Plasmid Transformation by Electroporation of *Leuconostoc paramesenteroides* and Its Use in Molecular Cloning

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In this report, we demonstrate the utility of electroporation as an efficient method for genetic transformation of *Leuconostoc paramesenteroides*. We optimized several factors which determine the transformation frequency, resulting in transformation efficiencies of up to $4 \times 10^7$ transformants per µg of pNZ12 DNA, which contains the promiscuous *Lactococcus lactis* pSH71 replicon. Slightly lower efficiencies were obtained with a deletion derivative of the broad-host-range plasmid pAMBl. These plasmids could be stably maintained in *L. paramesenteroides* NZ6009 for more than 100 generations, even in the absence of selective pressure. In order to show the use of the developed host-vector system, we cloned the *Lactococcus lactis* gene encoding phospho-β-galactosidase in *L. paramesenteroides*. Expression of this heterologous gene in *L. paramesenteroides* under control of *Lactococcus lactis* expression signals was evident from the presence, in transformants, of phospho-β-galactosidase activity and a specific phospho-β-galactosidase protein band on Western blots (immunoblots). In addition, we transformed a lactose-deficient derivative of *L. paramesenteroides* with a plasmid carrying a *Lactococcus lactis*-Escherichia coli lacZ gene fusion. The resulting transformants synthesized higher levels of β-galactosidase, indicating the efficiency of heterologous gene expression signals in *L. paramesenteroides*.

Bacteria of the genus *Leuconostoc* are gram-positive cocci that are phenotypically and ecologically related to group N streptococci (13). *Leuconostoc* spp. are widely used in the food industry; they are involved in the fermentation of vegetables, in wine making, and in the manufacture of fermented milk products, such as butter, buttermilk, and cheese (12). Their wide application as components of starter cultures in the dairy industry relies on their ability to produce CO₂ and the aroma component diacetyl from citrate. Furthermore, the heterofermentative conversion of lactose contributes to the formation of additional amounts of CO₂.

During the last few years, an increasing number of reports on genetic studies of *Leuconostoc* spp. have been published. It is known that many *Leuconostoc* dairy strains contain plasmid DNA (27), and the conjugal transfer of plasmid DNA from *Lactococcus lactis* to *Leuconostoc* spp. has been reported (30, 34, 35). The plasmids used in these studies encode readily identifiable properties, such as lactose fermentation, nisin production, and resistance to erythromycin and chloramphenicol. In addition, the transposon Tn919, conferring tetracycline resistance, has been transferred to *Lactococcus lactis* (18).

Until now, no host-vector system has been described for *Leuconostoc* species. The development of a plasmid-mediated DNA transformation system for *Leuconostoc* spp. is, however, a prerequisite for the application of recombinant DNA technology for both the fundamental genetic analysis and the practical improvement of these bacteria.

Electroporation has been used to transfer DNA into a variety of cell types by production of high-intensity electric fields of short duration, during which the cell is reversibly made permeable. Many reports have described transformation by electroporation of mammalian cells (28) and of plant and yeast protoplasts (11, 17). In addition, electroporation has been reported for gram-negative bacteria, such as *Escherichia coli* (9, 10) and *Campylobacter jejuni* (24), as well as for gram-positive bacteria, including the lactic acid bacteria *Lactobacillus casei* (5), *Lactococcus lactis* (16, 29), and *Streptococcus thermophilus* (32). Transformation by electroporation is easily and rapidly performed compared with transformation systems described for various lactic acid bacteria that involve protoplast preparation and complex regeneration media (14, 19). In the present communication, we describe the optimization of an electroporation procedure for *Leuconostoc paramesenteroides* and the use of the procedure in molecular cloning.

**MATERIALS AND METHODS**

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

Strain NZ6009 is a spontaneous mutant of *Leuconostoc* strain Lc9 and lacks an endogenous cryptic plasmid with an approximate size of 3 kilobase pairs. Its sensitivity to the Lcm9-specific bacteriophage BA-Lcm9 is the same as the sensitivity of the parent strain. Both strain Lc9 and its phage BA-Lcm9, which was used for strain identification in subsequent experiments, were obtained from B. Lébert, Nestec Research Laboratory, Vevey, Switzerland.

