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Improved Electroporation and Cloning Vector System for Gram-Positive Bacteria

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A protocol for transformation of intact *Enterococcus faecalis* cells by electroporation was developed through a systematic examination of the effects of changes in various parameters, including (i) growth conditions; (ii) composition of the electroporation solution; (iii) electroporation conditions, such as field strength and resistance; (iv) size, concentration, and purity of DNA used for transformation; and (v) conditions used to select for transformants. Key features of this protocol include the use of exponential-phase cells grown in inhibitory concentrations of glycine and the use of an acidic sucrose electroporation solution. Frequencies of $>2 \times 10^5$ transformants per μg of plasmid DNA were obtained for *E. faecalis* cells, whereas various strains of streptococci and *Bacillus anthracis* were transformed at frequencies of 10^3 to 10^4 transformants per μg of plasmid DNA with the same protocol. A novel *Escherichia coli-Streptococcus* and *Enterococcus* shuttle cloning vector, pDL276, was constructed for use in conjunction with the electroporation system. This vector features a multiple cloning site region flanked by *E. coli* transcription termination sequences, a relatively small size (<7 kb), and a kanamycin resistance determinant expressed in both gram-positive and gram-negative hosts. Various enterococcal and streptococcal DNA sequences were cloned in *E. coli* (including sequences that could not be cloned on other vectors) and were returned to the original host by electroporation. The vector and electroporation system was also used to clone directly into *E. faecalis*.

Because of their importance as pathogens and their widespread use in various industrial processes, considerable effort has been directed toward the development of modern genetic and molecular techniques to analyze and manipulate gram-positive bacteria. Our laboratories are involved in a variety of genetic studies of streptococci (for the purposes of this discussion, we include closely related organisms such as enterococci and lactococci in this group). One important aspect of this work involves the construction of cloning vectors and the improvement of methods to introduce recombinant DNA molecules, constructed in vitro, into host strains that are not naturally competent. Recombinant DNA manipulations in these organisms typically have involved initial cloning and molecular analyses in *Escherichia coli*, followed by reintroduction of the cloned DNA (or mutated derivatives thereof) into the original streptococcal host for studies of expression, complementation, gene replacement, etc. A number of shuttle vectors have been developed for these purposes (10, 20, 32).

Although host-vector systems such as these have greatly facilitated recombinant DNA studies in streptococci, there are still significant limitations to the potential application of this technology. Two problems associated with the streptococcal shuttle vectors described thus far are their rather large size and their limited number of useful cloning sites. Another significant problem relates to difficulty in generating random, representative gene banks of streptococcal DNA in *E. coli*. Although the inability to clone certain streptococcal genes in *E. coli* might relate to lethal effects of their protein products in a heterologous host, recent evidence (7) indicates that the failure of streptococcal gene transcripts to terminate in *E. coli* may account for many of the difficulties encountered in the generation of representative gene banks when using high-copy *E. coli* vector systems. Chen and Morrison (7) constructed an *E. coli* vector containing a multiple cloning sequence surrounded by strong transcription terminators and demonstrated its utility in the construction of representative gene banks from *Streptococcus pneumoniae* genomic DNA.

Further difficulties have been encountered in the reintroduction of cloned streptococcal DNA into the original or a closely related host. Usually, cloned DNA has been introduced into a naturally competent host, such as *Streptococcus sanguis* (21), *S. pneumoniae* (29), or *Streptococcus mutans* (18). Although this approach is simple and efficient, competent cell transformation often results in the generation of deletions in cloned genes during the conversion of the DNA to the single-stranded linear form required for uptake or during the subsequent regeneration of the double-stranded circular molecule in the transformed cell (3, 22). Alternative methods for the introduction of cloned DNA have been developed, including polyethylene glycol-mediated protoplast transformation (33) and electroporation of intact cells (6, 19, 35), spheroplasts (28), or protoplasts (14). These methods overcome the deletion problem, but the generation of protoplasts or spheroplasts and subsequent regeneration of cells can be tedious and difficult to reproduce. Many of the electroporation methods that have been reported for intact gram-positive cells have been inefficient or were applicable only to a particular strain.

