A convenient and reproducible method to genetically transform bacteria of the genus Bifidobacterium

Alessandra Argnani,† Rob J. Leer, Nicole van Luijk and Peter H. Pouwels

TNO Nutrition and Food Research Institute, Department of Molecular Genetics and Gene Technology, PO Box 5815, 2280 HV Rijswijk, The Netherlands

A protocol was developed for the introduction of foreign plasmid DNA into various Bifidobacterium strains. The method, which is applicable to all Bifidobacterium species tested so far, is based on electroporation of bacteria made competent by preincubation in electroporation buffer for several hours at 4 °C. Transformation of Bifidobacterium could be achieved with a plasmid vector originating from Bifidobacterium and with plasmid vectors from Corynebacterium, but not with vectors carrying replicons from Lactococcus or Lactobacillus.

Keywords: Bifidobacterium, electrotropisation, plasmid vector

INTRODUCTION

Bifidobacteria are Gram-positive, anaerobic, catalase-negative, fermentative rods, which are often Y- or V-shaped. Although there has been much confusion over the years as to the classification of this type of bacteria, there is now general agreement among taxonomists that bifidobacteria should be classified in the genus Bifidobacterium, which is now included in the family Actinomycetaceae. Bifidobacteria belong to a subclass of the Gram-positive bacteria, the genome of which is very GC-rich. Other genera of this group include Mycobacterium, Corynebacterium and Streptomyces. The GC content of bifidobacteria is between 55 and 64 mol% (Scardovi, 1986).

Bifidobacteria are among the most abundant species in the lower small intestine of man and animals. The distal ileum may contain 10^8–10^9 organisms per ml of intestinal content (Gorbach et al., 1967; Drasar et al., 1969). Bifidobacteria are also prominent in the human large intestine and are present at concentrations of 10^10 or more per g gut content, constituting 5–10% of total flora in the bowel (Mitsuoka, 1992).

It is widely believed that bifidobacteria have beneficial properties for their host, but convincing scientific data supporting this are scarce. Besides the assumed health-promoting properties of some Bifidobacterium species for humans, the economic importance of these microorganisms is beyond doubt. Bifidobacterium strains are widely used for the preparation of fermented milk products in many Asiatic and Western countries. To be able to fully exploit the potential of these organisms for practical application, detailed knowledge is required about such basic biological phenomena as cellular metabolism, gene expression, protein secretion, etc. However, studies on Bifidobacterium at the molecular level would be severely limited in the absence of an efficient transformation system. The availability of a system for genetic transformation would also enable strain improvement programmes to be carried out.

Although the electroporation technique has proven to be widely applicable to genetically transform bacterial strains from several genera of lactic acid bacteria, like Lactococcus, Pediococcus, Lactobacillus, Enterococcus (Harlander, 1987; Chassy & Flickinger, 1987; Luchansky et al., 1988; Holo & Nes, 1989; Cruz Rodz & Gilmore, 1990; Posno et al., 1991), and corynebacteria and brevibacteria (Chassy et al., 1988; Bonnassie et al., 1990), all Bifidobacterium strains so far examined have proved refractory to efficient and reproducible transformation. In this paper we describe the development of a system for efficient and reproducible genetic transformation of strains of the genus Bifidobacterium. The system is based on preincubation of the bacteria at low temperatures in electroporation buffer prior to electroporation, and on the use of plasmid vectors with a replicon from Actinomycetaceae.

METHODS

Bacterial strains and plasmids. The Bifidobacterium strains used were: B. animalis ATCC 27536, B. bifidum ATCC 15696 and B. infantis ATCC 27920 (American Type Culture Collection); B.
Table 1. Plasmids

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Markers</th>
<th>Size (kb)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>pDG7</td>
<td>Amp', Cm'</td>
<td>7.3</td>
<td>Matteuzzi et al. (1990)</td>
</tr>
<tr>
<td>pLB285</td>
<td>Amp', Cm'</td>
<td>7.7</td>
<td>Posno et al. (1991)</td>
</tr>
<tr>
<td>pGK12</td>
<td>Em', Cm'</td>
<td>4.4</td>
<td>Kok et al. (1984)</td>
</tr>
<tr>
<td>pECM2</td>
<td>Km', Cm'</td>
<td>10.3</td>
<td>Gift from J. Kalinowski</td>
</tr>
<tr>
<td>pEBM3</td>
<td>Km', Cm'</td>
<td>9.6</td>
<td>Gift from J. Kalinowski</td>
</tr>
</tbody>
</table>