Strain NZ6091 is a lactose-deficient derivative of strain NZ6009 that lacks β-galactosidase activity and that was isolated after plasmid curing by electroporation (unpublished results).

**Growth of bacteria.** *E. coli* and *Bacillus subtilis* strains were propagated in L broth at 37°C; all further manipulations of these bacteria were as described for *E. coli* by Maniatis et al. (22).

*L. paramesenteroides* and *Lactococcus lactis* strains were grown in complete MRS broth (Difco Laboratories, Detroit, Mich.) or in MRS broth containing lactose as the sole carbon source (MRS-lactose). For plating, MRS broth was supplemented with 1.5% agar; cells were plated by using a top-agar

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Table 1. Bacterial strains and plasmids used in this study

<table>
<thead>
<tr>
<th>Strain or plasmid</th>
<th>Relevant feature(s)</th>
<th>Reference or source</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>L. mesenteroides</em></td>
<td>Type strain</td>
<td>NCDO</td>
</tr>
<tr>
<td>NCDO 523</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>L. parmesenteroides</em></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NZ6009</td>
<td>Lact*, Cit*</td>
<td>This study</td>
</tr>
<tr>
<td>NZ6091</td>
<td>Lact*, Cit*</td>
<td>Lactose-deficient derivative of NZ6009 (unpublished results)</td>
</tr>
<tr>
<td><em>Lactococcus lactis</em></td>
<td>Cit*</td>
<td>NCDO</td>
</tr>
<tr>
<td>subsp. diacetylactis</td>
<td></td>
<td></td>
</tr>
<tr>
<td>NCDO 176</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>L. lactis</em></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MGI1363</td>
<td>Plasmid-free, Lact*</td>
<td>15</td>
</tr>
<tr>
<td>MG1820</td>
<td>Lact*</td>
<td>22</td>
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<tr>
<td><em>E. coli</em> MC1061</td>
<td>Plasmid-free</td>
<td>4</td>
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<tr>
<td><em>B. subtilis</em> 168</td>
<td>Plasmid-free</td>
<td>1</td>
</tr>
<tr>
<td>ΔpAMβ1</td>
<td>10.6 kb, Em*</td>
<td>36</td>
</tr>
<tr>
<td>pNZ12</td>
<td>4.3 kb, Cm*</td>
<td>7</td>
</tr>
<tr>
<td>pNZ36</td>
<td>6.8 kb, Cm*, pNZ12 with a 2.5-kb fragment containing the Lactocococcus lactis phospho-β-galactosidase gene</td>
<td>de Vos and Gas-son, J. Gen. Microbiol., in press</td>
</tr>
<tr>
<td>pNZ262</td>
<td>10.7 kb, Cm*, pNZ12 with a 6.3-kb fragment containing a Lactococcus lactis E. coli lacZ gene fusion</td>
<td>8</td>
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</table>

a kb, Kilobase pairs; Em*, Cm*, resistance to chloramphenicol and erythromycin, respectively. Cit* and Cit* and Lact* and Lact* indicate the ability and inability to ferment lactose and to ferment lactose, respectively.

b NCDO, National Collection of Dairy Organisms.

over (MRS broth containing 0.7% agar). When required, chloramphenicol or erythromycin (both purchased from Sigma Chemical Co., St. Louis, Mo.) were each added at 10 μg/ml. All lactic acid bacterium strains were cultivated at 28°C.

For the identification of strain NZ6009, the following characteristics were determined: (i) the ability to form CO₂ from glucose, (ii) the ability to utilize citrate (halo formation on citrate agar plates [12]), (iii) the formation of either D(-)-lactate or L-(+)-lactate from glucose (26), and (iv) sensitivity to vancomycin (Sigma) (27). For differentiation between *Leuconostoc* species, the ability to form acid from various carbohydrates was analyzed by using API test strips (API-50 CHL; API systems S.A., La Balme les Grottes, France). The ability to form dextran from sucrose was tested on sucrose agar plates (12).