In this communication we describe in detail the construction of a plasmid vector, pDL276, that can be used for

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TABLE 1. Plasmids and strains used in this study

Plasmid or strain	Relevant markers[a]	Bacterial host(s)	Source or reference
Plasmids			
pUC19	Amp^r Lac^+	*E. coli*	35
pCF10	Tet^r	*E. faecalis*	8
pWM402	Cam^r Tet^r	*E. coli*, streptococci	33
pVA380-1	None	Streptococci	24
pJDC9	Ery^r Lac^+	*E. coli*	7
pPL703	Kan^r	Bacilli	2, 26
pDL414	Kan^r	Streptococci	16
pDL269	Spc^r Amp^r	*E. coli*	This study
pDL270	Spc^r	*E. coli*	This study
pDL271	Spc^r Kan^r	*E. coli*	This study
pDL273	Spc^r	Streptococci	This study
pDL274	Spc^r Kan^r Lac^+	*E. coli*, streptococci	This study
pDL276	Kan^r Lac^+	*E. coli*, streptococci	This study
Bacterial strains			
E. coli DH5α	Lac^- Rec^-		Bethesda Research Laboratories, Inc.
E. faecalis OG1SSp	Str^r Spc^r		9
E. faecalis OG1RF	Rif^r Fus^r		9
E. faecalis JH2-2	Rif^r Fus^r		11
E. faecalis UV202	Rif^r Fus^r Rec^-		34
Streptococcus pyogenes DW1009			D. Wennerstrom (31)
S. sanguis FW213			P. Fives-Taylor (13)
Streptococcus agalactiae H36B (type Ib)			D. Wennerstrom (31)
B. anthracis UM23C1-1			J. Bartkus (2)

[a] Abbreviations: Lac^+, ferments lactose; Lac^-, unable to ferment lactose; Rec^-, recombination deficient. Other abbreviations refer to antibiotic resistance as follows: Amp^r, ampicillin; Tet^r, tetracycline; Spc^r, spectinomycin; Str^r, streptomycin; Rif^r, rifampin; Fus^r, fusidic acid; Cam^r, chloramphenicol; Ery^r, erythromycin; Kan^r, kanamycin.

cloning in *E. coli* or several gram-positive hosts and that can circumvent several of the limitations of vectors described previously. We also describe the development of a technique for electroporation of intact gram-positive bacterial cells that can be used for direct cloning or for shuttling cloned genes from *E. coli*. The method was developed specifically for the transformation of *Enterococcus faecalis* cells, but we have also found it to be effective for strains of several genera, including *Lactococcus*, *Streptococcus*, and *Bacillus*. Bartkus and Leppla (2) employed our method to introduce chimeric plasmids into *Bacillus anthracis* for genetic analysis of virulence gene expression. Holo and Nes (15) recently published a method for efficient transformation of lactococci grown in the presence of sucrose and high concentrations of glycine which shares several features with the method reported here. Our results, along with those reported by others (2, 15), indicate that a protocol similar to the one presented here may be a useful starting point for optimizing efficient electroporation procedures for a wide variety of gram-positive bacteria.

(A preliminary report of the pDL276 shuttle vector described here has been presented previously [17b].)

MATERIALS AND METHODS

Bacterial strains, plasmids, and culture media. The bacterial strains and plasmids used in this study are listed in Table 1. Various growth media were used for cultivation of streptococci, *E. faecalis*, and *B. anthracis* as described below. These included Todd-Hewitt broth (THB) and brain heart infusion (BHI), both from Difco; BYGT (BHI, yeast extract, glucose, Tris) as described by Dunny et al. (11); and M9-YE (M9 salts, Casamino Acids, yeast extract) as described by Dunny and Clewell (12). *E. coli* cells were cultivated in LB medium as described previously (9) or TB (12 g of tryptone, 24 g of yeast extract, and 4 ml of glycerol per liter; 0.017 M KH_2PO_4, 0.072 M K_2HPO_4) (30). Concentrations of antibiotics used in selective media were as follows: tetracycline, 10 μg/ml for enterococci and 5 μg/ml for *E. coli*; chloramphenicol, 10 μg/ml; erythromycin, 10 μg/ml for gram-positive hosts and 100 μg/ml for *E. coli*; spectinomycin, 1,000 μg/ml for gram-positive hosts and 100 μg/ml for *E. coli*; ampicillin, 100 μg/ml; kanamycin, 1,000 μg/ml for enterococci and streptococci, 100 μg/ml for *B. anthracis*, and 50 μg/ml for *E. coli*.

DNA isolation and analysis. Plasmid DNA was isolated from *E. coli* by the procedure of Birnboim and Doly (4). For plasmid screening of streptococci and enterococci, the procedure of Anderson and McKay (1) was followed. Preparation of larger quantities of plasmid DNA from these hosts was accomplished as described previously (17). Restriction endonucleases and DNA-modifying enzymes were used according to the instructions of the manufacturer (Bethesda Research Laboratories). DNA fragments were resolved on 0.8% agarose gels as described previously (17).

