

# Characterization of pRGO1, a Plasmid from *Propionibacterium acidipropionici*, and Its Use for Development of a Host-Vector System in Propionibacteria

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The complete nucleotide sequence of pRGO1, a cryptic plasmid from *Propionibacterium acidipropionici* E214, was determined. pRGO1 is 6,868 bp long, and its G+C content is 65.0%. Frame analysis of the sequence revealed six open reading frames, which were designated Orf1 to Orf6. The deduced amino acid sequences of Orf1 and Orf2 showed extensive similarities to an initiator of plasmid replication, the Rep protein, of various plasmids of gram-positive bacteria. The amino acid sequence of the putative translation product of *orf3* exhibited a high degree of similarity to the amino acid sequences of DNA invertase in several bacteria. For the putative translation products of *orf4*, *orf5*, and *orf6*, on the other hand, no homologous sequences were found. The function of these open reading frames was studied by deletion analysis. A shuttle vector, pPK705, was constructed for shuttling between *Escherichia coli* and a *Propionibacterium* strain containing *orf1* (*repA*), *orf2* (*repB*), *orf5*, and *orf6* from pRGO1, pUC18, and the hygromycin B-resistant gene as a drug marker. Shuttle vector pPK705 successfully transformed *Propionibacterium freudenreichii* subsp. *shermanii* IFO12426 by electroporation at an efficiency of  $8 \times 10^6$  CFU/ $\mu$ g of DNA under optimized conditions. Transformation of various species of propionibacteria with pPK705 was also performed at efficiencies of about  $10^4$  to  $10^7$  CFU/ $\mu$ g of DNA. The vector was stably maintained in strains of *P. freudenreichii* subsp. *shermanii*, *P. freudenreichii*, *P. pentosaceum*, and *P. freudenreichii* subsp. *freudenreichii* grown under nonselective conditions. Successful manipulation of a host-vector system in propionibacteria should facilitate genetic studies and lead to creation of genes that are useful industrially.

Propionibacteria, which have a wide range of probiotic activity, are used in making dairy foods, such as cheese, for the production of vitamin B<sub>12</sub>, tetrapyrrole compounds, and propionic acid (8, 15, 21), in bread baking, as starters for ensilage, and in some pharmaceutical preparations (35). To elucidate the biosynthetic pathways of vitamin B<sub>12</sub> and siroheme in *Propionibacterium*, we previously identified several genes coding for the enzymes involved in production of tetrapyrrole derivatives (*hemYHXBRL*) (11, 12) and vitamin B<sub>12</sub> (*cobA*, *cbiO*) (30).

Development of genetic manipulation in propionibacteria has progressed slowly due to a lack of detailed information on the genetics of the bacteria and a lack of an appropriate plasmid that can serve as a possible transformation vector. A number of plasmids from *Propionibacterium acidipropionici*, *P. freudenreichii*, and *P. jensenii*, ranging in size from 4.4 to more than 119 MDa, have been described (19, 24). However, neither analysis of a plasmid DNA sequence nor construction of a vector for propionibacteria has been reported. To establish a versatile vector system to facilitate genetic analysis and to allow the transfer of a gene of interest, we investigated the development of a host-vector system in propionibacteria.

We succeeded in determining the complete nucleotide sequence of plasmid pRGO1 from *P. acidipropionici* E214 (24). This is the first report of the complete nucleotide sequence of an endogenous plasmid from propionibacteria. On the basis of

the sequence information obtained, we were able to develop a host-vector system in propionibacteria.

## MATERIALS AND METHODS

**Bacterial strains and plasmids.** The *Propionibacterium* spp. strains used in this study were obtained from the Hiroshima University Type Culture Collection (HUT), the Institute for Fermentation, Osaka (IFO), the American Type Culture Collection (ATCC), and the Iowa State University Culture Collection. Plasmid pRGO1, prepared from *P. acidipropionici* E214, was kindly provided by B. A. Glatz. Fragments of pRGO1 were cloned in pUC18/19 (37), pMW218 (3), or pBluescriptII KS+ (Stratagene, La Jolla, Calif.). *Escherichia coli* JM109 (37) or JM110 (37) was used as the host strain. PSMT3, a plasmid containing the hygromycin B gene (*hygB*), was provided by A. Jaworsky.