**Plasmid DNA isolation.** Plasmid DNA isolations from *Lactococcus lactis* and *L. parmesenteroides* NZ6009 were performed on a small scale by using the alkaline lysis method (2) with the following modifications. Five milliliters of exponentially growing cells (A₆₀₀ of 0.5) was incubated in THMS buffer (30 mM Tris hydrochloride [pH 8.3], 3 mM MgCl₂, 25% sucrose) containing 2 mg of lysozyme per ml for 30 min at 37°C. The suspension was chilled, 2 volumes of a solution containing 0.2 N NaOH and 1% (wt/vol) sodium dodecyl sulfate (SDS) were added, and the procedure was continued as described for *E. coli* (23). For use in electroporation, plasmid DNA was purified by CsCl/ethidium bromide density gradient centrifugation (23).

Restriction endonuclease digestions were performed according to the suppliers’ recommendations (Bethesda Research Laboratories, Inc., Gaithersburg, Md., and Boehringer Mannheim, Federal Republic of Germany).

**Electroporation.** *L. parmesenteroides* NZ6009 was grown overnight in MRS broth supplemented with 0.1 mM DL-threonine (MRS-T). This culture was diluted 1:20 in MRS-T, and exponentially growing cells were harvested by centrifugation (6,000 × g, 20°C), washed twice with ice-cold electroporation buffer (5 mM potassium phosphate buffer [pH 7.4], 2 mM MgCl₂, 25% sucrose), and finally resuspended in ice-cold electroporation buffer to an A₆₀₀ of 2.0.

Subsequently, 0.8 ml of the cell suspension was transferred into a sterile Gene Pulser cuvette (Bio-Rad Laboratories, Richmond, Calif.) with an inter-electrode distance of 0.4 cm. Plasmid DNA in 10 μl of TE buffer (10 mM Tris hydrochloride [pH 7.5], 1 mM EDTA) was added, and the mixture was left to stand on ice for 10 min prior to electroporation.

High-voltage pulses were delivered with a Gene Pulser apparatus (Bio-Rad) by using the 25-μF capacitor. After 10 min on ice, the cells were diluted into 8 ml of MRS broth and kept at 28°C for at least 1 h to allow for the expression of the antibiotic resistance marker. Dilutions of cells were plated on MRS agar plates containing the appropriate antibiotic. Colonies were visible after 24 to 36 h of incubation at 28°C.

Control experiments to determine the survival of the cells and the occurrence of spontaneous antibiotic-resistant mutants were performed by plating cells either that had received no electrical pulse or that were electroporated without plasmid DNA, on media with or without an antibiotic.

**Enzymatic assays.** To determine the phospho-β-galactosidase and β-galactosidase activities in *Lactococcus lactis* and *L. parmesenteroides*, exponentially growing cells were used that were protoplasted in THMS buffer containing 2 mg of lysozyme per ml, washed twice in THMS buffer, and lysed in 50 mM sodium phosphate buffer (pH 7.2) containing 1 mM dithiothreitol. To obtain complete lysis, the mixture was sonicated three times for 5 each time at 4°C in a model W-375 sonifier (Heatstays Ultrasonic Inc., Plainview, N.Y.) at the maximal microtip setting. Aliquots were tested for phospho-β-galactosidase and β-galactosidase by using as substrates o-nitrophenyl-β-D-galactopyranoside -6-phosphate (ONPG-P) and o-nitrophenyl-β-D-galactopyranoside (ONPG), respectively (both purchased from Sigma Chemical Co.), as described by Okamoto and Morichi (25).

Protein content was determined by the method of Bradford (3) by using the Bio-Rad protein assay with bovine serum albumin as a standard.