Recombinant DNA methodology. The construction of pDL276 is described in Results and shown in Fig. 2. Most of the cloning of streptococcal DNA and enterococcal DNA described in Results involved subcloning fragments originally cloned in other vectors. For these experiments, the fragment to be subcloned was separated by agarose gel electrophoresis and purified from the gel with Gene-Clean (Bio101, La Jolla, Calif.) as described by the manufacturer. Ligation reactions were carried out as described by Christie et al. (9). For the cloning of the restriction enzyme fragments of pCF10, the procedure of Pecenka et al. (27) was used to enrich for inserts of the desired DNA fragment. Frozen competent DH5α cells (Bethesda Research Laboratories) were transformed with 50 to 200 ng of DNA from a ligation mixture as recommended by the supplier. *E. faecalis*

OG1RF was transformed as described below with 400 to 1,000 ng of ligated DNA. The identity of cloned DNA fragments was verified by agarose gel electrophoresis and hybridization to the original source of the cloned DNA as described by Christie and Dunny (8).

Electroporation. All experiments were carried out with a Bio-Rad Gene Pulser apparatus at a capacitance of 25 μF. The protocol given below was developed based on various experiments described in Results. The effects of variations in growth conditions and in the composition of culture media and electroporation solution were systematically examined. Most of the experiments used to develop this procedure made use of 0.4-cm cuvettes without the Pulse Controller unit. When 0.2-cm cuvettes with the Bio-Rad Pulse Controller unit were used, a resistance of 200 Ω and a voltage of 8 to 10 kV/cm were typically employed. Unless otherwise noted, all experiments were carried out with plasmid DNA purified by CsCl-ethidium bromide density gradient centrifugation as described previously (8). For the experimental results depicted in Tables 2 through 5 and Fig. 1, the number of transformant colonies as determined from counting a minimum of three or four plates was used to calculate frequencies, and the standard deviations were less than 25%. Each experiment was repeated two to four times with similar results.

The optimized electroporation protocol is as follows. (i) Grow cells for 12 to 15 h in BYGT or M9-YE medium plus glycine at various concentrations. Use a glycine concentration that gives 70 to 90% reduction in the A_{660} of the culture, as compared with a control grown for the same period of time in the absence of glycine. Because the extent to which the growth of a given strain is inhibited by glycine is somewhat variable, it is necessary to prepare overnight cultures at several different concentrations to ensure that a culture showing the optimal growth inhibition will be obtained. Useful ranges of glycine concentrations for selected *E. faecalis* strains grown in BYGT, in our hands are as follows: OG1SSp, 1.5 to 4%; OG1RF, 1.25 to 3%; JH2-2, 4 to 6%; UV202, 4 to 6%. The addition of glycine to group B streptococci and to noncompetent *S. sanguis* also improved transformation frequencies. Group A streptococci and *B. anthracis* transformed without glycine and actually lysed during electroporation when grown in glycine (see Table 5). (ii) Dilute the culture from step i into fresh medium (containing the same or a slightly higher glycine concentration) to bring the A_{660} to 0.05 to 0.08. Incubate for 60 min (90 min for slow-growing strains) at 37°C. (iii) Chill the culture on ice, harvest the cells by centrifugation, and wash the cells in 1/3 volume of chilled electroporation solution (0.625 M sucrose–1 mM $MgCl_2$, adjusted to pH 4.0 with 1 N HCl). (iv) Harvest cells from the wash and suspend them in 1/30 to 1/100 the original volume of electroporation solution. Incubate on ice 30 to 60 min. (Cells may be frozen in a dry ice-ethanol bath and stored for at least 1 year at −70 to −85°C at this point and thawed in an ice water bath just before use.) (v) Add cells from step iv to the electroporation cuvette (800 μl if using 0.4-cm cuvettes, 50 to 100 μl if using 0.2-cm cuvettes). Add DNA (<10 μl in H_2O or low salt buffer). About 300 ng of DNA added should ensure a successful transformation; the use of more than about 1 to 1.5 μg of plasmid DNA appears to be unecessary (Fig. 1). (vi) Electroporate immediately with the 25 μF setting on the Gene Pulser. When using the 0.4-cm cuvettes, the field strength should be 6,250 V/cm. When using 0.2-cm cuvettes and the pulse controller unit, the resistance should be set to 200 Ω and the field strength should be 8,750 to 10,000 V/cm for *E. faecalis*. (vii) Place cells on ice for 1 to 2 min and then dilute into 2 volumes of THB medium (plus inducing concentrations of antibiotics, if using an inducible resistance gene as a selective marker). Incubate for 90 to 120 min at 37°C. (viii) Spread the cells on selective THB agar plates containing 0.25 M sucrose.

