Transformation of alternan-producing strains of *Leuconostoc* by electroporation

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Received 9 March 2004; Revisions requested 18 March 2004; Revisions received 5 May 2004; Accepted 5 May 2004

Key words: alternan, electroporation, *Leuconostoc*, shuttle vector, transformation

Abstract

Alternan-producing *Leuconostoc mesenteroides* strain NRRL B-1355 and its glucansucrase-negative derivative NRRL B-21414 were transformed by electroporation using four Gram positive-Gram negative shuttle vectors. Optimal conditions were 400 n and 10 kV cm⁻¹, resulting in transformation efficiencies of up to 3.5 × 10⁴ per µg DNA. Relatively low copy numbers and native plasmids made it difficult to visualize the introduced plasmids on ethidium bromide-stained gels and, in some cases, on blot hybridizations. However, PCR analysis indicated that 95% of putative transformants carried plasmid sequences. Direct colony PCR was shown to work well for this system and also for transformants of *L. mesenteroides* subsp. cremoris.

Introduction

*Leuconostoc mesenteroides* is an important source of dextrans, defined as glucans in which α(1→6) linkages dominate (Leathers 2002). Most commercially manufactured dextran is the product of a single strain, *L. mesenteroides* NRRL B-512F. This dextran features a backbone of α(1→6) linked glucose with relatively few α(1→3) branch points. However, other strains of *L. mesenteroides* produce different types of dextrans (Jeanes et al. 1954). *L. mesenteroides* strain NRRL B-1355 produces the unique glucan, alternan (Cote 2002). Alternan is distinguished from a true dextran by a backbone structure of alternating α-(1→6) and α-(1→3) glucose linkages (Misaki et al. 1980, Seymour & Knapp 1980, Cote & Robyt 1982). Alternan consequently exhibits physical properties of high solubility and low viscosity that make the polysaccharide interesting for potential commercial applications (Cote 1992, Cote et al. 1997). A bioconversion method recently was described to modify alternan so that its solution viscosity properties more closely resemble those of gum arabic (Leathers et al. 2002a,b, 2003). A domestic alternative to gum arabic would be desirable because it is imported from Middle Eastern countries at unpredictable supply, cost, and quality. Oligosaccharides derived from alternan also show promise as prebiotics (Cote et al. 2003).

Strains of *L. mesenteroides* that produce alternan also produce at least equal amounts of dextran. However, mutants of strain NRRL B-1355 have been isolated that produce little or no dextran (Smith et al. 1994, Leathers et al. 1995, 1997b, 1998). One such strain, NRRL B-21138, was subjected to a second round of mutagenesis to produce strain NRRL B-21214, which produces neither dextran nor alternan and appears to have no glucansucrase activity (Leathers et al. 1997a). This strain is a potential host for the expression of cloned glucansucrase genes. However, DNA transformation has not been reported previously
Table 1. Transformation frequencies of Gram positive-gram negative shuttle vectors into *Leuconostoc mesenteroides* strain NRRL B-21414.

<table>
<thead>
<tr>
<th>Vector</th>
<th>Size (kb)</th>
<th>Drug for selection</th>
<th>Transformants/μg DNA</th>
</tr>
</thead>
<tbody>
<tr>
<td>pGK12</td>
<td>4.9</td>
<td>C</td>
<td>3.4 ± 1.1 x 10^4</td>
</tr>
<tr>
<td>pSA3</td>
<td>10.2</td>
<td>E</td>
<td>4.2 ± 1.2 x 10^1</td>
</tr>
<tr>
<td>pDB101</td>
<td>18.3</td>
<td>E</td>
<td>2.4 ± 0.4 x 10^2</td>
</tr>
<tr>
<td>pAMβ1</td>
<td>26.5</td>
<td>E</td>
<td>2.7 ± 0.3 x 10^2</td>
</tr>
</tbody>
</table>

C = Chloramphenicol. E = Erythromycin.

for alternate strains of *L. mesenteroides*. Such a system would be useful for basic and applied studies of natural and genetically modified glucansucrase genes in a homologous genetic background.

**Materials and methods**

**Strains and culture conditions**

*Leuconostoc mesenteroides* strains NRRL B-1355 and NRRL B-21414 were obtained from the ARS Patent Culture Collection at the National Center for Agricultural Utilization Research, Peoria, IL. *Leuconostoc mesenteroides* subsp. *cremoris* strain 44-4 was a dairy isolate (Wyckoff *et al.* 1991). Cultures were grown as previously described at 28 °C and 100 rpm in MRS- V8 broth (Wyckoff *et al.* 1991).