**Plasmid stability.** The stability of plasmid DNA in *L. parmesenteroides* NZ6009 was determined by serially passing the strains in MRS broth in the absence of an antibiotic. Cells were plated on MRS agar after 100 generations, and at least 200 individual colonies were picked and plated on selective and nonselective plates to determine the fraction of antibiotic-resistant colonies.

**Southern blot hybridizations.** Plasmid DNA was separated on a 0.8% agarose gel, and the DNA was transferred to Gene Screen Plus membranes and hybridized as described by the supplier (Du Pont, NEN Research Products, Boston, Mass.). Prehybridization was performed for at least 2 h in (pre-) hybridizing solution at 65°C.

Hybridization was performed overnight at 65°C by using 2
ml of hybridization solution and 0.3 μg of probe DNA, which was prepared by nick translation by using [α-32P]dATP (Du Pont, NEN Research Products) by the method of Maniatis et al. (23).

After hybridization, the filter was washed three times for 30 min each time at 65°C in 0.1× SSC and 0.1% SDS and subsequently autoradiographed by using XAR-5 film (Eastman Kodak Co., Rochester, N.Y.) and Du Pont Cronex Lightning-Plus intensifying screens at −80°C (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate).

Western blot (immunoblot) analysis. Whole-cell protein samples were prepared by incubating cells from 1.5 ml exponentially growing cultures in 500 μl of THMS buffer with 2 mg of lysozyme per ml for 1 h at 37°C. Cells were pelleted and resuspended in 100 μl of sample buffer (60 mM Tris hydrochloride [pH 6.8], 1% SDS, 10% β-mercaptoethanol, 1% glycerol, 0.01% bromphenol blue), and the lysates were heated at 100°C for 5 min. Samples (35 μl each) were applied to 12.5% polyacrylamide–SDS gels (20), which were run for 16 h at 35 V. The gels were blotted to nitrocellulose filters (BA85; Schleicher & Schuell, Inc., Keene, N.H.) in Tris-glycine buffer (25 mM Tris hydrochloride [pH 7.4], 192 mM glycine) containing 20% (vol/vol) methanol by using a blotting apparatus (Transblot; Bio-Rad) for 4 h at 300 mA.

Phospho-β-galactosidase was visualized by using rabbit antibodies against purified Lactococcus lactis phospho-β-galactosidase (8) and swine anti-rabbit peroxidase conjugate ( Dakopatts A/S, Glostrup, Denmark), with 1-chloro-4-naphthol as a substrate.

RESULTS

Strain identification. Various essential properties of strain NZ6009 are summarized in Table 2.

Because of the ability of this strain to form acid from arabinose, xylose, and mannitol and its inability to form dextran from sucrose, strain NZ6009 was classified as L. paramesenteroides. As observed in other Leuconostoc species, L. paramesenteroides contains plasmid DNA (27). Strain NZ6009 harbors four endogenous plasmids with molecular weights ranging from 35 to 1.8 kilobase pairs, as is shown in Fig. 1.

Electroporation of various plasmids into NZ6009. In initial experiments, L. paramesenteroides NZ6009 was transformed with 1 μg of DNA from the broad-host-range vectors pNZ12 and ΔAPM1 by using a single pulse of 6.25 kV/cm. The percentage of cell death under these conditions was less than 10%.

Transformation efficiencies per microgram of plasmid DNA ranged from 1 × 103 to 4 × 104 for pNZ12 and were a factor of 10 lower (2 × 103) for ΔAPM1. Cells of strain NZ6009 that had been electroporated in the absence of DNA did not produce colonies resistant to either chloramphenicol or erythromycin.

The plasmid content of transformants was analyzed by agarose gel electrophoresis of plasmid DNA isolated on a small scale; the presence of the donor plasmid DNA could be demonstrated in all antibiotic-resistant transformants. Examples of plasmid profiles from L. paramesenteroides NZ6009 transformants are shown in Fig. 2. Comparison of the intensities of the undigested and linearized DNA bands (Fig. 2, lanes A and C and B and D, respectively) of pNZ12 and pNZ36 with those of the endogenous plasmids indicates that pNZ12 and its derivative, pNZ36, have a higher copy number in L. paramesenteroides than do the endogenous plasmids detected.