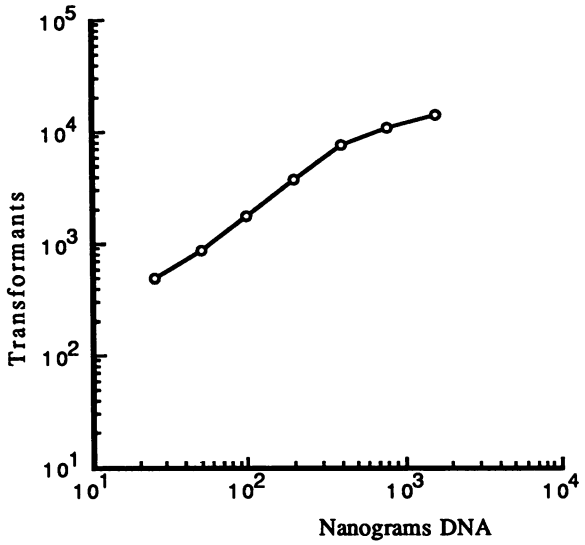

FIG. 1. Relationship of plasmid DNA concentration to transformation frequency. OG1RF cells were prepared for electroporation as described in footnote *a* of Table 3 (as for OG1SSp cells) and frozen at −85°C. Samples (0.8 ml) were thawed on ice and transformed with various amounts of gradient-purified pDL414 DNA with 0.4-cm cuvettes, no Pulse Controller, and 6,250 V/cm. Similar results were also obtained with pWM401 (data not shown).

RESULTS

Optimization of electroporation solution and growth conditions for *E. faecalis*. One of the first reports of electroporation of intact gram-positive bacteria, by Chassy and Flickinger (6), showed that optimal transformation frequencies were obtained when the time constants were in the range of 10 to 20 ms and when field strengths were such that approximately 50% of the cells were killed during the high-voltage pulse. These conditions were achieved with an electroporation solution consisting of *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid-buffered sucrose and $MgCl_2$. The current studies were initiated by determining empirically the composition of an electroporation solution that would produce similar results in *E. faecalis*. Exponential-phase cells were transformed with pDL414 DNA, with systematic variation of the following parameters relating to the composition of the electroporation solution: (i) pH (from <4 to 8, adjusted with either 1 N HCl or 1 N NaOH), (ii) sucrose concentration (0 to 1.2 M), (iii) $MgCl_2$ concentration (0 to 20 mM), and (iv) buffer (type and concentration). An unbuffered, acidic (pH 4.0) sucrose solution (0.625 M) containing 1 mM $MgCl_2$ consistently produced the highest frequencies of transformation.

Optimal growth conditions used to prepare *E. faecalis* cells for electroporation were determined by varying such parameters as medium composition, inoculum size, aeration, and phase of growth at the time of harvest. In a preliminary experiment, an overnight culture grown in M9-YE medium

TABLE 2. Effects of growth conditions on efficiency of transformation of *E. faecalis* by electroporation

Expt	Growth medium	Inoculum size[a] (%)	Growth period (h)	Transformants/μg of DNA
1[b]	M9-YE	20	1	8.0×10^3
	M9-YE	5	1	2.4×10^4
	M9-YE	20	2	4.5×10^3
2[c]	M9-YE	5	1	2.1×10^3
	BHI	5	1	3.0×10^2
	BYGT	5	1	4.8×10^3
	THB	5	1	6.0×10^2

[a] All inocula were from overnight cultures cultivated in the growth medium indicated. All growth media were supplemented with 20 mM DL-threonine.

[b] Electroporation was conducted with 4×10^8 OG1SSp cells suspended in 0.625 M sucrose–1 mM $MgCl_2$ adjusted to pH 4.5 with 1 N HCl. Transforming DNA (375 ng) was from pDL414.

[c] Electroporation was conducted with 5×10^8 OG1SSp cells suspended in 0.625 M sucrose–1 mM $MgCl_2$ adjusted to pH 4.0 with 1 N HCl. Transforming DNA was 200 ng of pDL414.

containing 10% threonine was diluted into fresh medium (20% inoculum, resulting in an initial A_{660} of 0.18), and samples were removed at 1-h intervals for electroporation. The cells from each sample were harvested, washed, and suspended to a cell density of 1.5×10^8 CFU/ml in an electroporation solution of 0.625 M sucrose and 1 mM $MgCl_2$ (pH 5.5) (this experiment was performed before the determination of the optimal pH for electroporation). Five hundred transformants per 200 ng of pDL414 DNA were obtained with cells grown for 1 h, 114 transformants were obtained for cells grown for 2 h, and about 40 transformants each were obtained for cells grown for 3, 4, or 5 h (the last two time points represent the end of the exponential growth phase and the entry of the culture into the stationary phase). Subsequent experiments (Table 2, experiment 1) confirmed that the highest frequencies were obtained by harvesting cells after 1 h of growth and that a smaller inoculum (5%, bringing the initial A_{660} to 0.05 to 0.07) would result in even higher transformation frequencies. The result of an experiment to test the effects of the medium composition on transformation frequencies is shown in Table 2 (experiment 2). Of the media tested, M9-YE and BYGT gave the best results. There were no differences in transformation frequency attributable to static growth versus growth with shaking or the maintenance of aerobic versus anaerobic conditions. Thus static growth with an air headspace was employed routinely for *E. faecalis* cultures used for electroporation.

