

Efficient Transformation System for *Propionibacterium freudenreichii* Based on a Novel Vector

J. P. M. JORE,^{1*} N. VAN LUIJK,¹ R. G. M. LUITEN,² M. J. VAN DER WERF,¹ AND P. H. POWELS¹

TNO Nutrition and Food Research, 3700 AJ Zeist,¹ and DSM Anti-Infectives, 2600 MA Delft,² The Netherlands

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A 3.6-kb endogenous plasmid was isolated from a *Propionibacterium freudenreichii* strain and sequenced completely. Based on homologies with plasmids from other bacteria, notably a plasmid from *Mycobacterium*, a region harboring putative replicative functions was defined. Outside this region two restriction enzyme recognition sites were used for insertion of an *Escherichia coli*-specific replicon and an erythromycin resistance gene for selection in *Propionibacterium*. Hybrid vectors obtained in this way replicated in both *E. coli* and *P. freudenreichii*. Whereas electroporation of *P. freudenreichii* with vector DNA isolated from an *E. coli* transformant yielded 10 to 30 colonies per μg of DNA, use of vector DNA reisolated from a *Propionibacterium* transformant dramatically increased the efficiency of transformation ($\geq 10^8$ colonies per μg of DNA). It could be shown that restriction-modification was responsible for this effect. The high efficiency of the system described here permitted successful transformation of *Propionibacterium* with DNA ligation mixtures.

The genus *Propionibacterium* can be divided into two groups, a group containing the classical (or dairy) propionibacteria and a group containing the cutaneous propionibacteria (6). Members of the first group, especially *Propionibacterium freudenreichii*, play an essential role in the manufacture of Swiss and related types of cheeses (12). Other industrial applications are found in the production of propionic acid and vitamin B₁₂ (5, 28). Of growing interest, but less well documented, are the probiotic properties ascribed to some propionibacterial strains (16, 21).

Strain improvement and, in general, study of this economically important group of bacteria would be greatly facilitated by the availability of a system for genetic modification. This report describes isolation and characterization of a 3.6-kb plasmid and successful use of this plasmid in the construction of a set of *Escherichia coli*-*Propionibacterium* shuttle vectors. Reproducible transformation of *P. freudenreichii* strains with these shuttle vectors was achieved by means of electroporation.

MATERIALS AND METHODS

Bacterial strains and plasmids. *Propionibacterium* strains were obtained from the Belgian Coordinated Collections of Microorganisms/LMG (Ghent, Belgium), from the American Type Culture Collection (Rockville, Md.), and from the Deutsche Sammlung von Mikroorganismen (Braunschweig, Germany). *P. freudenreichii* subsp. *freudenreichii* VTBI was obtained from DSM Food Specialties' industrial collection. For amplification of newly constructed shuttle vector DNA *E. coli* DH5 α was used. pBluescript SKII+ was obtained from Stratagene (La Jolla, Calif.).

Media and growth conditions. *E. coli* DH5 α was cultivated at 37°C in L medium (24) supplemented with 50 μg of ampicillin per ml if necessary. Propionibacteria were cultivated anaerobically at 30°C in MRS (7) or SLB medium (8) supplemented with an appropriate antibiotic when plasmid isolation was to be performed or in SLB medium when electroporation was to be performed.

Isolation of plasmid DNA from propionibacteria. Propionibacteria were cultivated in 5 ml of medium for 48 h. Plasmid DNA was extracted from the bacteria by a modified *E. coli* plasmid isolation procedure (4). Briefly, cells were washed in 25% sucrose–50 mM Tris-HCl (pH 8) and resuspended in 250 μl of TENS

(25% sucrose, 50 mM NaCl, 50 mM Tris-HCl, 5 mM EDTA; pH 8) containing 10 mg of lysozyme per ml. After 20 to 30 min of incubation at 37°C, cells were lysed by adding 500 μl of 0.2 N NaOH–1% sodium dodecyl sulfate and incubating the preparation on ice for 2 to 5 min. Then 400 μl of 3 M sodium acetate (pH 4.8) was added, and this was followed by 5 min of incubation on ice and extraction with phenol-chloroform. DNA was precipitated by adding isopropanol.