*brevi* 4 (laboratory collection); *B. breve* AS (gift from C. Romond (University of Lille); and *B. bifidum* U3, *B. infantis* U1, *B. longum* U2 and *B. longum* Wiesby 2 (gifts from Unilever, Vlaardingen, the Netherlands). None of these *Bifidobacterium* strains carried a plasmid. The plasmids used are listed in Table 1. Plasmid pDG7, obtained from Dr D. Matteuzzi and described in Matteuzzi et al. (1990), is an *Escherichia coli–Bifidobacterium* shuttle vector based on a small cryptic plasmid from *B. longum* B2577 (Sgorbati et al., 1982) and pJH101. Plasmids pEBM3 and pECM2, obtained from Dr J. Kalinowski, are *E. coli–Corynebacterium* shuttle vectors. Plasmid pLB285, described in Posno et al. (1991), is an *E. coli–Lactobacillus* shuttle vector capable of replication in various lactobacilli, and plasmid pGK12, described by Kok et al. (1984), is a broad-host-range vector that replicates in various Gram-positive and Gram-negative bacteria. All vectors contain the chloramphenicol resistance gene from pC194, which was used for selection in bifidobacteria.

Preparation of plasmid DNA. Plasmid DNA was isolated using a QIAGEN Plasmid Mini Kit according to a protocol supplied by the manufacturer. It was modified for *Bifidobacterium* by addition of lysozyme (Boehringer) to the resuspension buffer (30 mg per ml buffer) and incubation at 37 °C for 40 min. Small-scale isolation of plasmid DNA was performed following the protocol described in Sambrook et al. (1989), modified for *Bifidobacterium* by using lysozyme at 30 mg ml⁻¹ instead of 5 mg ml⁻¹, and incubating at 37 °C for 40 min.

Media and growth conditions. *E. coli* strain DH5α, used as host strain for propagating the shuttle vectors, was cultivated in L-medium (Sambrook et al., 1989) supplemented with the appropriate antibiotics: kanamycin (Sigma), final concentration 50 μg ml⁻¹, and ampicillin (Sigma), final concentration 50 μg ml⁻¹. Bifidobacteria were routinely cultivated in MRS broth (Difco) supplemented with 0.05% (final concentration) cysteine. HCl at 37 °C; for some of the *Bifidobacterium* strains anaerobiosis was essential for growth.

Preparation of bacteria for electroporation. An overnight culture of *Bifidobacterium* was used to inoculate fresh MRS broth supplemented with 0.05% (final concentration) cysteine. HCl and 0.5 M (final concentration) sucrose (Merck) and cultivated overnight at 37 °C (some strains anaerobically). This overnight culture was diluted 1:25 in fresh MRS broth supplemented with 0.05% cysteine and 0.5 M sucrose and cultured at 37 °C until an OD₆₀₀ of ~0.2 was reached. Bacteria were chilled on ice, harvested by centrifugation and washed twice with 0.5 M sucrose. Finally, they were resuspended in about 1/250 of the original culture volume of ice-cold 0.5 M buffered sucrose, dispensed in Eppendorf tubes and incubated at 0–37 °C for 0–25 h.

Electroporation. Plasmid DNA (0.5–1.5 μg) was mixed with 80 μl bacterial suspension in a pre-cooled Gene Pulser disposable cuvette (inter-electrode distance 0-2 cm; Bio-Rad). A high-voltage electric pulse was delivered with a Gene Pulser apparatus (Bio-Rad) by using the 25 μF capacitor and setting the pulse controller at 200 Ω parallel resistance, yielding a pulse duration of 3–4.6 ms. Following electroporation, bacteria were diluted with 800 μl MRS broth supplemented with 0.05% cysteine and 0.5 M sucrose. The bacteria were incubated for about 2.5 h at 37 °C to allow for recovery and expression of the antibiotic resistance marker and, finally, plated on MRS agar (1.5%, w/v) supplemented with 0.05% cysteine, 0.5 M sucrose and 10 μg chloramphenicol ml⁻¹ (final concentration; Boehringer). Plates were incubated anaerobically at 37 °C for 2 or 3 d, after which small-size or full-size colonies, respectively, were visible.