**Culture conditions, media, and reagents.** Propionibacteria were grown anaerobically at 32°C in sodium lactate broth (NLB) containing 1% sodium lactate, 1% yeast extract, and 1% Trypticase soy broth (13). *E. coli* was grown at 37°C in Luria broth (28). Ampicillin and kanamycin were added to appropriate media at concentrations of 100 and 30  $\mu$ g/ml, respectively. When required, hygromycin B (Wako Pure Chemical Industries, Osaka, Japan) was added at 250  $\mu$ g/ml for propionibacteria or at 100  $\mu$ g/ml for *E. coli*. Restriction enzymes and T4 DNA ligase, obtained from either Takara Shuzo Co. (Kusatsu, Japan) or Toyobo Co. (Osaka, Japan), were used according to the manufacturer's instructions. Reagent grade chemicals were obtained from Nacalai Tesque, Inc. (Kyoto, Japan) or Sigma Chemical Co. (St. Louis, Mo.).

**Manipulation of DNA.** Plasmid DNA from propionibacteria was prepared from an overnight culture by the alkali-sodium dodecyl sulfate procedure (5), with a slight modification. The propionibacterial cells were lysed with solution I containing lysozyme (20 mg/ml) or were disrupted with 0.5 g of glass beads (diameter, 0.1 mm) per 10 ml of culture by vortexing. After isopropanol precipitation, RNA was precipitated with LiCl<sub>2</sub> (2.5 M), and then plasmid DNA from the soluble extract was precipitated with isopropanol. Samples were treated with DNase-free RNase A (1 mg/ml) at 37°C for 30 min. The plasmid DNA was then precipitated with polyethylene glycol (28). *E. coli* plasmid DNA was prepared by the alkaline lysis method (28).

**Transformation of *Propionibacterium* species.** Transformation was performed on the bench top without any special anaerobic conditions. An overnight culture of propionibacteria was inoculated into fresh NLB to obtain an optical density at

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600 nm of about 0.05. The cells were grown at 32°C and harvested when the optical density at 600 nm reached about 0.8. Cells were washed once with 0.5 volume of 1 mM 2-[4-[2-hydroxyethyl]-1 piperazinyl]ethylene sulfonic acid (HEPES) buffer (pH 7.0). The cell pellet was suspended in 0.1 volume of 10% glycerol and incubated on ice for 30 min. After centrifugation, the pellet was suspended in 0.02 volume of 10% glycerol to obtain a cell concentration of about  $1.8 \times 10^{10}$  cells/ml. This suspension could be frozen in 100- $\mu$ l aliquots and stored at  $-80^\circ\text{C}$ . For transformation by electroporation, 100  $\mu$ l of the cell suspension was mixed with 1.0  $\mu$ l (1  $\mu$ g) of plasmid DNA in MilliQ water and transferred to a sterile cold 0.2-cm-gap cuvette. Transfer of plasmid DNA to propionibacteria was accomplished by electroporation by using an Electrocell Manipulator 600 (BTX Inc., San Diego, Calif.) and the following conditions: electric field strength, 6.0 kV/cm; capacitance, 25  $\mu$ F; and resistance, 129  $\Omega$ . After the pulse, the cells were diluted with 800  $\mu$ l of NLB and then incubated at 32°C for 8 h. An appropriate portion of the cell suspension was plated on NLB agar with a suitable antibiotic and incubated at 32°C for 4 days. *E. coli* cells were transformed by the method of Hanahan (10).

