New Genetic Techniques for Group B Streptococci: High-Efficiency Transformation, Maintenance of Temperature-Sensitive pWV01 Plasmids, and Mutagenesis with Tn917

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Three techniques were developed to improve the genetic manipulation of group B streptococci (GBS). We first optimized a protocol for transformation of GBS by electroporation, which provided transformation efficiencies of 10^5 CFU/ μ g. Variables that influenced the transformation efficiency were the glycine content of the competent cell growth media, the electric field strength during electroporation, the electroporation buffer composition, the host origin of the transforming plasmid, and the concentration of selective antibiotic at the final plating. Our transformation protocol provides an efficiency sufficient for cloning from ligation reactions directly into GBS, obviating an intermediate host such as *Escherichia coli*. Second, temperature-sensitive plasmids of the pWV01 lineage were shown to transform GBS, and their temperature-sensitive replication was confirmed. Lastly, the temperature-sensitive pWV01 plasmid pTV10K, which contains Tn917, was used as a transposon delivery vector for the construction of genomic Tn917 mutant libraries. We have shown, for the first time, that Tn917 transposes to the GBS chromosome and at a frequency of 10^{-3} /CFU. Furthermore, representative clones from a Tn917 library contained single transposon insertions that were randomly located throughout the chromosome. These techniques should provide useful methods for cloning, mutagenesis, and characterization of genes from GBS.

Group B streptococci (GBS, *Streptococcus agalactiae*) have been the leading bacterial agents associated with neonatal bacteremia, pneumonia, and meningitis during the last two decades in the United States and Western Europe (1). We have been primarily interested in identifying and characterizing the GBS traits important for its virulence. Several traits have been implicated, such as production of hyaluronidase, hemolysin, and CAMP factor and epithelial and endothelial cell adherence and invasion. The use of molecular biology techniques has provided direct means of confirming the role of the capsular polysaccharide in the pathogenesis of GBS infections, using isogenic strains for dissecting the influence of the capsule on host-bacterium interactions (12, 15, 28, 34).

Aside from Tn916 mutagenesis and cloning of genes into *Escherichia coli*, characterization of the GBS capsule genes and further identification of other virulence genes have been hampered by the lack of techniques for genetic manipulation of GBS that are available for other organisms. Mutagenesis with Tn916 has been limited, because its delivery has required conjugation, often generates multiple insertions per chromosome in GBS, and demonstrates restricted target site specificity. For example, we have been unable to isolate Tn916 mutants with mutations in hyaluronidase production, despite screening several genomic equivalents of Tn916-containing clones. This experience presumably reflects nonrandom transposition imposed by the 15-bp consensus target sequence for Tn916 insertion (31). These limitations and our desire for more direct

genetic manipulation of the organism prompted the adaptation of common molecular biological techniques for GBS.

The genus *Streptococcus* is closely related to the genera *Enterococcus* and *Lactococcus* (29). Significant advances in genetic manipulation have been made recently with selected members of these genera. Key examples include techniques for creating recombinant plasmid libraries for characterization of genes within *Streptococcus pneumoniae* (23) and Tn917 transposon mutagenesis in *Enterococcus faecalis* (32). These methods relied upon two tools: high-efficiency plasmid transformation and transformation with temperature-sensitive (ts) plasmid vectors. Developing these two tools and establishing Tn917 mutagenesis were the primary objectives of this study.

Plasmid transformation of GBS has been reported in two prior studies. First, Dunny et al. (8) showed transformation of GBS by an electroporation protocol, utilizing competent cells with weakened cell walls, optimized with *Enterococcus* and achieving an efficiency of 10^3 CFU/ μ g with GBS. In the second study, Ricci et al. (25) showed transformation of GBS to an efficiency of 10^4 CFU/ μ g without cell wall weakening; however, they used only one GBS strain, which was unencapsulated. We sought to optimize a transformation protocol which would allow the electroporation of encapsulated strains. As a result, our protocol demonstrates at least a 10-fold improvement in efficiency over the prior two reports, and we have successfully used it to establish ts plasmids in GBS and to begin characterizing Tn917 transposition in GBS.

MATERIALS AND METHODS

Bacterial strains and plasmids. Table 1 presents the bacterial strains and plasmids used in this study. The MICs of kanamycin, chloramphenicol, and erythromycin, used for selection after electroporation, were determined by spreading 10^8 CFU on 10-cm plates containing Todd-Hewitt agar with various dilutions of each antibiotic. Under these conditions, the kanamycin, chloramphenicol, and

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TABLE 1. Strains and plasmids used in this study