RESULTS AND DISCUSSION

The main purpose of this work was to develop a method that would allow transformation of *Bifidobacterium*. Initially, various protocols used for transformation by electroporation of other micro-organisms were tried, but without any success.

Preparation of recipient bacteria for electroporation

*Bifidobacteria* have a very thick and complex cell wall (Fischer, 1987). The presence of a thick (multi-layered) cell wall generally forms a barrier for the uptake of exogenous DNA molecules. This conclusion was inferred from experiments in which the efficiency of transformation of different strains of *Listeria monocytogenes* and that of corynebacteria was improved by treatment of the cells with penicillin G, glycine and D-amino acids, or muralytic enzymes (Powell et al., 1988; Bonnassie et al., 1990; Park & Stewart, 1990; Dunny et al., 1991). An attempt was made to modify the structure of the cell wall by preincubation of the bacteria in electroporation buffer. The occurrence of electrotransformation-competent *Lactobacillus bulgaricus* bacteria by pretreatment in a buffer of high ionic strength has been attributed to limited autolysis (Sasaki et al., 1987). Electrotransformation-competent bifidobacteria were obtained by cultivating the bacteria in MRS broth supplemented with 0.5 M sucrose, washing them in buffered sucrose as described in Methods, and storing them in the electroporation buffer at 4 °C. *Bifidobacterium* cells obtained with this treatment are completely viable and show no morphological changes. In preliminary experiments in which pretreated *B. animalis* was transformed with pDG7 in an electroporation buffer composed of 0.5 M sucrose + 1 mM ammonium citrate, pH 6, several hundred chloramphenicol-resistant colonies were found on selective plates.

To determine whether the composition of the electroporation buffer would affect the transformation efficiency, six different buffers were utilized (Table 2). A buffer containing 1 mM ammonium citrate, pH 6, gave the highest number of transformants. The presence of a high concentration of sucrose in the growth medium and electroporation buffer proved to be essential, as no transformants were observed when bacteria were cul-
activates in the absence of the osmotic stabilizer, or when the electroporation buffer contained no sucrose. An electric pulse of 10 kV cm\(^{-1}\) yielded a higher number of transformants than a pulse of 6 kV cm\(^{-1}\), irrespective of the buffer used.

To confirm that the antibiotic-resistant colonies were truly *Bifidobacterium* transformants, plasmid DNA was isolated and analysed by gel electrophoresis. As shown in Fig. 1, all chloramphenicol-resistant colonies tested contained a plasmid indistinguishable in size from the parent plasmid, pDG7. In addition, when plasmid DNA isolated from transformed cells was used to transform *E. coli*, transformants were obtained containing a plasmid with the same restriction enzyme profile as that of pDG7. Finally, the sugar metabolism profile of the transformants, analysed with an API test system, was the same as that of the untransformed *Bifidobacterium* strains (results not shown).

Since this work was completed, Missieh et al. (1994) have reported low-efficiency transformation by electroporation of a strain of *B. longum* using a plasmid vector from *B. longum* B2577 with properties similar to those of pDG7. The procedure described involves freezing of the bacteria at \(-135^\circ\text{C}\) for 1 h in sterile glycerol and storage at \(-70^\circ\text{C}\), prior to electroporation. We assume that freezing and thawing of bacteria mimics limited autolysis, which could explain the transformation of this bifidobacterial strain, although with low efficiency.