Also tested were the effects on transformation frequencies of the addition of threonine and glycine, which are known to inhibit cell wall synthesis in streptococci and other organisms (5, 23). Although growth of the cells in the presence of threonine had only minor effects on the transformation frequencies (data not shown), significant effects were observed with glycine (Table 3). A large increase in transformation efficiency was observed when the extent of glycine inhibition of the cells used for electroporation increased from 40 to 85% (Table 3). Because the extent of inhibition of each strain tested showed some day-to-day variation, it was necessary to grow a series of overnight cultures containing different glycine concentrations to obtain a culture showing a growth inhibition of 70 to 90% (as determined by differences in A_{660} relative to that of a control with no glycine).

TABLE 3. Effects of glycine on the growth of *E. faecalis* and transformation by electroporation[a]

% Glycine	% Inhibition of overnight growth	Transformants/μg of DNA
1.5	40	8.0×10^2
2.0	43	1.2×10^4
2.5	85	4.0×10^4

[a] OG1SSp cells were grown overnight in BYGT plus the indicated concentration of glycine, diluted to an A_{660} of 0.07 in the same medium, incubated for 1 h, washed, suspended in optimized electroporation solution, and transformed with 300 ng of pDL414 DNA as described in the legend to Fig. 1.

This culture was then used to inoculate (5%, by volume) a fresh culture, which was then incubated for 1 h in medium containing the same glycine concentration. If the overnight cultures were incubated for more than 12 to 15 h, a significant number of glycine-resistant mutants arose in the population and poor transformation efficiencies were obtained. Overnight growth in the presence of glycine was more efficient than additions of glycine only during the 1-h incubation period before harvest. Several other variables were also examined, such as the composition of the medium used to select transformed cells and the time of incubation for phenotypic expression after electroporation. The final protocol described in Materials and Methods represented the optimal conditions for transformation of *E. faecalis*, with the growth conditions and composition of the electroporation solution constituting the critical variables.

The highest transformation efficiencies occurred when there was a 20 to 50% reduction in the viable cell count after electroporation. Transformation frequencies dropped sharply with either more or less cell killing, but within the 20 to 50% range there was no strict correlation between amount of killing and transformation efficiency. Glycine concentrations resulting in 70 to 90% growth inhibition varied from strain to strain. For several commonly used *E. faecalis* strains, inoculation of a series of overnight cultures in the range of glycine concentrations listed in Materials and Methods generally yielded a culture with the appropriate level of inhibition. Cells prepared for electroporation in electroporation solution could be frozen in a dry ice-ethanol bath and maintained at −70 to −85°C. These frozen cells, if thawed in an ice-water bath and pulsed immediately, yielded the same (or higher) transformation frequencies as cells from the same batch before freezing. Cells of the more commonly used strains were prepared routinely from 300- to 600-ml cultures and stored frozen for future use.

Effects of DNA concentration and purity on transformation frequency. The relationship between plasmid DNA concentration and transformation frequency was assessed with gradient purified pDL414 DNA and *E. faecalis* OG1RF (Fig. 1). DNA concentrations between 25 and 500 ng/ml exhibited a concentration-dependent increase in transformation frequency, consistent with single-hit kinetics. Saturation was observed between 1 and 2 μg of transforming DNA per ml. More than 5×10^4 transformants per μg of pDL414 DNA were obtained under optimal conditions without the use of the Pulse Controller unit for the Gene Pulser. Smaller plasmids generally produced higher transformation frequencies, although plasmids greater than 30 kb in size also transformed. Plasmids prepared by the screening procedure of Anderson and McKay (1) transformed at approximately 5% the efficiency of comparable concentrations of gradient purified plasmid DNA (data not shown).

TABLE 4. Effects of various field strengths and time constants on transformation frequency of *E. faecalis*[a]

Field strength (V/cm)	Resistance (Ω)	Time constant (h)	Cells surviving	Transformants/ ml/μg of DNA
5,000	200	4.0	9×10^7	1.1×10^4
5,000	800	16.7	7×10^7	1.08×10^5
6,250	200	4.5	8.5×10^7	7.8×10^4
6,250	800	13.7	6.5×10^7	1.06×10^5
7,500	200	4.5	6.0×10^7	1.08×10^5
7,500	800	13.0	2.4×10^7	4.8×10^4
8,750	200	4.5	4.5×10^7	1.2×10^5
8,750	800	12.8	1.5×10^6	1.4×10^3
10,000	200	4.5	4.0×10^7	1.08×10^5
10,000	800	12.4	1.0×10^6	6.0×10^2

[a] Samples of 50 μl containing about 10^8 OG1RF cells in electroporation solution as described in footnote *a* of Table 3 were mixed with 50 ng of pWM401 DNA and electroporated as indicated.

