Transformation of Dairy *Leuconostoc* Using Plasmid Vectors from *Bacillus*, *Escherichia*, and *Lactococcus* Hosts

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**ABSTRACT**

Conditions that allow efficient genetic transformation of dairy *Leuconostoc* by electroporation were determined. The technique allowed transformation of *Leuconostoc mesenteroides* ssp. *cremoris*, *Leuconostoc mesenteroides* ssp. *dextranicum*, and *Leuconostoc lactis* with plasmid pNZ12. Optimized conditions resulted in transformation efficiencies of up to $2 \times 10^6$/µg of pNZ12 DNA for *L. cremoris* 44-4. Further, cloning vectors pNZ18, pGK12, pGK13, pGL3, pBD64, pGB301, pDB101, and pAMB1 from lactococcal, *Bacillus*, and *Escherichia* hosts were introduced and maintained by *L. cremoris* 44-4, extending the range of vectors functional in *Leuconostoc*. (Key words: *Leuconostoc*, transformation, plasmids)

Abbreviation key: EPB = electroporation buffer.

**INTRODUCTION**

Electroporation has been demonstrated to be an effective method for transforming a broad range of prokaryotic as well as eukaryotic organisms. Various Gram-positive lactic acid bacteria, organisms for which no natural competence system has been established, have been transformed by electroporation. A review by Chassey et al. (5) describes the species of bacteria that have been transformed using this technique.

Recently, two reports have been published that describe transformation of members of the genus *Leuconostoc* (7, 16). However, the electroporation buffers (EPB) and the voltage levels used in these studies resulted in absence or extremely low levels of transformation when applied to strains used in this study. These species form a separate phylogenetic subgroup, indicating a lack of close relatedness with other *Leuconostoc* (27). We, therefore, developed conditions for transformation of these *Leuconostoc* species. Optimization of voltage levels and buffer composition was made to obtain high frequency transformation in *Leuconostoc mesenteroides* ssp. *cremoris*, *Leuconostoc mesenteroides* ssp. *dextranicum*, and *Leuconostoc lactis*, the species most commonly used in dairy fermentations. Further, we report here the selection for and maintenance of several cloning and shuttle vectors successfully used in other Gram-positive bacteria. Because transformed *Leuconostoc* proved much more difficult to lyse during plasmid DNA isolation procedures, a modified lysis protocol for the transformants was developed.

**MATERIALS AND METHODS**

**Bacterial Strains and Media**

Strains and plasmids used in this study are listed in Tables 1 and 2. *Leuconostoc* strains were maintained in MRS-V8 broth (55 g/L of dehydrated MRS broth; Difco, Detroit, MI), and 250 mL/L of filtered V-8 juice (Campbell Soup Co.), lactococci were maintained in M17G (M17 with .5% glucose) (26) containing chloramphenicol or erythromycin at 5 µg/ml, and *Bacillus* and *Escherichia coli* strains were
TABLE 1. Recipient strains for transformation by electroporation, their source, and plasmid complements.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Source¹</th>
<th>Plasmid complement (kb)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Escherichia coli</em> V517</td>
<td>OSU</td>
<td>56, 76, 58, 53, 41, 32, 2.8, 2.1</td>
<td>(17)</td>
</tr>
<tr>
<td><em>Leuconostoc mesenteroides</em> sspp. cremoris 44-4</td>
<td>E. R. Vedamuthi</td>
<td>18.5</td>
<td>(24)</td>
</tr>
<tr>
<td><em>Leuconostoc mesenteroides</em> sspp. cremoris 19254</td>
<td>ATCC</td>
<td>61.5, 22.0, 19.5, 12.2, 6.2</td>
<td>This study</td>
</tr>
<tr>
<td><em>Leuconostoc mesenteroides</em> sspp. dextranicum 181</td>
<td>OSU</td>
<td>42.5</td>
<td>(21)</td>
</tr>
<tr>
<td><em>Leuconostoc mesenteroides</em> sspp. dextranicum 19255</td>
<td>ATCC</td>
<td>26.5</td>
<td>This study</td>
</tr>
<tr>
<td><em>Leuconostoc lactis</em> 19256</td>
<td>ATCC</td>
<td>17.1, 3.6</td>
<td>This study</td>
</tr>
</tbody>
</table>

¹OSU = Oregon State University, ATCC = American Type Culture Collection, Rockville, MD.