Strain or plasmid	Species	Lineage	Genotype or phenotype	Reference
Strains				
COH 31 r/s	GBS	Type III; <i>rif^r</i>		28
A909	GBS	Type IA		19
COH 1	GBS	Type III		33
OG1X	<i>E. faecalis</i>			32
DH5 α	<i>E. coli</i>		<i>supE44 ΔlacU169 (ϕ80 <i>lacZ</i>ΔM15) <i>hsdR17 recA1 endA1 gyrA96 thi-1 relA1</i></i>	9
JM83	<i>E. coli</i>		<i>F⁻ ara Δ(<i>lac-proAB</i>) <i>rpsH str</i> ϕ80<i>dlac</i>Δ(<i>lacZ</i>)M15 <i>thi</i></i>	9
RR1	<i>E. coli</i>		<i>F⁻ mcrB mrr hsdS20(r_B⁻m_a⁻) <i>leuB6 ara-14 proA2 lacY1 galK2 xyl-5 mtl-1 rpsL20 (Sm^r) supE44 λ⁻</i></i>	9
Plasmids				
pMV158		pMV158	pMV158 <i>ori</i> ; <i>tet</i>	4
pJS3		pMV158	pMV158 <i>ori</i> ; <i>cat</i>	2
pGBS1 ^a		pMV158	pMV158 <i>ori</i> ; <i>cat</i>	This study
pVE6007		pWV01	pWV01 <i>ts ori</i> ; <i>cat</i>	20
pG + host5		pWV01	pWV01 <i>ts ori</i> ; <i>erm</i>	3
pTV1OK		pWV01	pWV01 <i>ts ori</i> ; Tn917 <i>erm kan</i>	10
pTV32ts		pE194	pE194 <i>ts ori</i> ; Tn917- <i>lacZ erm cat</i>	35

^a pGBS1 is a derivative of pJS3 containing a multiple-cloning site (see Fig. 5).

erythromycin MICs for A909 were 500, 1, and 0.01 μ g/ml, respectively, and the MICs of kanamycin and chloramphenicol for COH 31 r/s were 500 and 1 μ g/ml, respectively.

Transformation of GBS. For preparing competent cells, 5 ml of M9 medium with 1% Casamino Acids and 0.3% yeast extract was inoculated from a fresh streak of GBS on Todd-Hewitt agar. After overnight growth, this culture was diluted 1:100 into 50 ml of the same medium with 0.6% glycine added (made from a sterile 10% glycine stock prepared by filtration). This culture was grown overnight and then expanded to 500 ml of the same medium prewarmed to 37°C. The resulting culture was harvested after 1 h by centrifugation at 10,000 rpm for 10 min in a Beckman J2-21 centrifuge ($\sim 9,000 \times g$), washed once in 150 ml of cold 0.625 M sucrose (pH 4), resuspended in 0.5 to 1 ml of the same buffer, and frozen in aliquots or used directly. After each of the centrifugation steps, the supernatants were decanted and then the residual supernatant was removed by pipetting. All glassware coming into contact with competent cells or solutions used to prepare them was autoclaved with water beforehand to ensure that all soap was removed to protect the weakened bacterial cells from lysis. The resulting competent cell suspension contained 10^{10} CFU/ml.

For electroporation, 1 μ g of plasmid DNA was added to 75 μ l of the cell suspension on ice and electroporated in a 0.1-cm cuvette at 1.25 kV (12.5 kV/cm), 600 Ω , and 25 μ F (in a Bio-Rad electroporator with pulse controller). The suspension was diluted immediately into 2 ml of Todd-Hewitt broth–0.25 M sucrose for 2 h of osmotic stabilization at 30 or 37°C (depending on the temperature sensitivity of plasmid replication) before a series of dilutions was plated to determine the final transformation efficiency. For pG+host5 transformation, the osmotic stabilization broth used for growth included 0.01 μ g of erythromycin per ml for induction during the second hour of recovery. All transformants were selected at 30 or 37°C on Todd-Hewitt agar containing antibiotics at the concentrations listed in Table 2, with the exception of tetracycline, which was used at 10 μ g/ml. The successful use of erythromycin selection at a final concentration of 0.1 μ g/ml required fresh erythromycin stock solutions (10 mg/ml in ethanol at -20°C) and the production of erythromycin-containing agar plates on a monthly basis.

Plasmid DNA isolation. For isolation of all plasmids used in this study, knowledge of their copy numbers was key to scaling culture volumes appropriately. For plasmid pGK12, a parent of the pWV01 plasmids used in this study, the copy numbers were 60 and 3 in *E. coli* and in *Lactococcus*, respectively (14). For pLS1 (the progenitor of pJS3 and pGBS1), the copy numbers were 24 and 4 in *S. pneumoniae* and in *E. coli*, respectively (16). Plasmid pJS3 was shown previously to replicate at ~ 90 copies/cell in pneumococci (5). Plasmids were isolated from *E. coli* by column chromatography (Qiagen, Inc.), and they were isolated from *Enterococcus* as described previously (32).