Effects of field strength and time constant on transformation frequency. The results described above were obtained at a field strength of 6,250 V/cm with time constants in the range of 11 to 18 ms in 0.4-cm cuvettes. Since optimal transformation frequencies were obtained at the highest attainable voltage, it seemed possible that these frequencies might be increased if even higher field strengths could be achieved. This was possible when the Bio-Rad Pulse Controller became available. Subsequently, the effects of increased field strengths on the efficiency of transformation were tested. Approximately 9×10^8 CFU of *E. faecalis* OG1RF and 600 ng of pWM401 DNA were mixed in 500 μl of electroporation solution; 50-μl aliquots (in 0.2-cm cuvettes) were pulsed at different field strengths and time constants by using the Pulse Controller to vary the time constant electronically. There was a broad range of conditions in which high transformation frequencies could be obtained (Table 4). This range could be defined by the product of the field strength and time constant.

Transformation was most efficient at high field strengths (7,500 to 10,000 V/cm) when short time constants were used. Increases in the time constant at these higher field strengths resulted in extensive killing of the cells and in lower transformation efficiencies. Voltages above 10,000 V/cm often resulted in arcing across the top of the cuvette and were not attempted further. Conditions equivalent to those used in earlier experiments with the 0.4-cm cuvettes without the Pulse Controller (6,250 V/cm and time constants of 13.7 ms) were nearly as efficient as the higher voltages obtained with 0.2-cm cuvettes and the Pulse Controller. However, in the latter situation smaller volumes were used. Consequently, higher DNA concentrations were possible with less total DNA. Preliminary results indicated that cells suspended in electroporation solution to three times the cell density used to produce the results shown in Table 4 yielded approximately three times the total number of transformants. Thus, from an economic point of view, the smaller cuvettes provide the maximum number of transformants while requiring fewer cells and less DNA.

Application of electroporation procedure to other bacterial species. The protocol described in Materials and Methods was optimized for the transformation of *E. faecalis*. However, it provided transformants at reasonable frequencies when applied to strains of other streptococcal genera as well as *B. anthracis* (Table 5). In addition to four of the more commonly used strains of *E. faecalis*, transformants were also obtained with a group A *Streptococcus* species and a group B streptococcal isolate as well as with a strain of *S. sanguis* (FW213) that is unable to achieve a state of natural competence. As noted in Materials and Methods, the addition of glycine to group B streptococci and noncompetent strains of *S. sanguis* also improved the transformation frequencies. Group A streptococci and *B. anthracis*, on the other hand, were transformable in the absence of glycine and actually lysed during electroporation when grown in the presence of glycine. The results obtained with these different organisms suggest that the method may be broadly applicable to the transformation of many different gram-positive bacteria, at least as a starting point for establishing optimal conditions for each genus and strain. This protocol has also been used, with minor modifications (30a), to transform *Lactococcus lactis* at frequencies comparable to those reported by Holo and Nes (15).

TABLE 5. Gram-positive bacteria transformed by electroporation

Organism	Plasmid	Transformants/ μg of DNA
E. faecalis OG1RF	pDL414	5×10^4
E. faecalis OG1RF	pWM401	1.2×10^5
E. faecalis OG1SSp	pDL414	1.6×10^4
E. faecalis JH2-2	pDL414	2.5×10^4
E. faecalis UV202	pDL414	4×10^3
S. pyogenes	pDL414	1.6×10^3
S. agalactiae	pDL414	4.8×10^3
S. sanguis	pDL414	1.4×10^4
B. anthracis	pPL703[a]	1×10^4

[a] Plasmid DNA was purified on a CsCl-ethidium bromide density gradient as described by Bartkus and Leppla (2).

Construction of an improved shuttle vector. The development of an electroporation protocol that appeared to facilitate transformation of several streptococcal species as well as species of other gram-positive bacterial genera was followed by the construction of a plasmid vector that should be useful in the cloning of virtually any streptococcal or enterococcal genetic determinant in *E. coli* and in the subsequent transfer of the cloned DNA back to an appropriate gram-positive host strain. The construction of the shuttle vector (Fig. 2) was initiated with a chimeric plasmid, pDL269. This plasmid consists of pUC19 with a streptococcal spectinomycin resistance gene cloned into the multiple cloning sequence. This resistance determinant is expressed constitutively in *E. coli* as well as in streptococci (17a). Plasmid pDL269 was digested with *Ava*II, self-ligated, and used to transform *E. coli* with selection for resistance to spectinomycin. A spectinomycin-resistant, ampicillin-sensitive clone contained a plasmid, pDL270, with a 220-bp deletion in the structural gene for β-lactamase. A 1.5-kb *Cla*I DNA fragment containing a kanamycin resistance gene of streptococcal origin was treated with the Klenow fragment of DNA polymerase I to create blunt ends and then inserted into *Ava*II-digested, Klenow-treated, pDL270 with T4 DNA ligase. This ligation mixture was used to transform *E. coli*. Selection for resistance to spectinomycin and kanamycin produced a clone containing plasmid pDL271. This plasmid was digested with *Pvu*II and *Nde*I, treated with Klenow fragment, and ligated to a gel-purified 3.5-kb fragment from pJDC9 containing the pUC19 multiple cloning sequence region flanked by strong *E. coli* transcription terminators as described by Chen and Morrison (7). The pJDC9-derived fragment was obtained by digestion of this plasmid with *Bal*I