The presence of donor plasmids in the transformants was confirmed in hybridization experiments as shown in Fig. 3. In all experiments, the strain identity of the transformants was confirmed by testing their sensitivity to the NZ6009-specific phage BA-Lcm9.

Electroporation conditions. To determine the conditions required for optimal electroporation of L. paramesenteroides NZ6009, the strain was electroporated under several different conditions by using pNZ12 DNA. Experiments were performed that varied the initial electrical field strength, the amount of plasmid DNA, and the source of plasmid DNA. A strong effect of the electrical field strength on electroporation efficiency was observed.

No transformants could be detected at the lowest voltage applied. Between 3.75 and 6.25 kV/cm, the efficiency of transformation (i.e., the number of antibiotic-resistant colonies per microgram of DNA) increased almost exponentially.

FIG. 1. Agarose gel electrophoresis of CsCl/ethidium bromide-purified plasmid DNA from L. paramesenteroides NZ6009. Numbers to the right indicate plasmid sizes (in kilobase pairs). On the left, plasmid designations are given.

### TABLE 2. Summary of characteristic features of L. paramesenteroides NZ6009 and comparison with those of Streptococcus lactis subsp. diacetilactis NCDO 176 and L. mesenteroides NCDO 523

<table>
<thead>
<tr>
<th>Strain</th>
<th>CO2 from glucose</th>
<th>Type of lactic acid from glucose</th>
<th>Vancomycin (μg/ml)</th>
<th>Citrate dissimilation</th>
<th>Growth in litmus milk</th>
<th>Dextran formation</th>
<th>Growth at 37°C</th>
</tr>
</thead>
<tbody>
<tr>
<td>NZ6009</td>
<td>+</td>
<td>D-(−)</td>
<td>&gt;500</td>
<td>+</td>
<td>−</td>
<td>−</td>
<td>+</td>
</tr>
<tr>
<td>NCDO 176</td>
<td>−</td>
<td>L-(+</td>
<td>&lt;10</td>
<td>+</td>
<td>+</td>
<td>−</td>
<td>+</td>
</tr>
<tr>
<td>NCDO 523</td>
<td>+</td>
<td>ND</td>
<td>&gt;500</td>
<td>−</td>
<td>Slight</td>
<td>−</td>
<td>−</td>
</tr>
</tbody>
</table>

* +, Reaction or growth observed; −, reaction or growth not observed; ND, not determined.
FIG. 2. Agarose gel electrophoresis of plasmid DNA isolated on a small scale from *L. paramesenteroides* NZ6009 transformants. Numbers on the left indicate sizes of molecular size standards (in kilobase pairs). Lanes: A and B, plasmid DNA from transformants harboring pNZ12, undigested and digested with *SalI*, respectively; C and D, plasmid DNA from transformants harboring pNZ36, undigested and digested with *EcoRI*, respectively. Asterisks mark the positions of the linearized pNZ12 (4.3 kilobase pairs) and pNZ36 (6.8 kilobase pairs).

with the field strength. At the highest voltage applied (6.25 kV/cm), efficiencies of $1 \times 10^3$ to $4 \times 10^4$ transformants per $\mu$g of DNA were observed. This corresponds to frequencies of about one transformant per $10^7$ recipient cells.

The relationship between the amount of transforming plasmid DNA and the number of transformants obtained by electroporation was examined and is presented in Table 3. In the range of 0.3 to 3.0 $\mu$g of plasmid DNA, a linear dose response was observed between DNA concentration and transformation efficiency, indicating that one molecule is sufficient to transform one host cell. The values in Table 3 indicate that saturation occurs at higher DNA concentrations.