For isolation of plasmid DNA from GBS, a 500-ml culture was grown to an optical density at 600 nm (OD_{600}) of 0.6 at 37°C, supplemented with powdered glycine to 4.5%, and grown for 1 h (2 h for 30°C cultures) to weaken the cell wall. The culture was centrifuged, and the pellet was resuspended in 7.5 ml of lysis buffer (6.7% sucrose, 50 mM Tris [pH 7.0], 10 mM EDTA) containing 40 mg of lysozyme per ml and incubated at 37°C for 30 min. At 1 min before the end of this incubation, the suspension was supplemented with 100 μ g of DNase-free RNase per ml, and 15 ml of fresh 1% sodium dodecyl sulfate (SDS)–0.2 N NaOH was added with gentle mixing. Then 7.5 ml of 3 M potassium acetate (pH 4.8) was added, with subsequent mixing, chilling on ice, and centrifugation. Plasmid DNA was extracted from the supernatant by either CsCl-ethidium bromide purification or Qiagen-500 column chromatography. The yield was 10 to 100 μ g/500 ml of

culture. Overly vigorous lysis was associated with plasmid degradation, and degradation could be anticipated if the bacterial suspension clarified completely upon addition of SDS-NaOH. For the miniprep analysis, the above protocol was scaled down for a 10-ml culture and 15% of the DNA yield was used for each mini-agarose gel lane.

Determination of plasmid segregation rates. A909(pG+host5) and A909 (pGBS1) cultures were grown at 30 or 37°C with 10 μ g of erythromycin or chloramphenicol per ml until the OD_{600} measured approximately 0.7, diluted 1:100,000 into fresh broth, and grown overnight without antibiotic selection. Initial and final concentrations of bacterial cells with and without plasmids were determined by quantitative plate counting on both selective and nonselective agar plates at 30 or 37°C. Plasmid segregation rates were derived from these titers through application of the following formula (adapted from reference 26): percent loss/generation = $[1 - (F_i/F_f)^{1/g}] \times 100$, where F is the frequency of the plasmid-mediated resistance phenotype in the population, f is final, i is initial, and g is the number of generations intervening between the final and initial titers (determined on nonselective media) according to the formula $g = [\log(\text{final titer}/\text{initial titer})]/\log 2$. Reported data represent three experiments.

Chromosomal DNA isolation. A 3-ml overnight culture of GBS was pelleted, resuspended in 200 μ l of lysis buffer (6.7% sucrose, 50 mM Tris [pH 7.0], 1 mM EDTA) and incubated with 25 U of mutanolysin and 100 μ g of DNase-free RNase per ml for 10 min at 37°C. Proteinase K and SDS were added to 800 μ g/ml and 0.5%, respectively, and incubation was continued for 30 min at 56°C. This suspension was vortexed once with an equal volume of phenol, and then chloroform-isoamyl alcohol 24:1 was added, followed by vortexing and centrifugation. The aqueous phase was extracted once again with phenol-chloroform-isoamyl alcohol and then with chloroform-isoamyl alcohol, ethanol precipitated, and resuspended in 50 μ l of TE buffer. The yield was approximately 50 μ g of DNA.

Southern analysis. A 4- μ g portion of *Hind*III-digested GBS chromosomal DNA was electrophoresed through 0.7% agarose and transferred to Micron Separations Inc. Magnagraph or Nitroplus membranes as specified by the manufacturer. The blots were hybridized to an *Eco*RI-*Pst*I fragment of pTV1OK bearing all of Tn917, labelled with [^{32}P]dCTP by using an Amersham random-primer labeling kit. Hybridization and washing were performed as specified by Micron Separations Inc. for stringent conditions appropriate for perfect DNA hybrids.

Derivation of genomic Tn917 libraries in GBS. Todd-Hewitt broth (10 ml) containing 2,000 μ g of kanamycin per ml and 10 μ g of erythromycin per ml was inoculated with A909(pTV1OK), grown at 30°C to an OD_{600} of 0.7, diluted into 1 liter of prewarmed 39°C Todd-Hewitt broth containing 10 μ g of erythromycin per ml, and grown to stationary phase at 39°C. Aliquots (1 ml) of the final culture were stored frozen for future characterization. The transposition frequency and rate of pTV1OK segregation were determined by comparing the number of Em^r Km^s and/or Em^r Km^r clones of the original 30 and 37°C cultures. Growth of the original A909(pTV1OK) clones to stationary phase was avoided to reduce the possibility of accumulating Tn917 insertions at chromosomal hot spots (35).

RESULTS

Optimization of plasmid transformation. The method for plasmid transformation of GBS, as reported by Dunny et al. (8), involves growth of the organism in glycine to weaken its

FIG. 1. Influence of glycine content in the competent cell culture medium upon transformation. The growth rate of COH 31 r/s decreased with increasing glycine concentration, even within the 0.4 to 1.0% range. Therefore, overnight growth to reach an OD_{600} of 0.1 ranged from 17 to 21 h. These cultures were then diluted 1:10, as in Materials and Methods, for the second growth step and permitted to reach an OD_{600} of approximately 0.01. The efficiency obtained in these early experiments is significantly lower overall than in subsequent experiments optimizing other variables, probably reflecting the low ODs of the final competent cell cultures, but it is equivalent to those reported by Dunny et al. (8). Optical densities in subsequent experiments were 10 times greater than these, perhaps reflecting the greater attention to handling weakened competent cells by improved glassware preparation (see Materials and Methods). Electroporation was done with plasmid pMV158 and 0.2-cm cuvettes at 11.5 kV/cm, 1,000 Ω , and 25 μ F.

cell wall followed by extensive washing in high-osmotic-strength buffer containing sucrose to lower the ionic strength of the bacterial suspension in preparation for electroporation with plasmid DNA. After electroporation, the bacterial suspension is incubated in high-osmotic-strength medium broth to stabilize the cell wall before being plated on selective agar plates. Using strain COH 31 r/s, we manipulated several technical variables to improve the efficiency of transformation, such as the culture conditions for competent cells, the transforming plasmid DNA, the electroporation parameters, and the final antibiotic selection of transformants.