General methods. The molecular biological techniques used in this study were described by Sambrook et al. (24). Restriction enzymes and T4 DNA ligase were purchased from New England Biolabs and GIBCO BRL. *Taq* polymerase was obtained from SphaeroQ (Leiden, The Netherlands). All enzymes were used according to the manufacturers' instructions.

Sequence analysis. The nucleotide sequence of plasmid p545, linearized by *Eco*RI and inserted in pBluescript SKII+, was determined by the primer walking strategy, starting from both ends. Overall, 25 primers were designed to cover the complete 3.5 kb, and each nucleotide was read at least two times in each direction. Finally, absence of a small *Eco*RI fragment could be ruled out by sequence analysis of noncloned p545 in the region surrounding the *Eco*RI site.

Sequencing was accomplished by using an Applied Biosystems model 373A automatic sequencer according to procedures provided by the supplier and fluorescent-dye-labeled dideoxynucleotides.

Isolation of a *Propionibacterium*-specific 16S rRNA promoter. On the basis of the sequence of 16S rRNA from *P. freudenreichii* DSM 20271 (= ATCC 6207) (GenBank accession number X53217), we chose an appropriate restriction enzyme (*Hind*III) and designed primers that enabled us to amplify an approximately 3-kb region encompassing the promoter by inverse PCR (18). From the PCR product a 0.6-kb *Sph*I-*Hind*III fragment directly upstream of the 16S rRNA coding sequence was isolated for further use.

Construction of *E. coli*-*Propionibacterium* shuttle vectors. To construct *E. coli*-*Propionibacterium* shuttle vectors, we used pBR322 in which the *Eco*RI-*Ava*I fragment encompassing the tetracycline resistance gene was replaced by a polylinker. In pBR322 Δ tetPL the sequence of this polylinker is as shown in Fig. 1, which includes restriction sites for *Eco*RI (restored), *Hind*III, *Sal*I, *Hpa*I, *Pst*I, *Sph*I, *Bam*HI, *Acc*65I, *Eco*RV, and *Bgl*II (*Ava*I is not restored). In the *Acc*65I site a 1.7-kb *Acc*65I fragment was cloned that encompassed the erythromycin resistance gene (*ermE*) from *Saccharopolyspora erythraea* NRLL2338 (3, 27), which yielded pBRES1. In pBRES1 the genes for erythromycin and ampicillin resistance were transcribed in opposite directions. The *Eco*RV site of pBRES1 was used for insertion of plasmid p545 from *P. freudenreichii* LMG 16545 linearized with *Bsa*BI, which resulted in pBRESP36B1 and pBRESP36B2; the difference between the latter two plasmids was the orientation of p545.

For insertion of *Alw*NI-linearized p545, a polylinker was introduced into

5' AATTCAAGCTTGTGACGTTAACTGACGGCATCGGGATCCGGTACCGATATCAGATCT 3'
GTTCCGAACAGCTGCAATTGGACGTCGGTACGCCATAGCCATAGCTAGAAGCC

FIG. 1. Sequence of the polylinker in pBR322 Δ tetPL.

* Corresponding author. Mailing address: TNO Nutrition and Food Research, P.O. Box 360, 3700 AJ Zeist, The Netherlands. Phone: 31 30 69 444 68. Fax: 31 30 69 444 66. E-mail: jore@voeding.tno.nl.

pBRES1 between the *Bgl*II site and the proximal *Acc*65I site, and the sequence of this polylinker was as follows:

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5' GTACCGGCCGCTGCGGCCAAGCTT 3'
   GCCCGCGACGCCGGTTCGAAC TAG
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The polylinker supplied restriction sites for *Acc*65I (restored), *Sfi*I, and *Hind*III (*Bgl*II was not restored). The resulting plasmid was designated pBRES2. Ligation of *Sfi*I-linearized pBRES2 with *Alw*NI-linearized p545 yielded pBRESP36A.

