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

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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*, electrotransformation, 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 10^5 – 10^7 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 micro-organisms 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.*

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Table 1. Plasmids

Plasmid	Markers	Size (kb)	Reference
pGK12	Em ^r Cm ^r	4.4	Kok <i>et al.</i> (1984)
pLP825	Amp ^r Cm ^r	7.7	Posno <i>et al.</i> (1991)
pDG7	Amp ^r Cm ^r	7.3	Matteuzzi <i>et al.</i> (1990)
pEBM3	Km ^r Cm ^r	9.6	Gift from J. Kalinowski
pECM2	Km ^r Cm ^r	10.3	Gift from J. Kalinowski

breve 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 carries 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 pLP825, 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 DH5 α , used as host strain for propagating the shuttle vectors, was cultivated in L-medium (Sambrook *et al.*, 1989) supplemented with the appropriate antibiotic: kanamycin (Sigma), final concentration 50 μ g ml⁻¹, and ampicillin (Sigma), final concentration 50 μ g 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 cultivated at 37 °C until an OD₆₉₅ 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 μ g) was mixed with 80 μ l 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 μ F 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 μ l 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 μ g 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-

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

The suspension buffer contained 0.5 M sucrose and the concentration of buffer was 1 mM. Pretreatment was for 14 h at 4 °C. The voltage used for electroporation was as indicated and the resistance was set at 200 Ω .

Buffer	Transformants per μg pDG7 DNA	
	6 kV cm^{-1}	10 kV cm^{-1}
Citrate, pH 5	65	740
Citrate, pH 6	130	2700
Phosphate, pH 4.5	200	940
Phosphate, pH 6	20	500
HEPES, pH 6	20	1700
HEPES, pH 7.4	0	660

tivated 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, Missich *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 °C for 1 h in sterile glycerol and storage at -70 °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 Ω . When the resistance was set at 100 Ω , the time constant obtained during electroporation was lower, resulting in a lower number of transformants. At 400 Ω ,

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 Ω .

Optimization of transformation of *B. animalis*

The effect of the duration and temperature of the preincubation step on the transformation efficiency of *B. animalis* is shown in Fig. 3. The highest transformation efficiency (9.4×10^4) 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 preincubation 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 Ω) 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 7×10^4 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 8×10^4 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 2×10^3 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

Fig. 1. Agarose gel electrophoresis of plasmid DNA extracted from transformants compared with plasmid pDG7 from *E. coli* used for transformation. Lanes: 1 and 14, 0.5 µg pDG7 (control) isolated from *E. coli*; 2 and 3, clones from citrate pH 5; 4 and 5, clones from citrate pH 6; 6 and 7, clones from phosphate pH 4.5; 8 and 9, clones from phosphate pH 6; 10 and 11, clones from HEPES pH 6; 12 and 13, clones from HEPES pH 7.4; 15, lambda/EcoRI/HindIII.

Fig. 2. Effect of voltage used for electroporation on the efficiency of transformation of *B. animalis* (expressed as the number of transformants per µg pDG7 DNA). Resistance was set at 200 Ω. Bacteria were incubated for 13–14 h at 4 °C in 0.5 M sucrose + 1 mM ammonium citrate, pH 6, prior to electroporation.

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*

Fig. 3. Relationship between temperature and duration of incubation of bacteria in electroporation buffer and the efficiency of transformation of *B. animalis* (expressed as number of transformants per µg pDG7 DNA). The suspension buffer contained 0.5 M sucrose + 1 mM ammonium citrate, pH 6. The voltage used for electroporation was 12 kV cm⁻¹. Resistance was set at 200 Ω. Preincubation temperature: 0 °C (◇), 4 °C (●), 14 °C (□) or 25 °C (○).

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

Table 3. Transformation of different bifidobacterial strains

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.