**Plasmids**

Gram positive–Gram negative shuttle vectors were isolated from *Bacillus* and *Lactococcus* hosts as previously described (Wyckoff *et al.* 1991). Plasmids pSA3 (Dao & Ferretti 1985), pDB101 (Behnke *et al.* 1979), and pAMβ1 (Clewell *et al.* 1974) bear an erythromycin resistance gene derived from *Streptococcus faecalis*. (Plasmid pSA3 also carries chloramphenicol and tetracycline resistance genes.) Plasmid pGK12 contains a chloramphenicol resistance gene and a distinct erythromycin resistance gene derived from *Staphylococcus aureus* (Kok *et al.* 1984).

**Electroporation**

General procedures for electroporation were previously described (Wyckoff *et al.* 1991). Fifty ml cultures of *L. mesenteroides* cells were grown to an optical density at 600 nm of 0.6 (approx. 1.4 x 10^9 cells ml⁻¹), washed twice in 25 ml ice-cold, sterile, low-conductivity distilled water, washed once in 5 ml ice-cold, sterile electroporation buffer (1 mM K₂HPO₄/KH₂PO₄, pH 7.4, 1 mM MgCl₂ and 0.5 M sucrose), and resuspended in 1 ml of the same buffer. Forty μl cell suspensions was mixed with 1 μg plasmid DNA in no more than 5 μl water in sterile, pre-chilled tubes and the mixture was transferred to pre-chilled electroporation cuvettes (Bio-Rad, Richmond, CA) having an interelectrode distance of 0.2 cm. Transformation was performed using a GenePulsar unit with a Pulse Controller (Bio-Rad) set at 25 μF capacitance. Transformed cells were immediately diluted into 1 ml of MRS-V8 medium and incubated for 1 h at 30 °C before plating on selective medium consisting of MRS-V8 agar amended with either chloramphenicol or erythromycin at 5 μg ml⁻¹ (Wyckoff *et al.* 1991).

**Plasmid isolation and analysis**

Plasmid DNA was isolated from 20 ml MRS-V8 cultures at OD₆₀₀ of 0.8 (approx. 1.9 x 10^9 cells ml⁻¹), following manufacturer’s protocol (Qiaprep spin columns, Qiagen, Santa Clarita, CA) with the following modifications. Cell pellets were resuspended in Buffer P1 containing 10 mg lysozyme ml⁻¹ and incubated at 37 °C for 30 min. Proteinase K was then added to give 0.2 mg ml⁻¹ and the samples were incubated at 55 °C for 30 min. After DNA isolation, contaminating chromosomal DNA was removed using Plasmid-Safe DNase (Epicentre, Madison, WI). Separation of plasmid DNA by agarose gel electrophoresis, DNA blotting, and hybridization was performed according to Hohn & Dejardins (1992). DNA blots were probed using purified PCR products of the Emf genes from pAMβ1 and pGK12 labeled with ^32^P, following manufacturer’s protocol (DECAprime II, Ambion, Austin, TX).

**PCR analysis of transformants**

Purified plasmids were analyzed by PCR per manufacturer’s protocol (HotStarTaq polymerase, Qiagen, Santa Clarita, CA). PCR samples were denatured at 95 °C for 45 s, annealed at 52–58 °C for 45 s, and extended at 72 °C for 75 s for 25–35 cycles followed by a final extension at 72 °C for 60 s. For detection of the erythromycin resistance (Emf) gene in pGK12 transformants, primers were 5’GGGTTATAATGAAAGACGAAA3’.
Fig. 1. Transformation frequencies of Leuconostoc mesenteroides strains NRRL B-1355 (○) and NRRL B-21414 (●) as a function of electroporation voltages.

(sense) and 5’GTAAACCTTTACAAACGA3’ (antisense), resulting in a 568 bp product. For detection of the presence of the Emf gene in pAMβ1, pDB101, and pSA3 transformants, primers were 5’TCATTGCTTGATGAAACTGA3’ (sense) and 5’CGACGAAACTGGCTAAAATA3’ (antisense), resulting in a 476 bp product.

Direct PCR of bacterial colonies

Alternatively, transformants were analyzed by direct colony PCR. Bacteria from individual colonies were transferred into microcentrifuge tubes containing 40 μl of 1x PCR buffer (HotStarTaq polymerase reaction buffer, Qiagen, Santa Clarita, CA) supplemented with 200 μg Proteinase K ml⁻¹ and 0.5% Tween 20. The samples were incubated at 55 °C for 1 h followed by incubation at 95 °C for 15 min. The samples were then spun to pellet cell debris. PCR reagents were added to the supernatants and PCR was performed as described above.

Results and discussion

Optimization of transformation conditions

Optimization studies for the transformation of L. mesenteroides strain NRRL B-1355 were performed using the shuttle vector plasmid pGK12 (Kok et al. 1984).