**DNA sequencing.** To determine the complete nucleotide sequence of plasmid pRGO1, overlapping fragments were subcloned in pUC18/19. The nucleotide sequences of both strands were determined by the dideoxy chain termination method (29) with an ABI PRISM 310 genetic analyzer (Perkin-Elmer, Foster City, Calif.) or an ALF DNA sequencing system (Amersham Pharmacia Biotech, Uppsala, Sweden). Sequence data were assembled and analyzed by using the GENETYX MAC program, version 8 (Software Development Co., Tokyo, Japan). Homology searches were carried out by using the BLAST (Basic Local Alignment Search Tool) program (1) and the DNA Data Bank of Japan (DDBJ).

**Stability of the plasmid in propionibacteria.** Propionibacteria carrying the plasmid were grown for 2 days in NLB without antibiotics (nonselective medium). The culture was diluted 1/50 with fresh NLB and propagated at 32°C for 2 days. After 10 serial transfers, cells were plated at an appropriate dilution on NLB solid medium and incubated for 4 days. Several hundred colonies were then picked randomly and replicated on NLB with and without hygromycin B. The plasmid DNAs in these colonies were analyzed by restriction enzyme digestion and gel electrophoresis. Percentages of stability were determined as follows: number of colonies grown on the selective medium/number of colonies grown on the nonselective medium  $\times$  100.

**Nucleotide sequence accession number.** The nucleotide sequence data reported here have been deposited in the DDBJ database under accession no. AB007909.

## RESULTS

**Nucleotide sequence of pRGO1.** To develop a vector system for propionibacteria, we searched various strains of *Propionibacterium* species for appropriate small plasmids. From among 50 strains obtained from stock cultures, including cultures from HUT, IFO, and the Iowa State University Culture Collection, plasmid pRGO1 (24) was selected, and its complete nucleotide sequence was determined (Fig. 1). Plasmid pRGO1 is 6,868 bp long. Its G+C content is 65.0%, which is within the range of G+C contents previously reported for the genus *Propionibacterium* (65 to 68%) (32). Six open reading frames (ORFs), designated *orf1* to *orf6*, were predicted by a computer frame analysis based on the G+C content of the three triplet positions of the genes of microorganisms with genomes rich in G+C (4) (data not shown). Two of the ORFs, *orf4* and *orf6*, have opposite orientations. The six ORFs have G+C contents ranging from 61.5 to 73.7%. The G+C content in the third position of the codon is 90 to 93% in *orf1*, *orf2*, and *orf6* and 81 to 83% in *orf3*, *orf4*, and *orf5*. These third-position G+C percentages are similar to those of several genes in *P. freudenreichii* (12). Predicted ribosome binding sites (Shine-Dalgarno [SD] sequences) (31) were found upstream from each initiation codon (ATG). Promoter consensus sequences ( $-35$  and  $-10$ ) similar to those of *E. coli* (26) were found upstream from each initiation codon except in the case of *orf2*.

*orf1* (nucleotides 2696 to 3604) is 909 nucleotides long and encodes a putative 33.9-kDa polypeptide with a calculated isoelectric point of 7.05. *orf2* (nucleotides 3604 to 3945) is 347 nucleotides long and encodes a putative 13.0-kDa polypeptide with a calculated isoelectric point of 10.26. The ATG initiation codon of *orf2* overlaps the TGA termination codon of *orf1*. The initiation codon of *orf2* is preceded by a predicted SD sequence, GGAGG, inside the *orf1* coding sequence, but no

promoterlike sequence was found. This arrangement suggests that *orf1* and *orf2* are transcriptionally coupled. Such overlapping of termination with initiation is observed in many bacteria (12, 18). The most likely initiation codon of *orf3* (nucleotides 6132 to 6674) is preceded by a potential promoter and SD sequence. *orf3* is 543 nucleotides long and encodes a putative 18.9-kDa polypeptide with a calculated isoelectric point of 11.34. *orf4* (nucleotides 21 to 611) and *orf5* (nucleotides 1075 to 1305) may code for putative 22.1- and 8.5-kDa polypeptides, respectively. A potential SD sequence and  $-35$  and  $-10$  promoter sequences are located upstream from the ATG initiation codon of these ORFs. *orf6* (nucleotides 1623 to 2258) may encode a 22.1-kDa polypeptide. A putative SD sequence, GGAGG, is located at an appropriate distance upstream of the initiation codon.