To enable stepwise deletion of p545-specific parts from pBRESP36A DNA, this DNA was first digested with *Sst*II and *Bcl*I, which resulted in 1.7- and 6.5-kb fragments. The 1.7-kb fragment was replaced by a synthetic duplex DNA. In this way, in effect, the 1.6-kb *Alw*NI-*Bcl*I fragment of plasmid p545 was deleted from the vector. The synthetic duplex DNA was designed to link *Sst*II and *Bcl*I ends and to supply a number of unique restriction sites; its sequence was as follows:

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5' GGAGATCTAGATCGATATCTCGAG 3'
   CGCCTCTAGATCTAGCTATAGAGCTCCTAG
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Thus, the following restriction enzyme recognition sites were supplied: *Sst*II (restored), *Bgl*II, *Xba*I, *Cl*aI, *Eco*RV, and *Xho*I (*Bcl*I was not restored). The ligation mixture was transferred to *E. coli*, and we selected a transformant that contained a vector having the expected composition. The vector was designated pBRESAΔS-B.

Electroporation. *P. freudenreichii* strains cultivated to the stationary growth phase were diluted 1:50 in fresh SLB medium. After incubation for about 20 h, the cells, which were in the exponential growth phase, were harvested and washed extensively in ice-cold 0.5 M sucrose. Electroporation of *P. freudenreichii* strains was performed with a Gene Pulser apparatus (Bio-Rad) by using a modified protocol developed for electroporation of bifidobacteria (2). Briefly, cells were washed once in ice-cold electroporation buffer (0.5 M buffered sucrose) and resuspended in electroporation buffer (about 1/100 of the original culture volume). Then 80 to 100 μ l of the suspension was mixed with DNA in a cooled electroporation cuvette, and an electric pulse was delivered at 200- Ω resistance and 25- μ F capacitance. Optimal electroporation results were obtained in 0.5 M sucrose buffered with 1 mM potassium acetate (pH 5.5) at 20 kV/cm. Immediately after the pulse 900 μ l of cold SLB medium containing 0.5 M sucrose was added, and after 2.5 to 3 h of incubation at 30°C, cells were plated on SLB agar plates containing 0.5 M sucrose and 10 μ g of erythromycin per ml. After 5 to 7 days of incubation at 30°C under anaerobic conditions, transformants could be detected.

Nucleotide sequence accession number. The nucleotide sequence of plasmid p545 has been deposited in the GenBank database under accession number AF291751.

RESULTS AND DISCUSSION

Initial transformation experiments. Our initial attempts to transform propionibacteria were aimed at *P. freudenreichii* type strain ATCC 6207. We used electroporation procedures developed in our laboratory for lactobacilli (15, 22) and for bifidobacteria (2) with *Corynebacterium-E. coli* shuttle vectors pECM2 and pEBM3 (gifts from J. Kalinowski), *Bifidobacterium-E. coli* shuttle vector pDG7 (17), *Lactobacillus-E. coli* shuttle vector pLP825, *Lactobacillus*-specific vector pLPE323 (14, 22), and broad-host-range *Lactococcus*-derived plasmid pGK12 (10). None of these attempts yielded any transformants. Since one of the possible explanations for this was the inability of propionibacteria to support replication of the vectors used, a set of new shuttle vectors based on a *Propionibacterium*-specific replicon was constructed.

Screening *Propionibacterium* strains for endogenous plasmids. Seventy-five *Propionibacterium* strains representing all four recognized species of dairy propionibacteria were screened for the presence of endogenous plasmids. In the majority of these strains no small endogenous plasmids could be found, in accordance with reports on other *Propionibacterium* strains (19, 20, 23). The following six strains were found to contain a 6- to 10-kb plasmid: *P. acidipropionici* ATCC 4875

(= DSM 20272) and LMG 16447, *P. jensenii* LMG 16453, *P. freudenreichii* LMG 16545, *P. freudenreichii* subsp. *freudenreichii* LMG 16546, and *Propionibacterium* sp. strain LMG 16550. All of these strains except ATCC 4875 also harbor one or more large (\geq 20-kb) plasmids. *P. freudenreichii* LMG 16545 and LMG 16546 were both found to contain a 3.6-kb plasmid; these strains were chosen for further study, and the plasmids which they harbor are designated p545 and p546, respectively. In Southern blot experiments in which cloned p545 (see below) was used as a probe, strong hybridization was observed with plasmid preparations from strains LMG 16545 and LMG 16546, indicating that plasmids p545 and p546 are closely related. No hybridization was observed with plasmid DNA from *P. acidipropionici* ATCC 4875 (results not shown); the plasmid of this strain has been described previously by Rehberger and Glatz as plasmid pRG01 (23).