To determine whether the source of plasmid DNA had any effect on the efficiency of *Leuconostoc* transformation, we transformed *L. paramesenteroides* NZ6009 with pNZ12 DNA isolated from *Lactococcus lactis* MG1363, *E. coli* MC1061, or *L. paramesenteroides* NZ6009. Similar frequencies, approximately $10^3$ transformants per $\mu$g of DNA, were obtained (data not shown), indicating that the source of DNA had no influence on the transformation efficiencies.

**Stability of plasmid DNA in *L. paramesenteroides* NZ6009.** After growth for 100 generations without selective pressure, no chloramphenicol- or erythromycin-sensitive colonies could be detected for strain NZ6009 containing either pNZ12 or ΔpAMβ1. All antibiotic-resistant colonies tested (24 of 24) appeared to contain the intact pNZ12 or ΔpAMβ1, as could be demonstrated by analysis of plasmid DNA content (data not shown). Growing the transformants mentioned above in the presence of the curing agent acriflavine (5 $\mu$g/ml) did not result in the curing of either pNZ12 or ΔpAMβ1 (data not shown), confirming the stability of these plasmids in *L. paramesenteroides*.

**Expression of the *Lactococcus lactis* phospho-β-galactosidase gene in *L. paramesenteroides*.** In order to test the utility of the established host-vector system for *L. paramesenteroides* NZ6009, the *Lactococcus lactis* phospho-β-galactosidase gene located on pNZ36 (Table 1) was introduced into this strain. The phospho-β-galactosidase activity of NZ6009 harboring pNZ36 (Table 4) was elevated only slightly as compared with the untransformed control (NZ6009). This is a consequence of relatively high background activity of strain NZ6009. However, the expression of the *Lactococcus lactis* phospho-β-galactosidase gene is evident in the lactose-deficient derivative NZ6091 containing pNZ36 (Table 4). The levels of phospho-β-galactosidase activity specified by pNZ36 were comparable in *Lactococcus lactis* and *L. paramesenteroides*.

Expression of phospho-β-galactosidase activity in transformants carrying pNZ36 was further demonstrated by Western blot analysis (Fig. 4). The protein extracts of NZ6009 harboring pNZ36 (Fig. 4, lane E) clearly show an additional band that migrates the same distance as the

**FIG. 3. Autoradiogram obtained from a Southern blot of plasmid DNA from *L. paramesenteroides* NZ6009 transformants harboring pNZ12 after hybridization with 32P-labeled pNZ12 DNA. Lanes: A, pNZ12 DNA digested with *SalI*; B and C, total plasmid DNA of NZ6009 harboring pNZ12, digested with *SalI* and undigested, respectively; D, total plasmid DNA of NZ6009 (not transformed). The arrow indicates the linearized 4.3-kilobase-pair pNZ12 plasmid band.**

**TABLE 3. DNA dependence of electroporation (effect of the concentration of pNZ12 on the transformation efficiency of *L. paramesenteroides* NZ6009).**

<table>
<thead>
<tr>
<th>$\mu$g of DNA</th>
<th>No. of transformants*</th>
<th>Transformation efficiency*</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.3</td>
<td>$2.5 \times 10^2$</td>
<td>$8.3 \times 10^2$</td>
</tr>
<tr>
<td>0.5</td>
<td>$4.2 \times 10^2$</td>
<td>$8.4 \times 10^2$</td>
</tr>
<tr>
<td>1.0</td>
<td>$6.5 \times 10^2$</td>
<td>$6.5 \times 10^2$</td>
</tr>
<tr>
<td>3.0</td>
<td>$2.1 \times 10^3$</td>
<td>$7 \times 10^2$</td>
</tr>
<tr>
<td>10.0</td>
<td>$3.6 \times 10^3$</td>
<td>$3.6 \times 10^2$</td>
</tr>
</tbody>
</table>

* Total number of antibiotic-resistant colonies observed.