**Similarities of Orf1 and Orf2 to proteins encoded by other plasmids.** An analysis of amino acid sequence homology revealed high levels of similarity between Orf1 and the replication proteins (RepA) of several plasmids from gram-positive bacteria, including pRBL1 from *Brevibacterium linens* (level of similarity, 63%) (2) (accession no. U39878), pXZ10142 from *Corynebacterium glutamicum* (69%) (accession no. X72691), and pAL5000 from *Mycobacterium fortuitum* (56%) (17, 23) (Fig. 2A). Orf1 also exhibited significant homology with the Rep proteins of plasmid pMB1 from *Bifidobacterium longum* (48%) (27) (accession no. X84655) and pBL8 from *B. linens* (15a) (accession no. Y11902), as well as with Rep proteins from ColE type plasmids (24 to 29%) (ColE2imm-K317, ColE2-CA42, ColE3-CA38, ColE4-CT9, ColE5-099, ColE6-CT14, ColE7-K317, ColE8-J, and ColE9-J), which are trans-acting factors required for autonomous replication (38).

The amino acid sequence deduced from *orf2* (Fig. 2B) is similar to the amino acid sequences of RepB proteins from pAL5000 (level of similarity, 39%), pXZ10142 (38%), and pMB1 (27%). The deduced Orf2 amino acid sequence also shows the characteristic helix-turn-helix (HTH) motif typically found in DNA-binding proteins (6, 20). The predicted helix 1 is homologous to lambda cII, and helix 2 is homologous to Nul-lambda (Fig. 3). The value calculated by the Dodd-Egan weight matrix method (7) had a high standard deviation (4.4), indicating that the amino acid sequence probably forms an HTH motif (18).

**Similarity of Orf3 to invertase/recombinase-like protein.** On the basis of the amino acid sequence alignment data, Orf3 most closely resembles DNA invertases involved in DNA rearrangement (9), such as Pin from *E. coli* (22) and plasmid pTF5 from *Thiobacillus ferrooxidans* (16) (accession no. U73041), PaeR7IN from *Pseudomonas aeruginosa* (34), and Cin from bacteriophage P7 (25). The levels of similarity of Orf3 to these DNA invertases range from 50 to 55%. The significant similarities include conserved regions of recombinases, such as the regions of recombinase 1 (YARVSTAEQ) and recombinase 2 (GDTLMVTRIDRLG) at amino acid positions 6 to 14 and 56 to 67, respectively (Fig. 4). As in many DNA-binding proteins (6), the amino acid sequence of the putative Orf3 DNA invertase forms an HTH motif (18) (Fig. 4), since the value calculated by the Dodd-Egan weight matrix method (7) had a high standard deviation, 4.48.

**Shuttle vector construction.** The high levels of homology between Orf1 and Orf2 of pRGO1 and the RepA and RepB proteins, which have been reported to play an important role in plasmid replication (2, 27, 33), led us to construct a shuttle vector for shuttling between *Propionibacterium* sp. and *E. coli*. A large fragment containing *orf1*, *orf2*, *orf5*, and *orf6* was obtained from pRGO1 by digestion with *EcoRI* and *BamHI*, and the fragment was ligated with *EcoRI*-*BamHI*-digested pUC18.





