gel</i> ^a		19
JH2-2	<i>rif fus</i>	<i>erm cat</i>	21
JH2-2(pIP501)	<i>rif fus</i>		25, 28
UV202	<i>rif fus rec</i>		30
FA2-2	<i>rif fus</i>		6
<i>S. sanguis</i>			
FC1	<i>tet</i> (Tn919)		11
<i>E. coli</i>			
DH1	F ⁻ <i>recA1 endA1 gyrA96 thi-1 hsdR17 supE44</i> λ ⁻		26
DH1(pSA3)	F ⁻ <i>recA1 endA1 gyrA96 thi-1 hsdR17 supE44</i> λ ⁻	<i>erm tet cat</i>	8
DH1(pAM910)	F ⁻ <i>recA1 endA1 gyrA96 thi-1 hsdR17 supE44</i> λ ⁻	<i>tet kan</i>	29
C600(pACYC184)	F ⁻ <i>thi-1 thr-1 leuB6 lacY1 tonA21 supE44</i> λ ⁻	<i>tet cat</i>	3, 12
DB11(pVA838)	F ⁻ <i>met thi gal nal rif hsdR</i> Em ^s	<i>erm cat</i>	24, J. Davies
<i>B. subtilis</i>			
BR151(pGB354)	<i>met trp lys</i>	<i>cat</i>	1; D. Behnke

^a *gel*, Gelatinase negative.

tion experiments, 500 ml of THB containing 2% glycine for strain OG1X (or isogenic equivalents) but 4% glycine for strain FA2-2 (or isogenic equivalents) (see Results) was inoculated with 0.5 ml of a full-grown overnight culture in the same medium. Cells were grown with gentle shaking to early log phase (a reading of 20 to 30 on a Klett-Summerson colorimeter, no. 54 filter), collected by centrifugation, suspended in 5 ml of THB plus 5 ml of glycerol, and stored frozen at -70°C in 1-ml samples. Since different batches of cells varied with regard to their susceptibility to the protoplasting agent (normally lysozyme), each batch of cells was tested for optimal time of lysozyme treatment. For this purpose, a 50-ml equivalent of the original culture (i.e., one of the 1-ml samples stored at -70°C) was thawed at 37°C, and the cells were collected by a 10-min centrifugation at 3,000 × g and suspended in 10 ml of TSCM buffer (TSCM is 100 mM Tris hydrochloride [pH 8], 500 mM sodium succinate [Mallinckrodt, Inc.], 40 mM CaCl₂, and 10 mM MgCl₂) containing 10 mg of bovine serum albumin (BSA;

U.S. Biochemical Corp.) and 1 mg of lysozyme (Sigma Chemical Co.) per ml. This suspension was incubated at 37°C; 100-μl samples were withdrawn every 30 min over a 3-h period and diluted up to 10⁻⁶ in TSCM buffer containing BSA at 100 μg/ml. Samples (10 μl) of those dilutions then were spotted onto protoplast agar plates (THB plates with 0.5 M sodium succinate, 100 μg of BSA per ml, and 0.8% agar).

After 24 h of incubation, the more rapidly arising colonies deriving from nonprotoplasted cells were marked and counted; after an additional 24 h of incubation, the small protoplast colonies were counted. Protoplast colonies were identified as having a small translucent center if illuminated from below. A time point which resulted in at least a 99.9% efficiency in protoplasting and more than 90% survival of protoplast-forming units, compared with the zero-time sample, was chosen as optimal for further experiments. This procedure generally involved a lysozyme exposure in the range of 60 to 120 min.

RESULTS

The optimized procedure for protoplast transformation of *S. faecalis* OG1X or FA2-2 is presented below. Factors found to be critical for this procedure are presented thereafter, followed by information demonstrating the usefulness of the procedure for shotgun cloning experiments.