In preliminary experiments, we first established transformation of GBS strain COH 31 r/s by the unmodified method of Dunny et al. (8). The concentration of glycine in the M9 growth medium for raising competent cells was critical for transformation. At concentrations below 0.4% glycine, transformation was very low, whereas above 1.0% glycine, growth of the cells was inhibited. Our next objective was to identify a glycine level within the 0.4 to 1.0% range that provided the maximal transformation efficiency. Figure 1 shows that 0.6% glycine in the culture medium provided the maximum transformation efficiency. This glycine level is lower than the optimum range of 1.25 to 6% observed for different strains of *Enterococcus faecalis* (8) and is also lower than the 2 to 3% glycine levels used by Holo and Nes (11) for lactococci.

We examined the effect of the osmotic strength and ionic composition of the electroporation buffer on transformation efficiency. A buffer containing sucrose as an osmotic stabilizer improved the transformation efficiency by an order of magnitude compared to that of distilled H_2O alone or a buffer containing 10% glycine (data not shown). We also found that 1 mM $MgSO_4$, as used in the method of Dunny et al. (8), reduced the transformation efficiency. We hypothesized that $MgSO_4$ reduced the transformation efficiency for GBS by reducing the pulse duration through an increase in the ionic strength of the buffer. This hypothesis was tested by preparing

FIG. 2. Influence of the $MgSO_4$ content in the electroporation buffer on the transformation efficiency and electrical time constant. Competent cells were prepared in a buffer composed of 0.625 M sucrose (pH 4.0) and then supplemented with different concentrations of $MgSO_4$ as shown prior to electroporation. Electroporation was performed with plasmid pJS3 and 0.1-cm cuvettes at 12.5 kV/cm, 600 Ω , and 25 μ F. The results represent two experimental trials; error bars depict sample standard deviation.

competent COH 31 r/s cells in a buffer containing only sucrose and then supplementing the buffer with increasing levels of $MgSO_4$ immediately prior to electroporation. Figure 2 shows that both the transformation efficiency and time constant (which is a measure of the pulse duration) declined with increasing magnesium concentrations in the range of 0 to 20 mM, confirming our hypothesis. Our final method uses 0.625 M sucrose (pH 4.0) as the electroporation and wash buffer, which is the buffer used by Dunny et al. (8) without $MgSO_4$. Competent cells can be stored in this buffer for 3 months at -80°C without a significant loss of competence (data not shown).

The electrical field strength required for maximal transformation of GBS was identified. Competent COH 31 r/s showed an increase in transformation efficiency by nearly an order of magnitude, with electroporation field strengths in the range of 8.5 to 12.5 kV/cm (Fig. 3). The maximal efficiency was observed

FIG. 3. Influence of electric field strength on transformation efficiency. Electroporation was performed with plasmid pMV158 and 0.2-cm cuvettes at 1,000 Ω and 25 μ F at the indicated field strengths. The results represent three trials: one with competent cells created with 0.6% glycine and two with competent cells created with 1.0% glycine. The results with all the trials were identical, except that the efficiencies with the latter competent cells were 1 log unit lower than those with the former cells.

FIG. 4. Influence of transforming plasmid concentration upon the total yield of transformants. Electroporation was performed with plasmid pMV158 and 0.2-cm cuvettes at 12 kV/cm, 1,000 Ω , and 25 μ F. The results represent two trials; error bars depict sample standard deviation.

in the range of 11.5 to 12.5 kV/cm. Field strengths higher than 12.5 kV/cm showed a tendency toward arcing under our conditions; therefore, we chose 12.5 kV/cm for our subsequent studies. The trend of higher efficiencies with higher field strengths is similar to the results of Holo and Nes (11), Dunny et al. (8), and Ricci et al. (25). Ricci et al. observed a clear optimum at 18 kV/cm, which is in a range we have not explored because of cuvette arcing. Arcing was also problematic for Holo and Nes (11) when using field strengths above 10 kV/cm. Ricci et al. (25) used a buffer composed of glycerol and water, which may have prevented arcing.

The influence of plasmid topology upon transformation efficiency was explored, specifically the transformability of the relaxed circular form, as would be generated in ligation reactions for generating recombinant plasmids. We derived covalent open circles from supercoiled pMV158 by linearization with *Hind*III and religation and then compared the transformation efficiencies of the ligation product and the supercoiled plasmid DNA. In two experiments, the supercoiled form transformed 2 log units more efficiently than did the relaxed circular form, at $(1.4 \pm 0.2) \times 10^5$ and $(4 \pm 2) \times 10^3$ CFU/ μ g, respectively. Although we did not subject the supercoiled plasmid DNA to mock treatment, which would have allowed us to attribute the difference in efficiencies entirely to a change in topology, these data do demonstrate that relaxed circular plasmid preparations, as generated by ligation, transform less efficiently than the parent supercoiled preparation does. We have confirmed that this efficiency is high enough for direct cloning into GBS, since we have recently generated several new recombinant plasmids through transformation of ligation reactions directly into GBS (data not shown).