SD

A T F S T I Q S Y R G H K G A G K A R P R A R R A A S I T D W E A  
 AGGCCACTTCTCCACCATCCAGAGCTACAGAGGACACAAGGGAGCCGCAAGGCTCGTCCCTGTCGCCCGCTGCTGCTTCTATCACCGATTGGGAGGC 3600  
 Orf2-->

\* M A D V Q H R V K R R G T A R E A A E R V G A S I R T A Q R W T S  
 ATGATGGCTGACGTCCAGCACCGCGTGAAGCGTCCGGGGCAGGCCCGGAGGCCGAGAACGTGTAGGGGCTCCATCCGAACCGCCAGCGGTGGACCT 3700  
 I P R E E W I T Q K A V E R E E I R A Y K Y D E G H T W G E T S R  
 CCATCCCCCGTGAAGAAATGGATCACTCAGAAGGCCGTGAGCGTGAGGAGATCCGGGCTACAAGTACGACGAGGGGCACACGTGGGGCGAGACCTCGCG 3800  
 H F G I A K T T A Q E R A R R A R R E R A A E A E K A A E E A E A  
 CCATTCGGGATCGGAAGACCACCGCCAGGAGCGGGCCCGGGCTCGAAGGGAGCGGGCGCGAAGCGGAGAAGCTGCCGAGGAGGCCGAGGCC 3900  
 A L R P T L F E G Q E Q G S A \*