Protocol for protoplast transformation experiments. Cells of one 50-ml culture equivalent (in 1 ml; see Materials and Methods) were protoplasted at 37°C (for the time found to be optimal, i.e., generally for 60 to 120 min; see Materials and Methods) in 10 ml of TSCM buffer containing lysozyme (1 mg/ml) and BSA (10 mg/ml). Protoplasts were collected by a 10-min centrifugation at 3,000 × g, washed once in 10 ml of TSCM buffer containing 100 μg of BSA per ml, and suspended by shaking (do not vortex) in 1 ml of the same buffer. For transformation, a 100-μl sample of the protoplasts was mixed with up to 100 μl of DNA in 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) (26), and the suspension was mixed into 1.8 ml of filter-sterilized 40% polyethylene glycol (molecular weight, 1,000; Sigma) in TSCM buffer. (For cloning experiments, the 100-μl sample of DNA

TABLE 2. Plasmid transformation of *S. faecalis* protoplasts

Plasmid	Source ^a	Size (kb)	Transformed <i>S. faecalis</i> strain	Selection by ^b (μg/ml):	Transformants/μg of DNA
pAM910	<i>S. faecalis</i>	8.7	OG1RF	Tc, 8	6.4 × 10 ³
pAM910	<i>S. faecalis</i>	8.7	UV202	Tc, 8	5.9 × 10 ²
pAM910	<i>S. faecalis</i>	8.7	JH2-2	Tc, 8	1.7 × 10 ³
pVA838	<i>E. coli</i>	9.2	OG1X	Em, 5	1 × 10 ⁴
pIP501	<i>S. faecalis</i>	30.0	OG1X	Cm, 5	6.3 × 10 ⁴
pSA3	<i>E. coli</i>	10.2	OG1X	Em, 5	2.1 × 10 ²
pGB354	<i>B. subtilis</i>	6.3	OG1RF	Cm, 5	6.1 × 10 ⁵
pGB354	<i>S. faecalis</i>	6.3	OG1RF	Cm, 5	8.9 × 10 ⁵
pAM401	<i>E. coli</i>	10.4	OG1X	Cm, 5	9.8 × 10 ⁵
pAM401	<i>S. faecalis</i>	10.4	OG1X	Cm, 5	1.4 × 10 ⁶
pAM401	<i>E. coli</i>	10.4	OG1X	Cm, 5	8.2 × 10 ²
pAM401	<i>S. faecalis</i>	10.4	OG1X	Cm, 5	1 × 10 ³

^a Plasmid DNA was isolated by isopycnic ethidium bromide-CsCl gradient centrifugation. The last two lines give results for plasmids prepared by the modified Ish-Horowitz and Burke procedure (20).

^b Tc, Tetracycline; Em, erythromycin; Cm, chloramphenicol.

added to the protoplasts contained about 5 μg of vector plus 5 μg of ligated chromosomal DNA.) Mixing of protoplasts, DNA, and polyethylene glycol was done by inverting the tube five times; this tube was incubated for 3 min at 0°C and then incubated for 5 min at 37°C. The mixture was then added to a centrifuge tube containing 10 ml of TSCM buffer (again containing 100 μg of BSA per ml). Protoplasts recovered therefrom by a 10-min centrifugation at $12,000 \times g$ were washed once in 10 ml of THB medium containing sodium succinate (135 mg/ml) and BSA (100 μg /ml). The protoplasts were suspended in 1 ml of the same medium (i.e., THB-sodium succinate-BSA) and incubated for 90 to 120 min at 37°C for phenotypic expression of transforming DNA. Finally, 20- to 100- μl samples were plated onto selective THB agar plates (containing, per liter, 8 g of agar, 135 g of sodium succinate, and 100 mg of BSA). After 2 to 3 days of incubation at 37°C, virtually all transformed protoplasts regenerated cell walls and gave rise to colonies.

Growth conditions of strains to be protoplasted. In the initial stage of this study, various growth media for the strains to be protoplasted were tested; these media included THB, brain heart infusion, Penassay broth, methionine assay medium, nutrient broth no. 2, and DM3. THB was found to be superior to all other media tested for two reasons: (i) it best supported the growth of several strains of *S. faecalis* with respect to generation time and cell yield, and (ii) regeneration of protoplasts to cell wall-containing cells was virtually 100% on THB-based agar plates. All other media gave less than 30% regeneration. Colonies arose within 2 days (compared with 8 to 10 days with DM3).