Lastly, we investigated the influence of plasmid DNA dosage upon the yield of transformants. Figure 4 demonstrates that in the range of 0.2 to 6 μ g of plasmid DNA per cuvette, the number of transformed cells per cuvette increased, whereas above 6 μ g, this number did not increase. Since these experiments were performed in 0.2-cm cuvettes, which hold 400 μ l of cell suspension, the optimal DNA concentration in Fig. 4 was 6 μ g/400 μ l, or roughly 1 μ g/75 μ l. Subsequently (and in our

final method), we used 0.1-cm cuvettes to conserve both plasmid and competent cell preparations. Since these cuvettes hold 75 μ l of cell suspension, we routinely use 1 μ g of plasmid DNA per cuvette. The linear relationship between the number of transformants and the mass of transforming plasmid DNA up to 6 μ g is similar to the observations of both Dunny et al. (8) and Ricci et al. (25). Above 6 μ g/400 μ l, the DNA may be present at saturating levels.

Transformation of conditional replication plasmids. Our optimized transformation method was used to transform GBS with plasmids which display conditional replication properties. We investigated the ability of ts derivatives of the parent plasmids pWV01 (pGHOST5, PVE6007, and pTV10K) and pE194 (pTV32ts) to transform and stably replicate in GBS at the permissive temperature of 30°C (see reference 7) for a discussion of the lineages of these plasmids). These plasmids have useful antibiotic resistance genes for selection in GBS, and pTV10K contains the transposon Tn917. Both plasmid lineages were chosen since they could be valuable as vectors for mutagenesis strategies. As a control, we included pGBS1, which is a derivative of pJS3 that contains a multiple cloning site and the replication origin from pMV158 (the derivation is shown in Fig. 5) and which replicates at both 30 and 37°C (Table 1). Representative strains from serotypes Ia (A909) and III (COH 31 r/s) were used as the recipients in these experiments. Table 2 shows the mean transformation efficiencies exhibited by these plasmids in experiments with three independent competent cell preparations for each GBS strain. The ranges in efficiency are also presented to show how competent cell preparations can differ.

Transformation efficiencies up to $\sim 10^5$ CFU/ μ g were observed with pVE6007 and pTV10K with both strains, whereas the control plasmid, pGBS1, transformed only strain COH 31 r/s with similar efficiency. In contrast, the pE194 plasmid derivative, pTV32ts, uniformly failed to transform either GBS strain.

The transformation efficiency of pVE6007 in GBS was strongly affected by the host species from which the plasmid was isolated. PVE6007 isolated from COH 31 r/s transformed both GBS strains 3 orders of magnitude more efficiently than did pVE6007 isolated from either of the *E. coli* strains DH5 α or JM83 (10^5 and $\leq 10^2$ CFU/ μ g, respectively). In contrast, the transformation efficiency of pGBS1 was not dependent on the host source when transformed into either COH 31 r/s or A909. (pGBS1 from *E. coli* DH5 α was not tested because, unlike pVE6001, pGBS1 does not replicate efficiently in this strain because its replication requires the *recA* gene product [16]). Likewise, the transformation efficiencies in COH 31 r/s of pMV158 DNA isolated from *E. faecalis* OG1X and from COH 31 r/s were similar [$(8 \pm 1) \times 10^4$ and $(8 \pm 2) \times 10^4$ CFU/ μ g, $n = 2$, for the two plasmid preparations, respectively]. These results suggest that transformation of certain plasmids can be influenced by the original plasmid host, perhaps reflecting differences in restriction/modification systems which are yet uninvestigated in GBS.

The transformation behavior of another pWV01 derivative, pG+host5, was somewhat different than expected from the above results. When it was transformed into strain A909, recovery of transformants was at least 4 orders of magnitude lower than that of pVE6007 from GBS and pTV10K from *E. coli*. COH 31 r/s was not used as a recipient because of its innate erythromycin resistance (Table 1). These experiments were first performed by selecting for transformed clones at an erythromycin concentration of 10 μ g/ml. Since pG+host5 differs from the other pWV01 plasmids by virtue of the resistance gene it carries, we explored whether this disparity in transformation efficiencies was related to the concentration of antibi-

FIG. 5. Schematic showing the derivation of plasmid pGBS1 by ligating *Hind*III-digested pUC19 with pJS3 and digesting this chimera (pLH1) with *Pvu*II to remove all but the 327 bp from the pUC19 portion containing the multiple-cloning site.