GCGCTCGCTCCGACACTCTTCGAGGGCCAGGAGCAAGGTTCTGCATGAGCAACCCGAGTCCTCGGGTAGACCGTCTGGCCGACGTTAAGCATGGCTGA 4000  
 AGCGGCCCGTGCCTGTGGGGTTTCACTGTCCACGGTGAAGCGTCCACCGTATGCCCTGGTGGCCACGGTCTACCGTTCATGACGCGTCATGGGTGATA 4100  
 CCCCTATCAGCGTTGATTTTCATGCGGTTTGTATGCCCGGGTGACACCCCTGATGCCCGTCCACCAATAACGTGGCGCTGCCATGACGTCCCACGGTG 4200  
 ACGCCCCCTGACGGGGGAAGTCCAAGAGCTGCGCGAGCGACTGGCCACCGTGGAGCATCGAGCCGAGCTAGCCGAAGCCATCCGGCCGAGGACAACA 4300  
 CACGATCGACGCCACCGCCTACCGCTTACGGGCTTAGAACCCGGCTCGACCCATAACAGCCCGCAACCGATGAGCCGGCTACCGCTCGCGAGCAACT 4400  
 CCGGTCCAGAACCGCCTAACCGTCCGAGCCACACCGCCGAGTTGGTGGCGTGGCTGACTGGTGGCGCTGACCGGCCCGGTCGTTCCGAGGGGAACC 4500  
 TCTCGCTGCGAGAGGACACAGCAGCCGGCTGTGCTGGTAGGGCATCCAGCAGCAGACCCCTCTGACCGGAGAAGTTCAAGGACTACGCAATTGCTGA 4600  
 CTACCGCCGAGCGGCAGCACACGATCGAGATGCTCAACGAACCGCACTACCGGCCCTTAGAAGGCCCAAGGCACGCTCACCTACCACGTGGATCACCA 4700  
 CGATCGCGCCGACAGCTATGGACCCCATCGCAAGATCAAACCCCTGAGCAGCCATCGCACCGCCCGCCGCGCAGCCGGAAGAAGCTCCGACGCCCT 4800  
 GCTGTCCGGACACGGCCTAACCGTCCGAGCCAGAACCGAGTCTGGTGGCTAAACCGAAGGCCCTTCATGTGAGAGCATAGTCTGACGTCCGGCAGTA 4900  
 GTCGTGCCCGCGGGGTAAACGCTACACAACGCTTAAAGAGCATCGGAGCAAGCTAACACAGGGGACTGATGAACAAAACACAAAATGGCCAGCGGTG 5000  
 GTAATTGCGCGATCTTGGCCGCCGAATGACCGCAACTGCCTATGCAGATTCTCTGGAACACCAGAATTACAGCCAGCGAGCAAAAGCGTCTCTTA 5100  
 CCCAGTACTCGGCCAAAACCTACACAACTGAATATAACCGATACGTTGAGACTTACGGAAGCGTACCAGCCGAAGCAGACATCAACGCAATATAGA 5200  
 ACGCTACTGAATCTGAGGACATCAAGTCAAACGGCTGCTCAGATGACTCGACATACCCCGCCAGGATACCGAAATCTACCGCAGCGACGCCCTGCC 5300  
 AGGTTCTCAATGTTTTCTGTCGCGAATTTGGATCACTAGGAGTGGTGTAGTATCGCTCTCCTTGAAGCAAGGAAGGGTGGTATGGCAACAGGGGG 5400  
 ACGAGGCTACCTGGAAGACTGTATACGACAATTCATAACGCTGGGCAATGGACACGATACAAGAACAACGGCGTAGACGCCAGCATGAAAAGCAGTA 5400  
 CATGTGCCACTTCAAGTACGGGATGGTGAAGACGCCATGGAATCTGGAGCCCAAGAAGGCTGCAGAGTCCAGTCAAGTGAAGTACTAGTTTCT 5500  
 CGGACCCGTAACAGGGGAACAGGGCTAGTCAGATAGGATCGATAATGGCGGACTATATTCCTCTATTGATATTCATTCTTGCATGATCTACGATTTT 5700  
 CTGGTAATAACTCTTGAAGACATGCGCGATTTAACAGCTATTCTATATTAGCAAAGGCTTCACTGGTCGAATCAGCGTAATCTTATAATTTGCCAT 5600  
 ACGCGTCATACTGGTTAGTCGCTGGAGATCCAGACGTTTGGGACTTCTATCGTCTTTATGGTGGATTCCAGTGGCTTTCTAGGAATAGTTTCAATAGT 5700  
 ACTGATGGCTAGCAGTAGAGTTGGGGACGAGTCTCGGGACTCCGGAGAACCACCAAGTCAGGGTCTCATGAGTGTGCGATAGCTTGGAGTGTCTACCA 6000  
 -35 -10  
 ATCTGGATATAGCTATATCGGTCGTTTGTGTTGATTCGCCAGTGGAGCCACCGGGGGGCGACACCGGGTGGCGAAACCCCTGGCAGAATTCGGAACG 6100  
 SD Orf3--> M S I T G Y A R V S T A E Q H E A A Q I D A L  
 GGACGGAATCGGGGCTCTACCGTTCCTGGCTATGAGCATCACTGGATATGCCAGAGTCTCCACCGCCGAGCAGCAGGAGCCGCCAAATCGACGCCCTC 6200  
 K A A G A D P I Y I D K A S G A T M A R P Q W R A C L A G L G P G D  
 AAGGCTGCTGGGGCCGACCCATCTACATCGACAAGGCTCCGGGGCCACCATGGCCCGCCCGCAGTGGCGGGCTGCCTGGCTGGCCCTGGGGCCCTGGGG 6300  
 T L M V T R I D R L G R S L A D L V A T L D A L G R R G V G F R S  
 ACACCCTCATGGTACTCGCATCGACCGACTCGGCCGATCCCTGGCCGATCTGGTGGCGACTGACCGCACTAGGCGCCGAGGTGTCGGGTTCGCCGTT 6400  
 L A E Q L D T T S P G G L A L F Q M A G V F A E F E R Q L I A A R  
 TTTGGCCGAGCAGCTCGACACCACAGCCAGGCCGGCTGGCCCTGTTCCAGATGGCCGGGTCTTCGCCGAATTCGAAGCCCAACTGATCGCCGCCCGC 6500  
 T R D G G L A A R A R A R G H R C P G R P C G A C C L T A E Q Q A S A R D M R A  
 ACCCGTGACGGACTGGCCGACCCGAGCCCGGACCGCCGGAGCCCGCCCTACCGCTGAGCAGGCCAGCCCGCTGATATGCCGG 6600  
 A G A S L G S I A R I L G V S T R T I T R V T T \*  
 CCGCCGGCCCTCGCTGGGATCGATCGCCAGAATCCTGGGCTCTCGACCCGACCATACCCCGTCCACTTGAGAGTGAGGTTGACCGTCTGACG 6700  
 GACCGTCAACCCGAACCGAAGACGACTGCGCCGAGCCCGAGCCCGCCCTTTGCCAGGACCACCAAGACCGGATCGACGAACGACTCTCGGATCG 6800  
 TTTCGGCCGACCGAGGTCGTCACACTGTTGGCACTATTAGTACCAGGGGAGTTCAACGTCCGGAC 6868