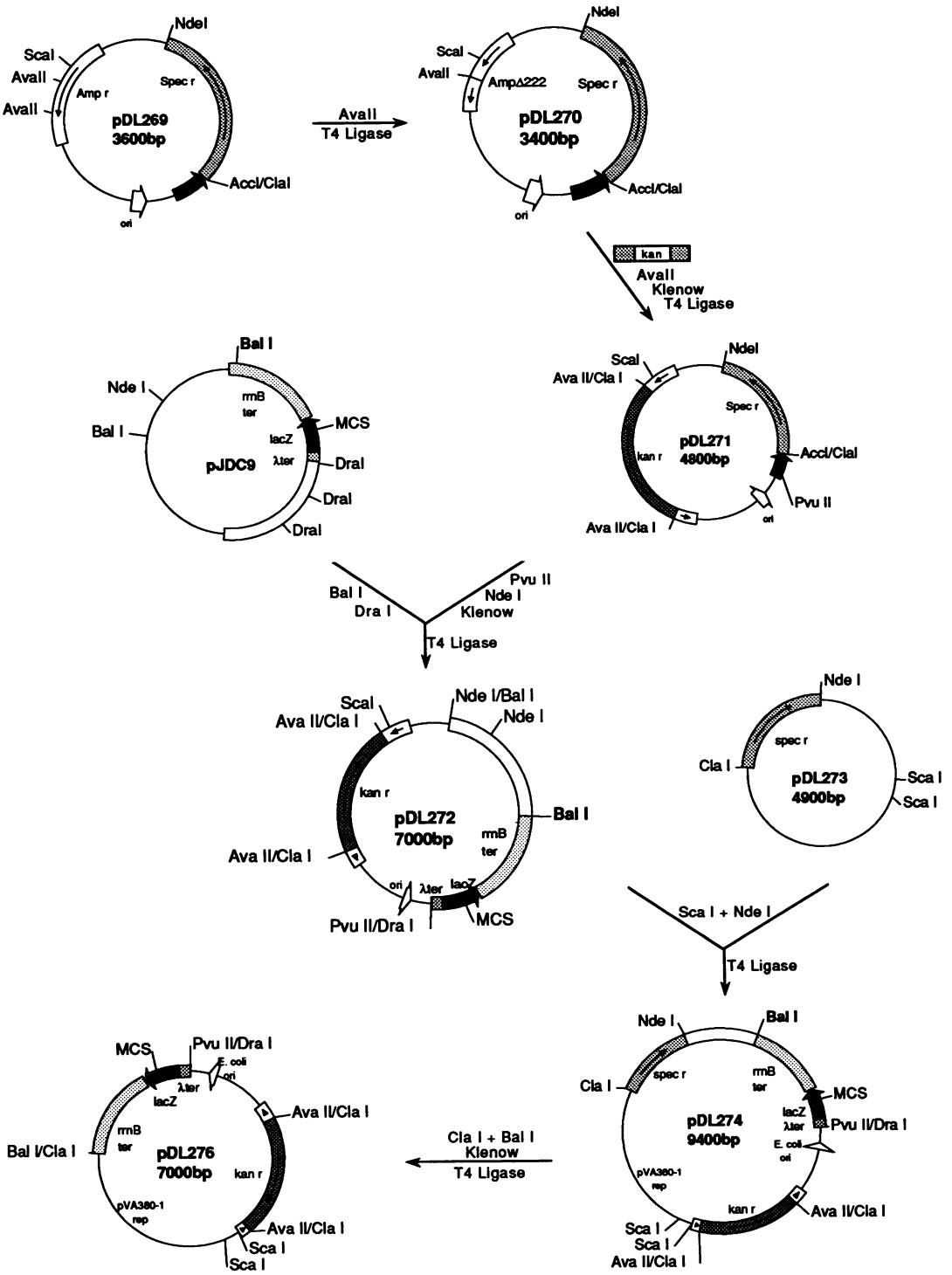

FIG. 2. Construction of pDL276. Construction details are described in Results.

and *Dra*I. Transformation of *E. coli*, with selection for resistance to kanamycin and counterselection for spectinomycin sensitivity, yielded a clone containing plasmid pDL272.