Plasmid DNA was isolated from *Leuconostoc* transformants grown under nonselective conditions by a modification of the procedure of Anderson and McKay (1). This modification included an additional incubation of the cells with a proteolytic enzyme following digestion of the cell wall by lysozyme. Proteinase K (Sigma Chemical Co., St. Louis, MO), 10 mg/ml in distilled water, was added to the cell suspension at a final concentration of 100 μg/ml and incubated at 55°C for 30 min. Alternatively, Pronase E (Sigma Chemical Co., St. Louis, MO), 20 mg/ml in distilled water, was added at 200 μg/ml and incubated at 37°C for 30 min. After proteolytic digestion, the remainder of the Anderson and McKay procedure was followed.

TABLE 2. Vectors transformed into *Leuconostoc* species by electroporation.

<table>
<thead>
<tr>
<th>Vector</th>
<th>Size (kb)</th>
<th>Host</th>
<th>Drug resistance</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>pNZ12</td>
<td>4.3</td>
<td><em>Escherichia coli</em> MC1061</td>
<td>CM¹ KM²</td>
<td>(9)</td>
</tr>
<tr>
<td>pNZ18</td>
<td>5.7</td>
<td><em>E. coli</em> MC1061</td>
<td>CM KM</td>
<td></td>
</tr>
<tr>
<td>pGK12</td>
<td>4.9</td>
<td><em>Bacillus subtilis</em> 8G5</td>
<td>CM EM¹</td>
<td>(15)</td>
</tr>
<tr>
<td>pGK13</td>
<td>4.5</td>
<td><em>B. subtilis</em> 8G5</td>
<td>CM EM</td>
<td>PC²</td>
</tr>
<tr>
<td>pGL3</td>
<td>5.0</td>
<td><em>E. coli</em> MC1061</td>
<td>CM KM</td>
<td></td>
</tr>
<tr>
<td>pBD64</td>
<td>4.9</td>
<td><em>B. subtilis</em> BD170</td>
<td>CM KM</td>
<td>(13)</td>
</tr>
<tr>
<td>pDBG101</td>
<td>18.3</td>
<td><em>Lactococcus lactis</em> JK101</td>
<td>EM</td>
<td>(3)</td>
</tr>
<tr>
<td>pGB301</td>
<td>10.1</td>
<td><em>Lactococcus lactis</em> JK301</td>
<td>CM EM</td>
<td>(2)</td>
</tr>
<tr>
<td>pAMβ1</td>
<td>26.5</td>
<td><em>Lactococcus lactis</em> 21003β</td>
<td>EM</td>
<td>(6)</td>
</tr>
</tbody>
</table>

¹Chloramphenicol.
²Kanamycin.
³Personal communication, W. M. de Vos.
⁴Erythromycin.
⁵Personal communication, J. Kok.
by centrifugation, washed twice with one-half original culture volume of ice-cold distilled water (Millipore, Bedford, MA), then washed once with .1 original culture volume of ice-cold EPB. The EPB was 1 mM \( \text{K}_2\text{HPO}_4\cdot\text{KH}_2\text{PO}_4 \), pH 7.4, containing 1 mM MgCl\(_2\) and either .5 \( M \) sucrose, .5 \( M \) maltose, or .3 \( M \) raffinose. Washed cells were resuspended in .02 original culture volume of ice-cold EPB and kept on ice until used.

Transformation was performed using a Gene-Pulsar unit combined with a Pulse Controller (Bio-Rad, Richmond, CA). Cuvettes with an interelectrode distance of .2 cm were employed. Forty microliters of concentrated cells were mixed with 1.0 \( \mu \)g of transforming DNA in a microfuge tube, transferred to cold electroporation cuvettes, and placed on ice for 5 min. The mixture was then given a single discharge from a 25-\( \mu \)F capacitor with various pulse controller and voltage settings. Immediately following the pulse, cells were resuspended in MRS-V8 broth to a final volume of 1 ml and incubated for 1 h at 30°C. Dilutions of the transformed cells were made in MRS-V8 broth and spread plated on MRS-V8 agar (MRS-V8 broth supplemented with 1.5% agar) containing chloramphenicol or erythromycin (Sigma) at 5 \( \mu \)g/ml. After 48 h of incubation at 30°C, transformation frequencies were calculated and isolated colonies selected for confirmation of transformation. Acquisition of the transforming plasmid was confirmed by agarose gel electrophoresis (18) of plasmid DNA isolated from putative transformation. Transformants were maintained in MRS-V8 broth containing chloramphenicol or erythromycin at 5 \( \mu \)g/ml. Controls receiving no DNA were included in each experiment. \textit{Escherichia coli} V517 plasmids (17) were used as molecular size standards.