otic used in the final selection media. We tested this hypothesis by examining the recovery of transformed clones after lowering the antibiotic concentration in the final selective agar. Erythromycin concentrations from 0.01 to 10 μ g/ml were compared to determine the level which allowed background growth of nontransformed A909 colonies after electroporation under mock conditions without plasmid DNA. Concentrations of <0.1 μ g/ml failed to inhibit the growth of nontransformed cells. The data in Table 2 show that at 0.1 μ g of erythromycin per ml, the efficiency of pG+host5 transformation increased by 3 orders of magnitude compared with that at 10 μ g/ml erythromycin. This observation emphasized the importance of minimizing the antibiotic level used in selection when attempting to establish a novel plasmid in GBS.

ts plasmid replication in GBS. Having established ts pWV01 plasmids in GBS, we investigated whether they would demonstrate temperature-dependent replication in GBS. The utility of the ts pWV01 plasmids for GBS was anticipated from their temperature-dependent replication in lactococci. In the latter genus, 33°C was the threshold for permissive versus nonpermissive replication of ts pWV01 plasmids (20). Since many GBS strains are mesophilic and will not grow at temperatures

above 41°C (21), the 33°C threshold would be ideal for controlling plasmid replication in GBS.

A909 bearing pG+host5 was chosen for the analysis of plasmid replication. The loss rates of the *Em*^r phenotype encoded by pG+host5, from an A909[pG+host5] broth culture grown in the absence of antibiotic selection at different temperatures, are as follows. At 36 and 38°C, pG+host5 segregated at rates of 51% $\pm$ 2% and 52% $\pm$ 2% per generation; by contrast, at 30°C, it segregated at only 5.3% $\pm$ 1.4% generation. Hence, pG+host5 demonstrated a dramatic loss in plasmid replication between 30 and 36°C, as observed in lactococci. In subsequent experiments with all pWV01 ts plasmids (pG+host5, pVE6007, and pTV1OK), a culture temperature of 30°C invariably supported plasmid replication whereas a culture temperature of 37 to 39°C cured the strain of the plasmid (data not shown).

pGBS1 (Table 1; Fig. 5) was included as a control for these studies of pG+host5 replication. In A909(pGBS1) cultures, the loss rate of *Cm*^r clones during growth in broth without antibiotic selection was determined. Surprisingly, high levels of segregation of pGBS1 were observed at both temperatures examined: 19% $\pm$ 6% per generation at 38°C and 36% $\pm$ 2% per generation at 30°C. This temperature dependence was the

TABLE 2. Comparative transformation of GBS by plasmids of three lineages

Plasmid/source	Lineage	Antibiotic selection ^a	Efficiency (CFU/μg) of transformation ^b for:	
			COH 31 r/s	A909
pGBS1/A909	pLS1	Cm, 10	1×10^5 : (3×10^5 , 1×10^4 , 2×10^4)	4×10^3 : (6×10^2 , 3×10^3 , 8×10^3)
pGBS1/JM83	pLS1	Cm, 10	9×10^4 : (3×10^5 , 6×10^3 , 1×10^4)	2×10^3 : (6×10^2 , 2×10^3 , 2×10^3)
pVE6007/COH 31 r/s	pWV01	Cm, 10	3×10^5 : (6×10^5 , 2×10^5 , 2×10^4)	2×10^5 : (2×10^5 , 3×10^5 , 2×10^5)
pVE6007/DH5-α	pWV01	Cm, 10	9×10^4 : (1×10^2 , 2×10^1 , 4×10^1)	9×10^1 : (2×10^2 , 5×10^1 , 4×10^1)
pVE6007/JM83	pWV01	Cm, 10	2×10^1 : (4×10^1 , 2×10^0 , 1×10^1)	7×10^1 : (2×10^2 , 2×10^1 , 1×10^1)
pTV1OK/RR1	pWV01	Km, 2,000	2×10^5 : (4×10^5 , 9×10^4 , 1×10^5)	1×10^5 : (3×10^4 , 1×10^5 , 2×10^5)
pG+host 5/ A909	pWV01	Em, 10	NA ^c	5×10^0 : (2×10^0 , 7×10^0 , 7×10^0)
pG+host 5/ A909	pWV01	Em, 0.1	NA ^c	2×10^3 : (2×10^2 , 2×10^3 , 2×10^3)
pTV32ts/OG1χ	pE194	Cm, 10	0: (0, 0, 0) ^d	0: (0, 0, 0)

^a Cm, chloramphenicol; Km, kanamycin; Em, erythromycin. Numbers indicate antibiotic concentrations (in micrograms per milliliter) in selective agar.
^b Efficiencies of plasmid transformation are shown for two GBS strains, utilizing the optimized transformation protocol. In each case, the mean efficiency is separated by a colon from values from trials of three independent competent cell preparations. Data shown are for three competent cell preparations of each strain.
^c NA, not applicable.
^d 0 means less than 10^{-8} viable cells.

reverse of that observed for the pWV01 plasmids. These observations suggested that pGBS1 could be used as a curable delivery vector in circumstances where ts pWV01 plasmids are unsuitable.