### Electroporation conditions

Fig. 2 shows the effect of variation of the applied voltage on the transformation efficiency of *B. animalis*. Optimal results were obtained when the voltage applied to the cuvette was 12 kV cm\(^{-1}\) and the resistance was set at 200 \(\Omega\). When the resistance was set at 100 \(\Omega\), the time constant obtained during electroporation was lower, resulting in a lower number of transformants. At 400 \(\Omega\), the viability of the bacteria was reduced, resulting in a decrease of the number of transformants (data not shown). For optimal results (with a time constant between 4 and 4.5) the volume of buffer in which the cells are resuspended after the washing steps should be about 2–3 times the volume of bacteria. A higher cell concentration lowers the transformation efficiency. In all other experiments with pDG7 DNA a voltage of 12 kV cm\(^{-1}\) was applied at a resistance of 200 \(\Omega\).

### Table 2. Effect of electroporation buffer composition on transformation efficiency of *B. animalis* ATCC 27536

<table>
<thead>
<tr>
<th>Buffer</th>
<th>Transforms per µg pDG7 DNA</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>6 kV cm(^{-1})</td>
</tr>
<tr>
<td>Citrate, pH 5</td>
<td>65</td>
</tr>
<tr>
<td>Citrate, pH 6</td>
<td>130</td>
</tr>
<tr>
<td>Phosphate, pH 4-5</td>
<td>200</td>
</tr>
<tr>
<td>Phosphate, pH 6</td>
<td>20</td>
</tr>
<tr>
<td>HEPES, pH 6</td>
<td>20</td>
</tr>
<tr>
<td>HEPES, pH 7-4</td>
<td>0</td>
</tr>
</tbody>
</table>

### Optimization of transformation of *B. animalis*

The effect of the duration and temperature of the pretreatment step on the transformation efficiency of *B. animalis* is shown in Fig. 3. The highest transformation efficiency \((9.4 \times 10^4)\) was found after incubation of the bacteria at 4 °C for 3.5 h. Incubation at lower or higher temperatures resulted in a reduction of the transformation efficiency. At temperatures over 14 °C the transformation efficiencies decreased rapidly. When the pretreatment step was carried out at 37 °C essentially the same results were obtained as at 25 °C (not shown).

### Transformation of different *Bifidobacterium* strains

Application of the pretreatment and electroporation procedure described above for *B. animalis* (pretreatment overnight at 4 °C in 1 mM citrate buffer, pH 6; pulse 12 kV cm\(^{-1}\) at 200 \(\Omega\)) to other *Bifidobacterium* species and strains yielded many transformants (Table 3). Although the efficiencies varied from strain to strain, several strains could be reproducibly transformed with high efficiency (up to \(7 \times 10^4\) per µg pDG7 DNA). Differences in transformation efficiency were observed after overnight pretreatment depending on the purity of the DNA preparations used for transformation. For example, *B. animalis* ATCC 27536 reproducibly yielded \(8 \times 10^4\) transformants per µg DNA with a newly isolated preparation of pDG7 DNA. No attempts have been made thus far to optimize the transformation efficiencies for strains other than *B. animalis* ATCC 27536.