**RESULTS**

Optimization of Transformation Protocol

Sucrose has been the most utilized osmotic stabilizer in EPB used for transformation of lactic acid bacteria (5, 7, 16) although raffinose also has been used (25). Initial experiments with \textit{Leuconostoc mesenteroides} ssp. \textit{cremoris} 44-4 indicated that the optimal voltage for transformation of this strain was near 8 kV/cm in EPB containing .5 \( M \) sucrose (Figure 1). Subsequent experiments with other \textit{Leuconostoc} strains yielded similar results. Substitution of either maltose or raffinose for sucrose in the EPB resulted in a 10-fold decrease in transformation frequency in \textit{Leuconostoc mesenteroides} ssp. \textit{cremoris} 44-4 with pNZ12 as the transforming plasmid (Figure 2). Conditions from these experiments, which gave optimal transformation frequencies in \textit{Leuconostoc mesenteroides} ssp. \textit{cremoris} 44-4 (8 kV/cm, 400 ohms, and sucrose-containing buffer), were employed in subsequent experiments.

![Figure 1](image1.png)

**Figure 1.** Transformation frequencies of \textit{Leuconostoc mesenteroides} ssp. \textit{cremoris} 44-4 with pNZ12 at increasing voltage levels. Gene pulsar settings were 400 ohms and 25 \( \mu \)F.

![Figure 2](image2.png)

**Figure 2.** Transformation frequencies of \textit{Leuconostoc mesenteroides} ssp. \textit{cremoris} 44-4 with pNZ12 in electroporation buffer containing sucrose (EPBS), maltose (EPBM), and raffinose (EPBR).
TRANSFORMATION OF LEUCONOSTOC

Figure 3. Plasmid profiles of Leuconostoc mesenteroides ssp. cremoris 44-4 transformants. Lane A, Escherichia coli V517; lane B, pNZ12; lane C, pNZ18; lane D, pGK12; lane E, pGK13; lane F, pGL3; lane G, pBD64; lane H, pGB301; lane I, pDB101; and lane J, pAMF1.

Table 4 shows other Leuconostoc strains that have been transformed using the vector pNZ12 and the corresponding transformation frequencies observed for each strain. Figure 4 is a photograph of the plasmid profiles of the transformants containing pNZ12.

Selection of Transformants

Selection with either chloramphenicol or erythromycin was possible for strains used in this study. No spontaneous antibiotic-resistant colonies grew on control plates containing 5 µg/ml chloramphenicol or erythromycin, and all presumptive transformants examined had acquired the transforming plasmid as determined by agarose gel separation. Chloramphenicol selection allowed for recovery of more transformants than selection by erythromycin in Leuconostoc. Selection of transformants for plasmids each containing both resistance genes (pGK12 and pGB301) was done on the same
TABLE 3. Transformation frequencies of various vectors in *Leuconostoc mesenteroides* ssp. *cremoris* 44-4.¹

<table>
<thead>
<tr>
<th>Vector</th>
<th>Transforms in DNA</th>
<th>Antibiotic selection</th>
</tr>
</thead>
<tbody>
<tr>
<td>pNZ12</td>
<td>2.0 × 10⁵ µg</td>
<td>CM²</td>
</tr>
<tr>
<td>pNZ18</td>
<td>2.0 × 10⁵ µg</td>
<td>CM</td>
</tr>
<tr>
<td>pGL1</td>
<td>7.0 × 10⁴ µg</td>
<td>CM</td>
</tr>
<tr>
<td>pGK12</td>
<td>8.2 × 10⁵ µg</td>
<td>CM</td>
</tr>
<tr>
<td>pGK13</td>
<td>1.0 × 10⁶ µg</td>
<td>CM</td>
</tr>
<tr>
<td>pBD64</td>
<td>2.3 × 10⁵ µg</td>
<td>CM</td>
</tr>
<tr>
<td>pGB301</td>
<td>1.5 × 10⁵ µg</td>
<td>CM</td>
</tr>
<tr>
<td>pDB101</td>
<td>9.7 × 10⁴ µg</td>
<td>EM³</td>
</tr>
<tr>
<td>pAMB1</td>
<td>6.0 × 10⁴ µg</td>
<td>EM</td>
</tr>
</tbody>
</table>

¹Gene pulsar settings: 8 kV/cm, 400 ohms, 25 μF and 1 mM 2-mercaptoethanol in the electroporation buffer.

²Chloramphenicol.

³Erythromycin.

transformed cells, resulting in at least 50% more chloramphenicol-resistant transformants produced than erythromycin-resistant transformants (data not shown). Neither the total number of cells nor the chloramphenicol-resistant cell count increased during incubation in MRS-V8 broth for up to 2 h following transformation (data not shown).