Transposition of Tn917 in GBS. Having verified the temperature-dependent replication of a representative ts pWV01 plasmid, we sought to exploit this phenotype to test for the transposition of Tn917 into the GBS chromosome. pTV1OK, which carries transposon Tn917 and the Km^r determinant external to the transposon (Fig. 6A), was transformed into A909. Km^r clones were tested for Em^r and for the presence of

pTV1OK by plasmid DNA analysis. Subsequently, we tested for the transposition of Tn917 from the plasmid to the chromosome by growing the cells in erythromycin-containing broth at the permissive temperature (30°C) followed by plating the broth cultures on erythromycin-containing Todd-Hewitt agar. Em^r isolates growing at 37°C which were Km^s were isolated and were shown to have lost the plasmid and carried Tn917 in the chromosome by Southern hybridization analysis with a Tn917-specific probe (Fig. 6B).

The transposition frequency (35) equals the number of Em^r

FIG. 6. Southern hybridization analyses of A909:Tn917 clones with a ³²P-labelled Tn917 probe. (A) Restriction enzyme map of pTV1OK. (B) HindIII digests of Em^r Km^s clones (lanes 1 to 9) compared to HindIII-digested pTV1OK/HIII as the control (lane 10). (C) Analysis of Em^r Km^s clones (lanes 12 to 20), with HindIII-digested pTV1OK/HIII as the control (lane 11).

CFU of the A909(pTV1OK) population at the nonpermissive temperature divided by the number of Em^r CFU at the permissive temperature. The transposition frequencies of six independent A909(pTV1OK) isolates were determined and gave a mean value of $(1.1 \pm 0.6) \times 10^{-3}$ CFU⁻¹. This value suggested not only that Tn917 was transposing to the chromosome but also that the transposition was occurring at a frequency sufficient for generation of transposon libraries in small culture volumes.

We investigated how randomly Tn917 inserted into the GBS chromosome and how many copies of Tn917 were present within the chromosomes of randomly chosen mutants. Transposon libraries were generated in A909(pTV1OK) by shifting the temperature of growth in selective media as discussed in Materials and Methods. Individual clones were analyzed for their antibiotic resistance phenotype, and genomic DNA was isolated from each for Southern hybridization analysis with a Tn917-specific probe.

We characterized four separate A909:Tn917 libraries and isolated two antibiotic resistance phenotypes. A total of 90 to 99% of the clones from each of the libraries were Em^r Km^s at 37°C, suggesting transposition of Tn917 and loss of the plasmid; 10% or less of the clones from each library were Em^r Km^r at 37°C, suggesting retention of the plasmid in some form. Southern hybridization analyses of representative samples of these two populations are shown in Fig. 6. Figure 6A is a map of pTV1OK and shows how *Hind*III restriction enzyme digestion creates (i) two Tn917 junction fragments, whose sizes reflect the *Hind*III restriction map of the plasmid (or, alternatively, the chromosomal segment) bearing Tn917, and (ii) one fragment internal to Tn917, whose size is independent of the plasmid or chromosomal location of Tn917. In Fig. 6B and C, the hybridizing bands corresponding to either the junction or internal fragments are identified in *Hind*III digests of DNA samples from randomly selected A909:Tn917 library clones, representing the two populations described above. *Hind*III-digested pTV1OK DNA was included as a control. Em^r Km^s clones (Fig. 6B) contained a pair of junction fragment bands which were unique for each clone. This pattern demonstrates that one copy of Tn917 was present in a unique chromosomal site within each mutant and also suggests that insertion of Tn917 into the A909 chromosome was random.

The pattern of junction fragment bands of the Em^r Km^r class (Fig. 6C) differs in two ways from that of the Em^r Km^s class. First, two pairs of hybridizing bands now appear for each clone (with the exception of two clones [discussed below]). Second, one of these pairs of bands appears consistently among all the clones; the same pair of bands appears in the control pTV1OK-*Hind*III digest; this observation demonstrates retention of the plasmid episomally in these clones. The other pair of bands varies in size among the clones, as in the Em^r Km^s class above, demonstrating chromosomal Tn917 insertion unique to each clone. Two clones in Fig. 6C, lanes 16 and 19, show only one junction fragment band distinct from the pair comigrating with the pTV1OK-*Hind*III junction fragment bands; we suggest that an additional fragment band escaped detection by virtue of its size. Nonetheless, the hybridizing bands that do appear are consistent with single chromosomal Tn917 insertions located uniquely within the genome of these clones.

DISCUSSION

This study provides advances in molecular technology for the genetic manipulation of GBS. First, efficiencies of 10^5 CFU/ μ g of DNA have been demonstrated with an improved

transformation method, achieving a 10- to 100-fold improvement over those in previous reports of GBS transformation (8, 25). Second, we have shown that ts plasmids of the pWV01 lineage can replicate in GBS and have verified their ts replication. Finally, one of these plasmids, pTV1OK, has been used to deliver Tn917, a transposon novel to GBS, and its transposition has been shown to be efficient and random and to generate single insertions per chromosome.