FIG. 1—Continued.

The resulting plasmid, digested with *StuI*, was ligated to the *hygB* fragment from plasmid pSMT3 digested with *DraI-SmaI*. The noncoding region downstream from *orf2* of pRGO1 was deleted by digestion with *NheI*, and self-ligation created shuttle vector pPK705 (Fig. 5).

**Development of transformation system in propionibacteria.**

When erythromycin- or chloramphenicol-resistant genes in a vector derived from pRGO1 were used, no transformant of propionibacteria was obtained. When the *hygB* gene was used as a marker, however, transformants were obtained. Since small colonies were sometimes observed in the background on plates containing 100 µg of HygB per ml, 250 µg of HygB per ml was used on the selective plate. Plasmid pPK705 prepared from a transformant of *P. freudenreichii* subsp. *shermanii* IFO12426 was used for optimization of transformation. Transformation was performed by electroporation under the following conditions: amount of plasmid DNA, 0.1 to 6 µg; electric field strength, 6 to 12 kV/cm; and resistance, 129, 246, 480, or

720 Ω (Table 1). From the growth rate of strain IFO12426, the postincubation time after pulse treatment of the cells was estimated to be 8 h at 32°C. The highest transformation efficiency (1.0 × 10<sup>6</sup> CFU/µg of DNA) was obtained when competent cells and 1.0 µg of plasmid DNA were exposed to an electric field strength of 10.0 kV/cm with a resistance of 129 or 246 Ω. The transformation efficiency decreased when the cells were exposed to a high resistance or high field strength or both due to an increase in the time constant leading to cell death (less than 30% survival) after the pulse treatment. Transformants were confirmed to carry plasmid pPK705 by restriction enzyme digestion and gel electrophoresis (data not shown).

**Expansion of transformation host range.** The source of plasmid DNA usually influences the efficiency of transformation, presumably due to differences in restriction and modification of the DNA. This effect was investigated by employing shuttle vector pPK705 prepared from *E. coli* JM109 or *P. freudenreichii* subsp. *shermanii* IFO12426 (Table 2). Plasmid DNA