### Transformation of *Bifidobacterium* with vectors from *Corynebacterium*

Since from a phylogenetic point of view *Corynebacterium* spp. are related to *Bifidobacterium*, we investigated the ability of two plasmids with a *Corynebacterium* replicon (pEBM3 and pECM2) to transform *B. animalis*. Both plasmids yielded transformants containing a plasmid with a structure indistinguishable by restriction enzyme analysis from that of the parent plasmid (not shown). Approximately \(2 \times 10^3\) transformants were obtained per µg DNA, after overnight pretreatment of bacteria at 4 °C in 1 mM citrate buffer, pH 6, +0.5 M sucrose; electroporation 10 kV cm\(^{-1}\). These results clearly demonstrate that *Corynebacterium* plasmids can replicate in *B. animalis*. Since the intensity of the plasmid band visualized by staining of the gel after electrophoresis of miniscreen DNA was comparable to that of pDG7 in *Bifidobacterium* transformants, the copy numbers of the *Corynebacterium* vectors
do not seem to differ greatly from that of pDG7. All attempts to transform Lactobacillus casei with pDG7 or pEBM3/pECM2 have failed, suggesting that these plasmids cannot replicate in this organism. Interestingly, a plasmid from Corynebacterium diphtheriae, pNG2, has been shown to replicate autonomously in Corynebacterium, Mycobacterium and E. coli (Messerotti et al., 1990). Our observation that the Corynebacterium plasmids pEMB3 and pECM2 replicate in B. animalis, while plasmid pNG2 can replicate in Mycobacterium, might suggest that replication functions of some plasmids from bacteria of the so-called GC-rich branch of the evolutionary tree of the bacterial kingdom are recognized in different hosts, as are replication functions of some plasmids of the AT-rich branch (Gruss & Ehrlich, 1989; Del Solar et al., 1993). In support of this view, we have observed that the presumed replication protein of the Corynebacterium plasmid pXZ10142 (EMBL/GenBank accession number X72691) is strikingly similar to that of the Mycobacterium plasmid pAL5000 (Ranes et al., 1990) and also, but to a lesser extent, to those of plasmids ColE3 from E. coli (Yasueda et al., 1989) and pJD1 from Neisseria gonorrhoeae (Korch et al., 1985; Fig. 4). The similarity of the replication protein of pXZ10142 with the other three proteins ranges from 46% to 57%, but is considerably higher in the regions between amino acids 110–200, 220–237 and 251–320. These proteins show no resemblance to replication proteins of plasmids of the AT-rich branch. Nucleotide sequence analysis might reveal
The sequences were aligned with the GCG software program Pileup. Identical and similar amino acids in the proteins of pAL5000 and pXZ (pXZ10142) are indicated by x and *, respectively. Regions of similarity in all four proteins are shaded. The four plasmids are described in the text.

### Table 3. Transformation of different bifidobacterial strains

<table>
<thead>
<tr>
<th>Strain</th>
<th>Transformants per µg pDG7 DNA</th>
</tr>
</thead>
<tbody>
<tr>
<td>B. animalis ATCC 27536</td>
<td>5 x 10⁴</td>
</tr>
<tr>
<td>B. brevis 4</td>
<td>1.3 x 10⁴</td>
</tr>
<tr>
<td>B. brevis AS</td>
<td>2 x 10⁴</td>
</tr>
<tr>
<td>B. bifidum U3</td>
<td>3 x 10⁴</td>
</tr>
<tr>
<td>B. bifidum ATCC 15696</td>
<td>7.4 x 10³</td>
</tr>
<tr>
<td>B. infantis U1</td>
<td>2.5 x 10⁴</td>
</tr>
<tr>
<td>B. infantis ATCC 27920</td>
<td>4 x 10⁴</td>
</tr>
<tr>
<td>B. longum U2</td>
<td>2.6 x 10⁴</td>
</tr>
<tr>
<td>B. longum Wiesby 2</td>
<td>7 x 10⁴</td>
</tr>
</tbody>
</table>

Prior to electroporation, bacteria were incubated overnight at 4°C in 0.5 M sucrose + 1 mM ammonium citrate, pH 6. The voltage used for electroporation was 12 kV cm⁻¹ and the resistance was set at 200 Ω.

### Plasmid vectors from lactococci and lactobacilli do not replicate in Bifidobacterium

Despite several attempts, we have been unable to transform B. animalis with plasmid pLP825, carrying a replicon from L. plantarum (Posno et al., 1991) or with the broad-host-range Lactococcus plasmid pGK12 (Kok et al., 1984). These two plasmids, which have a GC content of ~35 mol%, are typical representatives of a family of AT-rich plasmids shown before to replicate in a variety of organisms of the AT-rich branch (Gruß & Ehrlich, 1989; Del Solar et al., 1993). In contrast to the family of AT-rich plasmids, plasmids from Bifidobacterium, Mycobacterium and Corynebacterium are GC-rich (GC content 55–65 mol%). The inability of AT-rich plasmids like pLP825 and pGK12 to replicate in B. animalis, and presumably in other Bifidobacterium strains, may be explained by assuming that the AT-rich expression signals of the replication functions are not, or are inefficiently, recognized by the host enzymes.

### ACKNOWLEDGEMENTS

We thank Hanny Hessing for valuable suggestions with regard to the comparison of protein sequences. Part of the results presented in this paper have been described in patent application EP 94201746.

### REFERENCES


Received 30 June 1995; revised 29 August 1995; accepted 6 September 1995.