A potential *E. coli-Streptococcus* and *Enterococcus* shuttle vector was constructed by the insertion of a streptococcal replication origin into pDL272. The latter was digested with *Sca*I and *Nde*I and ligated to similarly digested pDL273, which contained the replication region of the streptococcal plasmid pVA380-1 (24) and the spectinomycin resistance determinant described above. The ligation mixture was used to transform a competent culture of *S. sanguis* Challis. A spectinomycin- and kanamycin-resistant clone was obtained that contained the chimeric plasmid pDL274. The ability of

this plasmid to replicate in *E. coli* was then demonstrated by transformation of strain DH5α. The size of pDL274 was reduced further by digestion with *Cla*I and *Bal*I, treatment with Klenow fragment, blunt end ligation, and transformation of *E. coli* with selection for resistance to kanamycin. A 6.9-kb plasmid, designated pDL276, was obtained from one of the transformant clones derived from this experiment. This plasmid contained the origin region common to the pUC plasmids, the streptococcal kanamycin resistance gene expressed constitutively in streptococcal and *E. coli* host strains, the essential replication functions of pVA380-1, and the multiple cloning sequence region of pUC19 flanked by strong transcriptional terminators. This plasmid was used to transform *S. sanguis* Challis by natural transformation and other streptococcal species by electroporation. It was also used as a vector for the cloning of a number of streptococcal-enterococcal genetic determinants, as described below.

Use of electroporation and pDL276 for cloning in *E. coli*, enterococci, and other gram-positive bacteria. Plasmid pDL276 has been employed in our laboratories for several cloning and sequencing applications which will be described in detail in subsequent publications. These results will be summarized briefly here to illustrate the potential applications of the system. The 7.4-kb *Eco*RI c and 4.2-kb *Eco*RI e fragments and a 7.2-kb *Sal*I-*Xba*I fragment of the pheromone-inducible conjugative *E. faecalis* plasmid pCF10 (8) have been cloned and used for sequence analysis in *E. coli* (15a, 15b). Chromosomal genes from group B streptococci encoding surface proteins (4a) and pigment (31a) have been cloned in *E. coli* and transferred to *E. faecalis* by electroporation; the cloned gene products were expressed in both hosts.

To test the feasibility of using this system for direct cloning into *E. faecalis*, pCF10 DNA was digested with *Hinc*II, and a 5-kb fragment believed to contain a *tetM* tetracycline resistance determinant (8) was purified by agarose gel electrophoresis. This DNA was ligated to *Sma*I-digested pDL276. The ligated DNA was transformed into strain OG1RF by electroporation, and several tetracycline-resistant transformants were analyzed by restriction enzyme digestion and Southern hybridization. These analyses (not shown) indicated that the 5-kb fragment had, in fact, been inserted into pDL276 and cloned directly into *E. faecalis*.

DISCUSSION

The electroporation system and shuttle vector described here have facilitated a number of experiments involving cloning and transfer of streptococcal genes in our laboratories. The electroporation system for *E. faecalis* is easier and in our hands more efficient than protoplast transformation. The vector has facilitated cloning and sequencing manipulations that were either impossible or very difficult with previously existing vectors. For example, the *Eco*RI c and e fragments of pCF10, which have been cloned into the low-copy-number shuttle vector pWM402 (9), could not be cloned in pUC vectors or other vectors useful for sequencing. These same fragments were stably maintained in *E. coli* and in *E. faecalis* when inserted into pDL276, which utilizes the same replicon as the pUC family of plasmids (35). On the basis of the relative intensity of plasmid DNA bands on ethidium bromide-stained agarose gels, we estimate that the copy number of pDL276 and pDL276-derived chimeric plasmids is four to five times that of pWM402 in both *E. coli* and *E. faecalis* hosts and similar to the unamplified copy number of pUC19 in *E. coli*. Although we have not examined the reason for our success in cloning with pDL276, our results, combined with those of Chen and Morrison (7), would indicate that the presence of transcription terminators may be very important in stabilizing the insertion of streptococcal DNA in these vectors.

During the past 1 to 2 years, numerous reports of electroporation of various gram-positive microbes have appeared in the literature (6, 15, 28). In agreement with the recent report of Holo and Nes (15), we found that glycine enhances susceptibility of enterococci and certain streptococci to transformation by electroporation. However, among gram-positive bacteria that are either insensitive or overly sensitive to cell wall inhibition by glycine, the other critical parameters for successful electroporation described here may be of equal importance in the development of gene transfer systems. Also, since pDL276 appears to have a fairly broad host range, this vector may have considerable utility for recombinant DNA manipulations in gram-positive and gram-negative bacterial hosts.

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