Further studies showed that the efficiency of protoplasting various strains of *S. faecalis* was affected significantly by two factors: (i) only cells harvested during early-log-phase growth could be protoplasted with more than 99% efficiency, and (ii) the addition of slightly inhibiting concentrations of glycine to the growth medium greatly facilitated the protoplasting of *S. faecalis*, but the optimum amount varied from strain to strain. For example, for OG1 derivatives, a glycine concentration of 2% was sufficient, whereas up to 4% glycine had to be used for strain FA2-2. Suitable glycine concentrations were those resulting in an optical density value for a fully grown overnight culture which was about 75% that of a culture grown without glycine.

Optimization of protoplasting efficiency. An important observation made here and previously (27, 29) is that the growth of *S. faecalis* protoplasts is inhibited in the presence of nonprotoplasted cells (i.e., rapidly arising colonies not derived from true protoplasts). Our results, based on plating of various dilutions of cells, indicate this inhibiting agent to be a substance excreted by nonprotoplasted cells. It therefore proved to be important that protoplasts be transformed very efficiently to overcome this effect. Indeed, only preparations of cells protoplasted to at least 99.9% showed a sufficiently high number of recoverable transformants after plating. Experiments in which more than 10^4 nonprotoplasted cells were plated per selective plate resulted in the recovery of no transformants. The potency of the inhibiting substance excreted by nonprotoplasted cells was quite high, since growth of protoplast colonies was inhibited within a 1-cm radius around colonies of nonprotoplasted cells.

An overlong treatment of cells with the protoplasting agent was found not to be desirable either, because the ability of protoplasted cells to regenerate cell walls decreased with the length of this treatment (data not shown). In our hands, the best results were obtained if the protoplasting

FIG. 1. Effect of polyethylene glycol (PEG; molecular weight, 1,000) concentration on efficiency of transformation of *S. faecalis* OG1X protoplasts with pGB354.

treatment gave more than 99.9% conversion of cells to protoplasts and the decrease in the viable count was less than 10%.

Protoplasting agent. Various experiments (data not shown) indicated the following. (i) Mutanolysin might be used as a protoplasting agent; it was found, however, that various batches of a commercial source varied greatly in activity and sometimes contained small amounts of DNase activity. (ii) The use of lysozyme gave highly reproducible results, and loss of viable counts was much lower than with mutanolysin. (iii) As a first approximation, we recommend a 90-min treatment of a 50-ml culture equivalent of OG1X cells with 10 mg of lysozyme and 100 mg of BSA in 10 ml of TSCM buffer; for FA2-2, a 180-min treatment with 40 mg of lysozyme gave good results. (iv) Thawing cells which were stored at -70°C at 37°C makes them more susceptible to lysozyme than thawing on ice.

Use of polyethylene glycol. The data for optimizing the system with respect to treatment with polyethylene glycol are summarized as follows. (i) Polyethylene glycol preparations with molecular weights of 1,000 or 6,000 gave very similar results; for ease of handling, we recommend the use of polyethylene glycol 1,000. (ii) Polyethylene glycol solutions must be sterilized by filtration; heat sterilization should be avoided. A 10-min treatment of protoplasts with filter-sterilized polyethylene glycol resulted in 90% killing, whereas only 1% of protoplasts survived the same treatment with autoclaved polyethylene glycol. (iii) The optimal concentration of polyethylene glycol used in *S. faecalis* protoplast transformation experiments is 40% (Fig. 1); a 25% solution was about 10-fold less efficient. A few transformants (no. 1 to 10) arose in the absence of any polyethylene glycol. (iv) Since treatment of protoplasts with a 7.5% polyethylene glycol solution results in no measurable loss of viability, treatment with polyethylene glycol for transformation was stopped by the addition of 5 volumes of TSCM buffer.

Handling and plating of protoplasts. Under the conditions used in this study, protoplasts proved to be quite stable; the survival rate during all centrifugation steps was essentially 100%. It was found, however, that protoplasts should be

FIG. 2. Effect of DNA concentration on transformation of *S. faecalis* OG1RF protoplasts.

suspended gently, since 95% were killed by vortexing them for 2 min.