Additional Electroporation Parameters

Cells frozen in EPB at -70°C could be stored for subsequent use. However, a gradual loss in transformability was observed. A 10-fold decrease in transformation frequency of *Leuconostoc mesenteroides* ssp. *cremoris* 44-4 occurred over a 90-d storage period (data not shown).

The addition of 2-mercaptoethanol to the EPB in 1 mM concentrations increased the transformation frequencies of some of the strains. Strains *Leuconostoc mesenteroides* ssp. *cremoris* 44-4, *Leuconostoc mesenteroides* ssp. *cremoris* 19254, and *Leuconostoc mesenteroides* ssp. *cremoris* 19255 all exhibited minor increases in transformation frequencies in the presence of the reducing agent (data not shown).

All *Leuconostoc* strains in this study contained a native plasmid or plasmids (Table 1). No change in mobility or curing of the native plasmids was noted for any of the *Leuconostoc* upon acquisition of a transforming plasmid (Figures 3 and 4).

**DISCUSSION**

Conditions for transforming several *Leuconostoc* species have been determined. Higher voltage pulses were required than indicated by Luchansky et al. (16) or David et al. (7). Differences in EPB compositions may influence the conditions required for optimal transformation. Negligible cell death was observed in this study, which is in accordance with results obtained by David et al. (7).

We have shown that a variety of replicons can be introduced into and maintained in *Leuconostoc*. These replicons originated in Gram-positive microorganisms of the *Lactococcus*, *Streptococcus*, and *Bacillus* genera.

Transformation frequencies observed in this study were higher when sucrose was used as the osmotic stabilizer in EPB (Figure 2). Somkuti and Steinberg (25) used raffinose as the osmotic stabilizer in EPB when electroporating *Streptococcus thermophilus*, and Otts and Day (22) reported that maltose was the osmotic stabilizer of choice when regenerating prokaryotic *Leuconostoc mesenteroides*. However, substitution of these sugars for sucrose decreased the frequencies of transformation for the strains used in this study.

Selection for chloramphenicol resistance allowed for greater recovery of transformants.

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**TABLE 4. Transformation frequencies of *Leuconostoc* strains using pNZ12.¹**

<table>
<thead>
<tr>
<th>Strain</th>
<th>Transforms/µg pNZ12</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Leuconostoc mesenteroides</em> ssp. <em>dextranicum</em> 181</td>
<td>1.0 × 10²</td>
</tr>
<tr>
<td><em>Leuconostoc mesenteroides</em> ssp. <em>cremoris</em> 19254</td>
<td>1.0 × 10¹</td>
</tr>
<tr>
<td><em>Leuconostoc mesenteroides</em> ssp. <em>dextranicum</em> 19255</td>
<td>6.0 × 10⁴</td>
</tr>
<tr>
<td><em>Leuconostoc lactis</em> 19256</td>
<td>1.2 × 10³</td>
</tr>
</tbody>
</table>

¹Gene pulsar settings: 8 kV/cm, 400 ohms, and 25 µF.
This may have been due to the need for a longer expression period for the erythromycin resistance gene than for the chloramphenicol resistance gene and may be corrected by lengthening the incubation period between transformation and plating. Although a 1-h expression period was used in this study, a period of ≥ 2 h may improve frequencies with erythromycin selection.

Reducing agents increased transformation frequencies observed in some strains of *Leuconostoc*. Gilliland and Speck (11, 12) studied the mechanism of inactivation of microorganisms by electrohydraulic shock and indicated that free radicals generated during the discharge of a capacitor into a buffer may be involved. When the reducing reagent 2-mercaptoethanol was included in the EPB, the number of survivors did not increase, but for some strains, increased transformation frequencies occurred when the reducing agent was present. Dilution of the cells in growth medium immediately after pulsing may also provide benefits similar to a reducing agent by reducing the concentration of free radicals or by providing substrates for oxidation reactions. Significant decreases in transformation frequencies have been observed in electrotomnformed *E. coli* strains that were not diluted in media immediately following transformation (10).

There is considerable interest among several research laboratories on genetic exchange between *Leuconostoc* and lactococci (7, 8, 14, 23). Lactococci, widely used in milk fermentations, suffer from plasmid instability, especially for the proteinase gene (19). We have noted, as have David et al. (7), that plasmids introduced into *Leuconostoc* are extremely stable, and the advantages, therefore, of introducing proteinase and lactose genes into these organisms are obvious. Such *Leuconostoc* also may be more phage resistant than lactococci. Hopefully the improved electrotomnformation system described here will facilitate these develop-
ments leading to improved Leuconostoc for milk fermentations.

ACKNOWLEDGMENTS

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REFERENCES