Glycine-mediated cell wall weakening during the growth of competent cells is the crux of our transformation protocol, and we believe that the manner of glycine treatment holds the key to future improvements. We observed that as the glycine concentration increased from 0.2 to 1.0% in the experiments in Fig. 1, the transformation frequency increased from 5×10^{-5} to 5×10^{-4} (data not shown); however, the cells grew more slowly at increased glycine concentrations. These observations suggest that the optimum efficiency at 0.6% glycine reflected a balance of increasing transformation frequency and decreasing cell viability with increasing glycine concentrations. The implication is that if cell viability could be improved, the transformation efficiency would also increase. This could be achieved by including sucrose during the culture steps in the presence of glycine, as did Holo and Nes (11). They found that inclusion of sucrose elevated both the growth rate and the transformation efficiency, the latter by 2 to 5 log units depending on the glycine level. These investigators demonstrated a transformation efficiency of 10^7 CFU/ μ g with lactococci, 2 orders of magnitude higher than our efficiency with GBS, and their expanded use of sucrose could explain the difference.

We have preliminary evidence that the density or growth phase of the final competent cell culture also influences the transformation efficiency. If cells are grown according to the optimized protocol but with a variable duration during the final culturing step in glycine before harvesting, they will increase from an initial OD of about 0.01 to an OD of 0.9 in about 6 h; however, cells harvested at ODs of 0.4 to 0.9 provide transformation efficiencies of 5×10^3 to 5×10^4 CFU/ μ g. In contrast, cells harvested in the OD range of 0.1 to 0.25 show transformation efficiencies of greater than 1×10^5 . This is the OD range reached by cells grown for 1 h, as in the optimized protocol. Ricci et al. (25) also observed an efficiency optimum at a low OD of 0.4 with their unencapsulated strain grown in the absence of glycine. These results suggest that the early- to mid-log-phase growth state provides the optimal number of competent cells for transformation.

With experience in transforming several strains of GBS, we have observed that COH 31 r/s is transformed more efficiently than A909 or COH1 by the optimized method (data not shown) (Table 2, pGBS1). This difference could signal an influence of encapsulation upon transformation efficiency, since COH 31 r/s is less encapsulated than the other two strains. Alternatively, this difference in efficiency could suggest that the glycine concentration in the medium requires adjustment for each strain, as recommended by Dunny et al. (8).

The successful transformation and replication of pWV01 plasmids derivatives in GBS indicates that plasmids derived from this vector, initially developed for use in lactococci (18) for recombination, analysis of expression, and heterologous expression, will be useful in GBS too. The ability of ts pWV01 plasmids to replicate in GBS, other species of streptococci (10, 24, 30), and enterococci (20) suggests their broad utility as well.

Our inability to transform pTV32ts into GBS is consistent with the absence of reports of establishing members of the pE194 lineage in streptococcal species. However, the pE194 plasmid pTV32ts has been used in *E. faecalis* (32). In lacto-

cocci, transformation by pE194 plasmids has been observed, but it tends to be accompanied by recombination with the chromosome (6, 13). We have observed Tn917 transposition into or recombination with the chromosome of COH 31 r/s at a very low frequency following electroporation by pTV32ts. This observation suggests that since this plasmid does not appear to replicate in GBS, it could serve as a suicide vector.

We observed Tn917 transposition in GBS to be efficient and random and to generate single insertions per chromosome. In some species, Tn917 transposition events appear to cluster in chromosomal hot spots several kilobases in size. This observation comes from the analysis of auxotrophic Tn917 mutants of *Bacillus subtilis* (35) and from pulsed-field gel electrophoresis of *L. lactis* Tn917 mutants (13). Our observations cannot exclude Tn917 preferences for large regions of the GBS chromosome. However, the variety of mutant phenotypes that we have begun to isolate from the A909:Tn917 libraries provides further evidence of random transposition. These phenotypes include variants in beta-hemolysin production (20), capsule expression, binding to fibronectin, and invasion of eucaryotic cells (27). Finally, we have preliminary observations that transpositions into a target plasmid with a specific region of nonessential DNA occur randomly throughout this region in either orientation (17). Our experience with Tn917 coupled with its replication of the delivery plasmid and efficient transformation shows inherent advantages over the use of a conjugative transposon such as Tn916.

Plasmid pTV10K persisted episomally in our Tn917 libraries at a frequency 1 to 2 log units higher than we predicted by calculation from pG+host5 segregation rates. This observation could be attributed either to a difference in the segregational loss rates of pG+host5 and pTV10K or to library enrichment for mutations accommodating pTV10K maintenance at the nonpermissive temperature; additional support for the latter hypothesis comes from the observation that the Em^r Km^r Tn917 clones examined grew well at the nonpermissive temperature in broth containing kanamycin at 2,000 µg/ml. Whatever the explanation, the frequency of plasmid retention in the GBS Tn917 libraries has not limited the usefulness of this plasmid in delivery of Tn917.

GBS has been one of the more difficult organisms to manipulate genetically. As the need for understanding the molecular pathogenesis of infections caused by this organism has increased, so has the demand for molecular technology in this organism. Additional tools for GBS, such as plasmid-cloning vectors, plasmids with reporter genes, and transposons capable of identifying promoters and secreted genes, are being developed.

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