Strain	Transformants per µg pDG7 DNA
<i>B. animalis</i> ATCC 27536	5 × 10 ³
<i>B. breve</i> 4	1.3 × 10 ⁴
<i>B. breve</i> AS	2 × 10 ²
<i>B. bifidum</i> U3	3 × 10 ²
<i>B. bifidum</i> ATCC 15696	7.4 × 10 ³
<i>B. infantis</i> U1	2.5 × 10 ²
<i>B. infantis</i> ATCC 27920	4 × 10 ⁴
<i>B. longum</i> U2	2.6 × 10 ³
<i>B. longum</i> Wiesby 2	7 × 10 ⁴

pAL5000	1	MGWPLSEISD	LVLVSDHTPV	INRGLSPSVS	HVADETEQLW	LEFWPLASDD	50
pXZ							
ColE3				MSAVL	QR.	F REK	LEHKEYCTND
pJD1				MSQLY	TQPDLELQER	LEHKEYCKDF	
pAL5000	51	LLEGYYRQSR	ASALGKRYTE	ANPTALANLL	VVDVDPHDA	LRALSARGSH	100
pXZ		MPSRISWSST	STSRTHSCVR	CGTETAG.		G L...	TFWLKT
ColE3		* * * * *	* * * * *				
pJD1		FAYGVRLPK	NIALARFTQ	QNOPHALYWL	PEDVORTGAS	I...	DMSDR
		KEAPMLVRBY	AAAIKRRYIQ	VNPSHLRVFM	LEFDLYEGAG	L...	AWEDN
pAL5000	101	PLENA...IV	GNRANGHANA	VWALNAPVPR	TEY ARRKP	AYMAACBGL	150
pXZ		PFKRARTRRV	GARGAIYPHR	VROAQA...		I AYAAVTEGL	
ColE3		* * * * *	* * * * *				
pJD1		NCP APNITY	KNPRNGHAHL	LYALALPVRT	AP DASASAL	RYAAATERAL	
		NLP MPAAWA	INRENGGAHL	AYALSAPVLT	AEYGGROKAL	RYLAALAAAY	
pAL5000	151	RRAVDGDGRSY	SGLITKNPCH	IANETE.WLH	SD...LYTL	SHI.F.ABLG	200
pXZ		RRSVDDQDKY	SGLITKNPCH	TANDSH.WVT	DK...LYTL	DELRFLKET	
ColE3		xx xxxxxx*x	xx*x*x*x				
pJD1		CEKLGADVNY	SGLICKNPCH	PEWQEVNRE	EP...YTL	DELADYLDLS	
		KAKLRGDVGF	VSLITKNPCH	PHW...LTLRG	VPDAIRGYDL	EYLADFPVLD	
pAL5000	201	ANMEPPPRWRQ	QTTYKAAPT	LGRENALFDS	VRLWAYRPA	MRIYLPTRNV	250
pXZ		GFMPPEPWKK	..TRRSFPG	LGRENALFES	ARSWAYRE..	IRHHFPG.DP	
ColE3		* xxx *x	* * * * *	xxxxxxx*x	x xxxx		
pJD1		ASAR...RSV	...DKNYG	LGRENHLEK	VRKNAYR.AI	...RGGWPF	
		KFKFYIGRSN	...VEAVG	LSRNCIVFNL	VSRNAHKNVL	AFKQGGYTV.	
pAL5000	251	DGLGRAIYAE	CHARNARFPC	NDVCPGELPD	SEVRATANSI	WRWITTKSRI	300
pXZ		DGLGRSQAT	AQALNQEL	..FS.EPLPV	AEVDQIARSI	HRWITTKSRM	
ColE3		xxxxx x x	* * * * *		xx xx xx	xxx xxxxx	
pJD1		SQLDAVIGR	VEVMYASLEV	...FLSP	AECAIGKSI	AKYT...ERKF	
		QQWLKEVHYQ	CMRVNGDFEV	...FMWE	KEYVCISKSI	ANWVWYKFDI	
pAL5000	301	WAD.GE.VV	YEATLSARGS	AISRKGAAAR	TAASTVARRA	KSASAMEALL	350
pXZ		WTD.G.PAV	YEATFTTQIA	TRGKRSAEHR	WGTTNAERIE	REFIND....	
ColE3		x x x x	xxxxx *x	xxxxx x			
pJD1		..SPEGFSAV	QAARG..RRG	GTKSKRAAVP	TSARSLKPWE	ALGISRATYY	
		AASRRRFEEL	QAHRNLRKLT	TINAGRTKII	TEL.....		
pAL5000	351						361
pXZ							
ColE3		RKLKCDPDLA	K				
pJD1							

Fig. 4. Comparison of the deduced amino acid sequence of the replication protein genes of plasmids from different bacteria. 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.

whether *Bifidobacterium* plasmids like pDG7 show similarity to *Corynebacterium* and *Mycobacterium* plasmids and form part of a larger plasmid family.

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.

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