* Number of transformants per microgram of DNA.
TABLE 4. Specific phospho-β-galactosidase and β-galactosidase activities of L. paramecserenteroides (NZ6009 and NZ6091) and L. lactis strains (MG1363 and MG1820) containing plasmids carrying the Lactococcus lactis phospho-β-galactosidase gene (pNZ36) or the Lactococcus lactis—E. coli lacZ gene fusion (pNZ262)

<table>
<thead>
<tr>
<th>Strain</th>
<th>Phospho-β-galactosidase</th>
<th>β-Galactosidase</th>
</tr>
</thead>
<tbody>
<tr>
<td>NZ6009</td>
<td>42</td>
<td>2.3 × 10^3</td>
</tr>
<tr>
<td>NZ6009(pNZ36)</td>
<td>75</td>
<td>2.4 × 10^3</td>
</tr>
<tr>
<td>NZ6091</td>
<td>&lt;0.1</td>
<td>&lt;0.1</td>
</tr>
<tr>
<td>NZ6091(pNZ36)</td>
<td>92</td>
<td>0.7 × 10^2</td>
</tr>
<tr>
<td>NZ6091(pNZ262)</td>
<td>&lt;0.1</td>
<td>&lt;0.1</td>
</tr>
<tr>
<td>MG1363</td>
<td>79</td>
<td>1.0 × 10^4</td>
</tr>
<tr>
<td>MG1363(pNZ36)</td>
<td>79</td>
<td>1.0 × 10^4</td>
</tr>
<tr>
<td>MG1363(pNZ262)</td>
<td>&lt;0.1</td>
<td>&lt;0.1</td>
</tr>
<tr>
<td>MG1820</td>
<td>2 × 10^3</td>
<td>1.0 × 10^4</td>
</tr>
</tbody>
</table>

* Specific activities are expressed as nanomoles per minute per milligram of protein.

purified phospho-β-galactosidase (lane D). This band is also present in the phospho-β-galactosidase-positive Lactococcus lactis MG1820 (lane A) and in MG1363 harboring pNZ36 (lane C), whereas such a band could not be detected in the phospho-β-galactosidase-deficient Lactococcus lactis MG1363 or in L. paramecserenteroides NZ6091 (lanes B and F, respectively).

Expression of the E. coli β-galactosidase gene in L. paramecserenteroides. The plasmid pNZ262 is a pNZ12 derivative containing a gene fusion between the Lactococcus lactis SK11 proteinase transcription and translation initiation signals and the E. coli lacZ gene (8). The lactose-deficient L. paramecserenteroides strain NZ6091 appeared to be devoid of β-galactosidase activity and was therefore chosen as the subject of transformation with pNZ262 DNA.

The β-galactosidase activity of L. paramecserenteroides transformants was comparable to the specific activity observed in Lactococcus lactis MG1363 carrying pNZ262 (Table 4) and only slightly lower than the activity in wild-type, lactose-proficient L. paramecserenteroides NZ6009. However, the presence of the lacZ gene in NZ6091 did not allow strain NZ6091 to grow on MR5-lactose (data not shown).

DISCUSSION

The results presented here show that L. paramecserenteroides NZ6009 can be transformed efficiently by means of electroporation with two unrelated broad-host-range plasmids. The conjugal transfer of pAMB1 to L. dextranicus and L. cremoris was already demonstrated previously (30). indicating the functionality of its replicon in Leuconostoc species. The successful transformation of L. paramecserenteroides by pNZ12 DNA exemplifies the wide host range of this vector. The replication of pNZ12 is derived from the cryptic Lactococcus lactis NCDO 712 plasmid pSH71 and is functional in various streptococcal hosts, as well as in B. subtilis (7), Lactobacillus casei (5), Staphylococcus aureus (7), and E. coli (7).