Analysis of plasmids p545 and p546. Restriction enzyme analysis of p545 and p546 DNA revealed identical restriction patterns (data not shown). Because of the assumed identity, only one of these plasmids, p545, was sequenced; the sequence was 3,555 bp long. A BLAST search in GenBank (1) revealed that two open reading frames (ORFs) (ORF1 and ORF2, comprising 303 and 85 amino acids, respectively) showed significant homology to (putative) replication proteins from a number of plasmids, including pAL5000 (11, 25), from *Mycobacterium fortuitum*. ORF1 and ORF2 were 28 to 30% identical and 34 to 38% similar to pAL5000 replication proteins *repA* and *repB*, respectively. As found for the other plasmids, the two replication proteins in p545 showed translational coupling. Such coupling was also suggested by L. Meile (personal communication) for *Propionibacterium* plasmid pLME108 (accession number AJ006662).

In pAL5000 a minimal replicon could be defined, and this replicon consisted of the translationally coupled *repA* and *repB* genes and a 435-bp “*inc* region” located upstream from *repA* and containing the origin of replication (25). Although a similar origin could not be found in p545, it was deemed likely that sites not interrupting the two ORFs or the approximately 500-bp upstream region would not interfere with replication of the plasmid. Analysis of p545 DNA for suitable unique restriction sites yielded two likely candidates, a *Bsa*BI site and an *Alw*NI site (Fig. 2A). These sites were used to introduce an *E. coli*-specific replicon and a selection marker for *Propionibacterium*, as described in Materials and Methods.

Transformation of *P. freudenreichii* strains by electroporation. By using *P. freudenreichii* ATCC 6207, LMG 16545, and VTB1 as host organisms, low but reproducible transformation efficiencies, 10 to 30 transformants per μ g of plasmid DNA, were obtained (Table 1). Within limits, the type of buffer and the actual voltage applied had only modest effects. The sizes and restriction patterns of plasmid DNA isolated from *P. freudenreichii* transformants were indistinguishable from those of the input DNA, indicating that replication took place without detectable alteration of the plasmid DNA. Southern blot hybridization confirmed that the vectors were present as autonomously replicating DNA; chromosomal integration was never observed. Moreover, the assumption that *Bsa*BI and *Alw*NI sites in p545 were located outside the replication region proved to be correct. Finally, the replicon was found to be active irrespective of the polarity relative to the selection marker