Protoplasts grown in THB broth supplemented with 0.5 M sodium succinate had an optical density doubling time about 1.5-fold longer than that of nonprotoplasted cells; 90 to 120 min of pregrowth in this medium without antibiotics was found to be sufficient for phenotypic expression of transforming DNA.

Since the inhibiting effect of nonprotoplasted cells on growth of protoplast transformants is observed even on selective plates, it was found to be important not to plate more than a 100- μ l sample per plate.

Plasmids used for transformation. The transformation system was optimized (as described above) with pGB354, a high-copy-number derivative of the broad-host-range streptococcal plasmid pIP501 (2). Under the above-defined optimal conditions, various plasmids were tested for their ability to transform protoplasts. The results are summarized in Table 2. The *E. coli*-streptococcal shuttle vectors pAM910, pVA838, and pSA3, as well as pIP501 and pGB354, are all seen to transform with different efficiencies. In all cases, rapid-preparation screening of at least 10 transformants, with the aid of an appropriate restriction enzyme, confirmed that the plasmids maintained their original molecular weights. For a given plasmid, preparations from *E. coli*, *S. faecalis*, or *B. subtilis* transformed with similar efficiencies; this result suggests that a significant restriction barrier does not exist between these strains. Concentrations of erythromycin and tetracycline in the selective osmotic stabilizing agar had to be carefully controlled. Due to the fact that high numbers of cells (10^8 protoplasts per plate) and 2 to 3 days of incubation are required in the system, problems with background growth of nontransformed protoplasts might arise if concentrations of antibiotics given in Table 2 are not used. This possibility is especially true for tetracycline. Since we never observed any background growth on chloramphenicol-containing plates, we prefer this type of selection in transformation experiments.

The dependence of protoplast transformation on plasmid DNA concentration was examined; a linear dependence was found for a range between 10 and 500 ng of plasmid pGB354 per ml of transformation mixture (Fig. 2). The observation that transformation was not linear for DNA amounts higher than 500 ng was made repeatedly. It is not known if this is due to contaminating inhibitors in the plasmid preparations.

By using 150 ng of pGB354 and 1/10 to 10 times the amount

of protoplasts used in the previous experiment, only a fourfold difference in number of transformants was observed (data not shown). This result was taken to mean that protoplast transformation is primarily dependent on the DNA concentration. When pGB354 was linearized by exposure to an appropriate restriction enzyme, the efficiency of transformation was reduced by 4 orders of magnitude compared with that for uncleaved (circular) molecules (data not shown).

Making use of the *Bcl*I site of pGB354, a shotgun cloning experiment was performed with *Bcl*I-cleaved chromosomal DNA of *S. faecalis*. After protoplast transformation in OG1RF, the rapid preparation for plasmids described above was used to screen for clones with inserts in pGB354. About 40% of randomly selected transformants were found to have inserts. Eight of those transformants, with inserts in the size range of 1 to 12 kilobases (kb), were grown for approximately 100 generations in the presence of chloramphenicol; the *Bcl*I and *Hind*III restriction patterns of the resident plasmids were unchanged compared with those of the original isolates, implying that the integrity of the original chimeras was maintained.

Construction of pAM401. The shuttle vector pAM401 was constructed as follows. The *E. coli* vector pACYC184 (3) (encoding resistance to tetracycline and chloramphenicol) was digested with *Ava*I, and the streptococcal plasmid pGB354 (2) (encoding chloramphenicol resistance) was digested with *Sph*I (in each case, a single restriction site was present). Equimolar amounts of these linearized plasmids were mixed, treated with T4 DNA polymerase to generate blunt ends, and ligated with T4 DNA ligase. The resulting mixture was transformed into *S. faecalis* OG1X protoplasts selecting for chloramphenicol resistance. Plasmid DNA representing a mixture of transformants was isolated by the rapid-preparation method (see Materials and Methods) and used to transform *E. coli* DH1, selection being with chloramphenicol and tetracycline. Plasmid DNA (isolated by the rapid-preparation method) of a mixture of those transformants was used for protoplast transformation in *S. faecalis* OG1X (again selecting with chloramphenicol), and that cycle was repeated three times. Plasmid DNA of one of the final transformants was isolated by two isopycnic ethidium bromide-CsCl gradient centrifugations and characterized by restriction analysis.