Plasmid pNZ12 was chosen for initial experiments designed to establish optimal conditions for the electroporation of L. paramecserenteroides NZ6009. The transformation efficiencies obtained in L. paramecserenteroides NZ6009 amounted to 1 × 10^3 to 4 × 10^4 transformants per µg of pNZ12 DNA. A linear dose response was observed up to an amount of 3.0 µg of pNZ12 DNA per 2 × 10^9 cells. Saturation occurred when higher DNA concentrations were used. Minor variations between experiments were observed that may be attributed to differences in growth phases of the cell preparations (24). Our experimental outline was based on earlier data on electroporation of lactic acid bacteria (5, 29, 32), showing that the voltage applied is the most significant parameter.

Application of pulses up to 6.25 kV/cm, which represented the maximum voltage that could be reached, resulted in a linear increase in the number of transformants, indicating that higher voltages might give rise to an even larger number of transformants (9). However, preliminary experiments using cuvettes with a 0.2-cm inter-electrode distance, resulting in a higher electrical field strength, showed that transformation efficiencies do not exceed 4 × 10^3/µg of pNZ12 DNA. This indicates that the efficiencies obtained by using 6.25 kV/cm may be the maximal efficiencies obtainable with this strain under the given experimental conditions.

Efficiencies of transformation in L. paramecserenteroides NZ6009 were lower than those achieved by transformation of Lactococcus lactis (29) and Lactobacillus casei (5). Results of the electroporation of Streptococcus thermophilus (32) and very recently published results on the electroporation of L. lactis and L. dextranicus (21) showed comparable transformation efficiencies. However, the system for the electroporation of these Leuconostoc strains has not been optimized or used to study the expression of new genes in this species. L. paramecserenteroides NZ6009 cells were very resistant to high electrical pulses, with only a 10% decline in the number of CFU at 6.25 kV/cm. The apparent large number of surviving cells may not reflect the actual number of viable cells but could be due to electroporation-induced rupture of cell chains, giving rise to more CFU.
Leuconostoc spp. are heterofermentative and do not possess a lactose phosphotransferase system (6). Therefore, the phosphotransferase system-specific phospho-β-galactosidase activity, which hydrolyzes the intracellular phosphorylated lactose, is absent in this genus. Instead, Leuconostoc spp. hydrolyzes the internalized lactose by using a β-galactosidase. Little is known about the sugar uptake system in this organism, but it may be mediated by a proton motive force-dependent permease system, which might function in a manner analogous to that in other heterofermentative organisms (33). Since the lactosenucleotidase of lactose is completely metabolized by Leuconostoc spp. (6), a permease acting as a lactose-galactose antipporter, as has recently been proposed for Streptococcus thermophilus (27a), is not likely to exist in these bacteria.

Transformants containing pNZ36 showed specific phospho-β-galactosidase activities comparable to the specific activity seen in Lactococcus lactis MG1363 (pNZ36). However, these activities could be assayed accurately only in a β-galactosidase-negative derivative (NZ6091). Recent experiments have shown that this strain does not contain the lactose plasmid detected in the wild-type strain (unpublished results). Background activity of NZ6091 might be due to phosphatase activity that converts ONPG-P to ONPG, which subsequently can be hydrolyzed by β-galactosidase (32).

The E. coli lacZ gene located on pNZ262 was introduced into NZ6091, and transformants could express the E. coli β-galactosidase by using the Lactococcus lactis SK11 protease transcript and translation initiation signals. Comparing the levels of β-galactosidase activity in both a lactose-deficient Lactococcus lactis strain and a Leuconostoc strain, we can conclude that lactococcal expression signals can be efficiently recognized by L. paramesenteroides. The fact that pNZ262 results in high levels of β-galactosidase synthesis in L. paramesenteroides NZ6091 but does not complement for its lactose-deficient phenotype suggests that this strain also lacks other components of the lactose pathway.

The results described here demonstrate the possibilities of heterologous gene expression in L. paramesenteroides by using established lactocic acid bacterium cloning and expression vectors. Furthermore, the results provide opportunities for further genetic studies in Leuconostoc spp., including the analysis of genes encoding important dairy functions, such as lactose and citrate metabolism.

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LITERATURE CITED