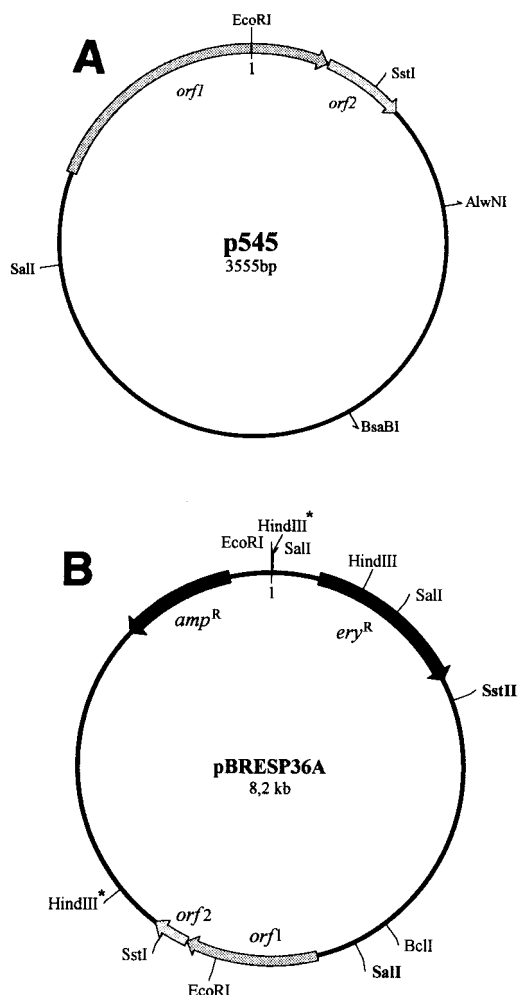


FIG. 2. Limited restriction map of plasmid p545 (A) and of one of its derivative shuttle vectors (B). The positions of the two translationally coupled ORFs are also indicated. The restriction enzyme recognition sites in boldface type indicate the 1.8-kb p545-specific region that can be deleted without disturbing replication. *HindIII* sites used for deletion of the pBR-specific part are indicated by asterisks.

and *E. coli* replicon (pBRESP36B1 versus pBRESP36B2). From Table 1 we also concluded that activity of the p545 replicon may be limited to *P. freudenreichii* strains, since electroporation of other *Propionibacterium* species did not yield any transformants.

Restriction-modification. In an attempt to increase the efficiency of transformation, an electroporation experiment was performed with plasmid DNA isolated from a *P. freudenreichii* ATCC 6207 transformant. A 10^6 - to 10^7 -fold greater transformation efficiency compared to that obtained with DNA isolated from *E. coli* DH5 α was observed (Table 1); i.e., there were $\geq 10^8$ transformants per μg of DNA, suggesting that one or more likely several restriction-modification systems were present in ATCC 6207. Indeed, when plasmid DNA isolated from an ATCC 6207 transformant was used to transform ATCC 6207 again after passage through *E. coli* DH5 α , the same low frequency of transformation that was initially observed was obtained, ruling out the possibility that the high

TABLE 1. Transformation efficiencies in *Propionibacterium* strains with DNA isolated from *E. coli* or *P. freudenreichii* ATCC 6207^a

Recipient strain	Transformation efficiency (no. of transformants/ μg of DNA)	
	DNA isolated from <i>E. coli</i>	DNA isolated from <i>P. freudenreichii</i>
<i>P. freudenreichii</i> strains		
ATCC 6207	10–30	$\geq 10^8$
VTB1	10–30	$\geq 10^8$
LMG 16545	10–30	$\geq 10^8$
<i>P. acidipropionici</i> strains		
DSM 13572	0	0
DSM 20727	0	0
<i>P. jensenii</i> DSM 20535	0	0
<i>P. thoenii</i> DSM 20276	0	0

^a Electroporation was performed at 20 kV/cm, 200 Ω , and 25 μF . The vectors used for transformation were pBRESP36A, pBRESP36B1, and pBRESP36B2.

frequency of transformation obtained with ATCC 6207-derived plasmid DNA was caused by a mutation in the plasmid DNA.

In the same way the existence of restriction-modification systems in *P. freudenreichii* LMG 16545 and VTB1 could be shown. Moreover, since the same increase in efficiency was observed when ATCC 6207-derived plasmid DNA was used to transform ATCC 6207, LMG 16545, or VTB1, it is plausible that the restriction-modification systems in these strains are identical. Analysis of the type and specificity of the restriction-modification system is currently under way; preliminary results indicate that no type II restriction enzymes are present. Again, no transformants were obtained upon electroporation of other *Propionibacterium* species (Table 1).

Towards identification of a minimal replicon in p545. Since a selectable derivative of plasmid p545 (i.e., pBRESP36A) (Fig. 2B) and a highly efficient transformation procedure were available, experiments were performed to determine which parts of the p545 plasmid are essential for replication in propionibacteria by transferring pBRESP36A and deletion derivatives of pBRESP36A to propionibacteria.

As might be expected, the *E. coli*-specific part of pBRESP36A was not involved, since deletion of this part from the shuttle vector by partial digestion with *HindIII* and religation (Fig. 2B) did not impair replication in propionibacteria.