As expected, this plasmid, called pAM401 (Fig. 3), had lost the *Ava*I and *Sph*I sites used in construction. The relative orientation of the two parent plasmids was based on the *Hind*III fragments being 6.4 and 4 kb in size (data not shown). (Among eight additional clones tested, we found no shuttle vector having the opposite relative orientation.) Furthermore, we observed by restriction analyses (data not shown) that pAM401 has two *Bcl*I sites and one *Bst*EII site, which is located within the pGB354-derived chloramphenicol resistance determinant (1). pAM401 contains the following unique restriction sites (all within the gram-negative replicon of the shuttle vector): *Eco*RI and *Nco*I in the chloramphenicol resistance determinant and *Eco*RV, *Bam*HI, *Sph*I, *Sal*I, and *Nru*I in the tetracycline resistance determinant. Whereas cloning into all these sites can be screened for in *E. coli* by insertional inactivation, the unique *Xba*I site can be used for forced cloning in conjunction with any unique site in the *tet* gene.

Experiments in which the stability of pAM401 in both hosts was tested showed that the plasmid was more stable in *E. coli* DH1 than in *S. faecalis* OG1X. For those experiments, the respective strains were grown without antibiotic

FIG. 3. Map of the shuttle vector pAM401. A/S indicates the location of the *Ava*I and *Sph*I sites used for joining pACYC184 and pGB354. The dark line represents the segment derived from pACYC184 (with restriction sites around the *tet* gene, drawn enlarged for clarity); the light line represents pGB354-derived DNA. Locations for replication region (*rep*), copy control region (*cop*), and chloramphenicol resistance (*cat*) for pGB354 were taken from Behnke and Gilmore (1). Restriction sites and locations of *tet* and *cat* in pACYC184 were as described in the Boehringer Mannheim catalog "Biochemicals for Molecular Biology 1985."

selection for 30 generations in THB and then plated on nonselective THB agar. The fraction of chloramphenicol-resistant strains after unselected growth in THB was determined by streaking 100 of those colonies in parallel on drug-free and chloramphenicol-containing THB agar plates. The percentage of chloramphenicol-resistant strains obtained was 73% for DH1(pAM401) and 16% for OG1X(pAM401). In similar experiments, we observed values of 76% for OG1X(pGB354) and 100% for OG1X(pIP501). Because of the instability, strains carrying pAM401 or its derivatives were always grown under selective pressure.

To examine the utility of pAM401 and the optimized transformation system for direct shotgun cloning in *S. faecalis*, we attempted to clone the chromosome-borne tetracycline resistance determinant of *S. sanguis* FC1 (the resistance determinant is actually located on the conjugative transposon Tn919, which is homologous with Tn916 [11]). Chromosomal DNA of *S. sanguis* FC1 was digested with the restriction endonuclease *Hinc*II and ligated with *Eco*RV-treated pAM401. After transformation of *S. faecalis* OG1X protoplasts selecting with chloramphenicol and tetracycline, we were able to isolate a clone harboring the tetracycline resistance determinant. Restriction enzyme analyses and Southern hybridization experiments (making use of homologous regions of Tn916 as a probe) showed the resultant plasmid, designated pAM409, to contain a 5.3-kb insert which contains the *tet* determinant in accordance with published data (11; C. Gawron-Burke, R. Wirth, M. Yamamoto, S. Flannagan, G. Fitzgerald, F. An, and D. Clewell, in S. Hamada, S. Mikalek, H. Kiyono, L. Menaker, and J. McGlee (ed.), *Proceedings of the International Conference on Cellular, Molecular and Clinical Aspects of Streptococcus mutans*, in press) (data not shown).

DISCUSSION

Smith (29) showed that protoplast transformation with *S. faecalis* could be accomplished, but with low efficiency.