Highest transformation frequencies over a range of voltages were obtained using a resistance of 400 Ω (data not shown). As shown in Figure 1, efficiencies of greater than 10⁴ transformants per μg DNA were obtained at voltages of 8–10 kV cm⁻¹, with highest frequencies most reproducibly obtained at 10 kV cm⁻¹. Decay time constants under these conditions were 7–8 ms. Optimal conditions for transformation of mutant strain NRRL B-21414 were similar to those for parental strain NRRL B-1355 (Figure 1). Controls subjected to electroporation without plasmid DNA
showed no colonies on selective medium. On non-selective medium, these controls showed survival rates of $35 \pm 8\%$ for strain NRRL B-1355 and $34 \pm 11\%$ for strain NRRL B-21414.

Optimal transformation conditions, efficiencies, and survival rates obtained here for alternan strains of *L. mesenteroides* differ from those reported for closely related species and genera. In a previous study of dairy isolates, *L. mesenteroides* subsp. *cremoris* was transformed optimally at $8 \text{ kV cm}^{-1}$ and only poorly transformed at $10 \text{ kV cm}^{-1}$, with efficiencies of up to $2 \times 10^6$ transformants per $\mu g$ pNZ12, with little or no cell death due to electroporation (Wyckoff et al. 1991). Under identical conditions, the transformation frequencies other dairy isolates varied from $1 \times 10^1$ to $6 \times 10^3$ transformants per $\mu g$ DNA (Wyckoff et al. 1991). Optimal transformation of the wine species, *Leuconostoc oenos* (now considered *Oenococcus oeni*) was reported at $2 \text{ kV cm}^{-1}$, with yields of up to $10^3$ transformants per $\mu g$ DNA and survival rates of $20\%$ (Dicks 1994). *Leuconostoc paramesenteroides* (now considered *Weisella paramesenteroides*) was optimally transformed at $6.25 \text{ kV cm}^{-1}$, producing up to $4 \times 10^3$ transformants per $\mu g$ DNA with less than $10\%$ cell death due to electroporation (David et al. 1989).

**Transformation frequencies for additional shuttle vectors**

Using the optimal transformation conditions described above, shuttle vectors pSA3, PDB101, and pAMβ1 were introduced successfully into *L. mesenteroides* strain NRRL B-21414 (Table 1). Transformation efficiencies were considerably lower for these larger plasmids, although transformation efficiency did not appear to be a strict function of plasmid size. These results are consistent with the findings of Wyckoff et al. (1991).

**Plasmid profiles and verification of the Em$^r$ gene in transformants**

Figure 2A illustrates the plasmid profiles obtained from putative *L. mesenteroides* transformants grown in the presence of erythromycin. Strain NRRL B-21414 carries six native plasmids, making it difficult to visualize introduced plasmids in simple ethidium bromide-stained gels. Only transformants containing pDB101 and pGK12 appeared to show extra bands suggesting the acquisition of the transforming plasmid. This gel was subsequently blotted and probed using the Em$^r$ gene from the corresponding plasmids (Figure 2B). Hybridizations showed the presence of plasmids pAMβ1, pDB101, and pGK12 in transformants. For unknown reasons, plasmid pSA3 was not clearly revealed by this method. However, PCR analysis using Em$^r$-specific primers verified the acquisition of all four plasmids in putative transformants (Figure 2C).

**Reproducibility of the transformation procedure**

*L. mesenteroides* strain NRRL B-21414 was transformed with pDB101 and plasmid DNA was isolated from twelve independent transformants grown under erythromycin selection (Figure 3A). All twelve showed acquisition of the plasmid as judged by hybridization (Figure 3B) and PCR analysis (Figure 3C). Similar PCR results were obtained from transformants of pAMβ1, pGK12, and pSA3 (data not shown).
Rapid screening of transformants by direct colony PCR

To rapidly screen putative transformants for the presence of introduced plasmids without purification of the plasmid DNA, a direct colony PCR procedure was used as described in Materials and methods. Figure 4 shows the analysis of pGK12 and pAMβ1 transformants of *L. mesenteroides* strain NRRL B-21414. Results compared favorably with those obtained with purified plasmid DNA from transformants, and confirmed the reproducibility of the transformation protocol. Overall, more than 95% of independent transformants tested by both methods were shown to possess the expected Em<sup>r</sup> gene. This method also was used successfully to verify the presence of pAMβ1 DNA in transformants of *L. mesenteroides* subsp. *cremoris* (Figure 4). This procedure could have general utility for other lactic acid bacteria as a rapid means of identifying specific plasmids, particularly when copy numbers are limited.

Acknowledgements

The authors thank Trina Hartman and Melinda S. Nunnally for expert technical assistance.

References