Since *P. freudenreichii* VTB1 and ATCC 6207 could be successfully transformed with vector pBRESA Δ S-B (see Materials and Methods), we concluded that the 1.6-kb region between *AlwNI* and *BclI* in p545 is not essential for replication of the plasmid. Further deletion of the 240-bp p545-specific *SalI*-*BclI* fragment (achieved by ligation of the 1.3-kb *SalI*-*SstI* fragment and the 6.6-kb *SstI*-*XhoI* fragment of pBRESA Δ S-B, isolated from a *Propionibacterium* transformant) did not impair replication in propionibacteria either; transfer of the ligation mixture to *P. freudenreichii* ATCC 6207 yielded numerous transformants. Analysis of a number of transformants showed that they all carried the expected deletion variant of pBRESA Δ S-B. The newly derived plasmid was designated pBRESA Δ S-S. In effect, we showed that all essential information for replication of p545 in propionibacteria is located on a 1.7-kb fragment and that the other 1.8 kb can be deleted without obviously disturbing replication of the plasmid.

TABLE 2. Antibiotic resistance genes used in this study

Gene	G+C content (%)	Origin	Promoter	<i>Propionibacterium</i> transformants
<i>cat</i>	29	pC194 (<i>Staphylococcus aureus</i>)	Original	—
			16S rRNA	—
<i>cat</i>	53	pACYC184 (<i>Escherichia coli</i>)	Original	—
			<i>ermE</i>	—
			16S rRNA	(+) ^a
<i>cml</i>	63	pEBM3 (<i>Corynebacterium striatum</i>)	Original	+
<i>ery</i>	34	pAMβ1 (<i>Enterococcus faecalis</i>)	Original	—
<i>ery</i>	71	pIJ488 (<i>Saccharopolyspora erythraea</i>)	Original (= <i>ermE</i>)	+

^a Only as a secondary selection marker.

Electroporation of *P. freudenreichii* strains with vectors carrying other selection markers. Vectors were constructed in which the erythromycin resistance gene present in the pBRESP36 series of shuttle vectors was replaced by a different selection marker or into which a second selection marker was introduced (Table 2). Vectors carrying as a single selection marker the erythromycin resistance gene from *Enterococcus faecalis* plasmid pAMβ1 (13) or the chloramphenicol resistance (*cat*) gene from *Staphylococcus aureus* plasmid pC194 (9) with either its own promoter or with a *P. freudenreichii*-specific rRNA promoter (see Materials and Methods) did not yield any transformants upon electroporation of *P. freudenreichii* strains. Given the high G+C content of *ermE* and the low G+C contents of the other selection markers (34% for *E. faecalis* and 29% for *S. aureus*), it is tempting to speculate that genes with low G+C contents are poorly expressed in propionibacteria if they are expressed at all. Therefore, the chloramphenicol resistance (*cat*) gene from pACYC184 (G+C content, 53%) was introduced as a second selection marker into pBRESP36B2, and this marker carried either its own promoter, the *ermE* promoter, or the *P. freudenreichii*-specific 16S rRNA promoter. The vectors obtained in this way all conferred chloramphenicol resistance to *E. coli*, but after introduction into *P. freudenreichii* by electroporation, primary selection of transformants with chloramphenicol proved to be impossible. Only after primary selection with erythromycin could chloramphenicol be used as a selective agent, and this occurred only when the 16S rRNA promoter was present.

Introduction of the chloramphenicol resistance gene (*cml*) from *Corynebacterium striatum* (26) into pBRESP36B2 yielded a vector that could be directly selected for with chloramphenicol. However, although the G+C content of *cml* (63%) is comparable to that of *P. freudenreichii*, this may not be the only reason, since *cml* provides resistance by expelling chloramphenicol, whereas *cat* provides resistance by acetylating chloramphenicol.

Analysis of vector stability. *P. freudenreichii* transformants containing pBRESP36A, pBRESP36B1, or pBRESP36B2 were cultivated for about 25 generations in medium without erythromycin and subsequently plated on solidified medium with and without erythromycin. No gross differences in the number of colonies were observed, indicating that the vector is segregationally stably maintained (≤5% loss after 25 generations without selection). In addition, structural stability was studied by cultivation in selective medium for about 25 generations, plating on solidified medium, and analysis of plasmid DNA from a number of colonies. No deletions were observed,

indicating that the vectors were also structurally stably maintained.

Conclusion. A reproducible and highly efficient host-vector system for *P. freudenreichii* has been developed. To our knowledge, this is the first time that such a system has been described.

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