Whereas he obtained less than 100 transformants per μ g of DNA with pAM910 DNA, our optimized system resulted in between 10^3 and 10^4 transformants per μ g with the same plasmid and *S. faecalis* strain. Since the previously described system used 4 h of pregrowth of protoplasts under nonselective conditions compared with 90 min in the optimized system, the actual increase in efficiency of transformation might be even higher (i.e., the optimized system minimized the multiplication of sibling transformants). We cannot attribute the higher efficiency of the optimized system to the alteration of one specific parameter, but found several modifications to be important.

In accordance with a *B. subtilis* protoplast transformation system (4), the original *S. faecalis* method used two growth media and three buffers for the storage of cells, the generation of protoplasts, and the washing of protoplasts. Since we felt that reduction of physiological stress to cells might result in higher efficiency of transformation, we used only one growth medium and one type of buffer. (Composition of the buffer used here was taken from Katsumata et al. [22].) A systematic search for a growth medium revealed THB to be optimal. Regeneration of protoplasts to cell wall-containing cells was relatively rapid and virtually 100%.

The system described here used higher concentrations of glycine in the growth medium, compared with the previous system (up to 4% for FA2-2 compared with 0.2%). These concentrations made the cells much more susceptible to lysozyme and reduced the treatment time needed to generate protoplasts, thus probably reducing physiological stress on the cells.

The fact that filter-sterilized polyethylene glycol inhibits *S. faecalis* protoplasts much less than autoclaved preparations clearly contributes to the higher survival rate obtained here. The chemical nature of substances toxic to protoplasts produced during autoclaving of polyethylene glycol solutions is unknown; however, it is noteworthy that a similar effect has been reported by Yaokum (31) in connection with protoplast generation involving *E. coli*.

Derivatives of the conjugative broad-host-range plasmid pIP501 (17) offer significant versatility for use in *S. faecalis* protoplast transformation. Transformation with pIP501 was found to be relatively efficient (6.3×10^4 transformants per μ g of DNA; Table 2), indicating that plasmids at least 30 kb in size can be successfully transformed. pGB354, the small (6.4-kb), high-copy-number derivative of pIP501 (2) resulted in nearly 10^6 transformants per μ g of DNA. Since pGB354 has no sites for some of the most often used restriction endonucleases, like *Eco*RI, *Bam*HI, *Sal*I, *Pst*I, and others, it was an ideal starting replicon for the construction of an *E. coli*-*S. faecalis* shuttle vector. The *E. coli* vector pACYC184 was chosen for this construction because of its small size (4 kb), good characterization, and the fact that it does not have a beta-lactamase gene. (The occurrence of beta-lactamase determinants in streptococci remains uncertain; thus we avoid the introduction of such determinants on ethical grounds.) The resulting vector, pAM401, was very efficient in protoplast transformation (Table 2), regardless of whether the DNA was prepared from *E. coli* or *S. faecalis*.

The method described here was successful for the transformation of at least two *S. faecalis* strains (i.e., with OG1- or JH2-2-related backgrounds), but with different efficiencies (Table 2). Our experiments have shown that the protoplast transformation system for *S. faecalis* OG1X can be as efficient as transformation of CaCl_2 -treated *E. coli*. Our ability to insert a tetracycline resistance determinant from *S. sanguis* into *S. faecalis* attests to the utility of direct cloning

in this system. We furthermore emphasize the fact that transformation can be performed with plasmid DNA isolated by a rapid-preparation procedure (Table 2, last two lines). We have been able to isolate plasmids from 1.5 ml of an overnight culture of *E. coli*, analyze 25% of that material by restriction enzyme digestion and agarose gel electrophoresis, and use the remaining material for protoplast transformation of *S. faecalis* within 9 h.

Finally, we point out that the shuttle vector pAM401 described here should be useful for cloning experiments in a variety of different gram-positive species because of the broad host range of the gram-positive replicon used for its construction. pIP501 has been shown to transfer between at least nine streptococcal species (10, 16, 25), *Lactobacillus casei* (13), *Staphylococcus aureus* (10, 28), and *Pediococcus* spp. (14).

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