|          |             |              |            |                |             |               |    |       |    |      |     |       |   |
|----------|-------------|--------------|------------|----------------|-------------|---------------|----|-------|----|------|-----|-------|---|
| A        |             |              | * * * *    | **             | ****        | *             | ** | *     | ** | **** | **  | ***** | * |
| pRGO1    | MATTTVDEQW  | EQVWLPRWP    | LASDDLAAGI | YRMARPSALG     | VRYIEVNPQA  | ISNLLVVD      | CD |       |    |      |     |       |   |
| pRBL1    | M STASTETW  | GQMWLPLWP    | LASDDLQGI  | YRTSRHNALE     | LRYIEANPQS  | LSNLLVVD      | ID |       |    |      |     |       |   |
| pXZ10142 | MRPSTRPEGR  | FEQLWLPLWP   | LASDNLRDGI | YRTSRDKALD     | KRYIETNPDI  | SNLLVVD       | ID |       |    |      |     |       |   |
| pAL5000  | VSH VADE    | FEQLWLPYWP   | LASDDLLEGI | YRQSRASALG     | RRYIEANPTA  | LANLLVVD      | VD |       |    |      |     |       |   |
|          |             | ** *         | ***        | * *            | ***         | *****         |    | ***** | ** | * *  | **  | *     |   |
| pRGO1    | HPDAAMRAAV  | WDRHDWLPNA   | IVENPD     | NGH AHAVWALEAA | IPRTEYAHRK  | PIAYAAAVTE    |    |       |    |      |     |       |   |
| pRBL1    | HPDALMRAM   | WNRKAWQPN    | VVENPA     | NGH AHAVWALAE  | VTRTEYARRK  | PLAYAAAVTE    |    |       |    |      |     |       |   |
| pXZ10142 | KQDALLRSM   | WDRDGWRPNA   | VVENPXLNGH | AHAVWALAE      | FTRTEYARRK  | PXAYAAAVTE    |    |       |    |      |     |       |   |
| pAL5000  | HPDAALRALS  | ARGSHPLPNA   | IVGNRA     | NGH AHAVWALNAP | VPRTEYARRK  | PLAYMAACAE    |    |       |    |      |     |       |   |
|          |             | ****         | ****       | * **           | ****        | *             | *  | *     | *  | *    | *   | *     | * |
| pRGO1    | GLRRSVDGDA  | SYAGLITKNP   | EHPAWNTTWC | TDHLYRLAEL     | DTHLDAAGLM  | PAPSWRRR      |    |       |    |      |     |       |   |
| pRBL1    | GLRRSVDGDK  | GYSGLITKNP   | THDQWEASWL | TDHLYNLDEL     | TEHLTVSDFM  | PPDSWQRTK     |    |       |    |      |     |       |   |
| pXZ10142 | GLRRSVDGDK  | GYSGLITKNP   | EHTAWDSHWV | TDKLYTLDEL     | RFWLEETGFM  | PPESWKKTR     |    |       |    |      |     |       |   |
| pAL5000  | GLRRAVDGDR  | SYSGLMTKNP   | GHIAWETEWL | HSDLYTLSHI     | EAEELGAN    | M PPRWRQTT    |    |       |    |      |     |       |   |
|          |             | *            | ***        | **             | *           | *             | *  | *     | *  | *    | *   | *     | * |
| pRGO1    | NPVGLGR     | NCAIFETART   | WA YRDARRI | RQRHEYPTAE     | DSADLH      | AV IASTVEALNA |    |       |    |      |     |       |   |
| pRBL1    | RKNPVGLGR   | NCTLFETVR    | WDVYRVARTI | RKRNEHPTPE     | DRHDLEA     | AI VNLCOGMNST |    |       |    |      |     |       |   |
| pXZ10142 | RKSPIGLGR   | NCALFESARS   | WA YREI    | RHHFGDP        | DGLGRSIQAT  | AQALNQEL      |    |       |    |      |     |       |   |
| pAL5000  | YKAAPTPLGR  | NCALFDSVRL   | WA YRPALM  | RIYLPTRNV      | DGLGRAIYAE  | CHARNAEFPC    |    |       |    |      |     |       |   |
|          |             | **           | *          | *              | **          | *             | *  | *     | *  | *    | *   | *     | * |
| pRGO1    | GYSEPLPAR   | EAGIAASI     | HRWITHRFYG | WIDSHTVNEA     | TFSTIQSYRG  | HKGAGKARP     |    |       |    |      |     |       |   |
| pRBL1    | FSEALPAS    | EIRATIRSF    | YKWIITRYTG | WLDSTTSQS      | KSAAYHRNTG  | RKGGKGGV      |    |       |    |      |     |       |   |
| pXZ10142 | FSEPLPAV    | AEVDQIARSI   | HRWIITKSRM | WTDGPAVYWA     | TFSTIQASTR  | GKSAEHRWGT    |    |       |    |      |     |       |   |
| pAL5000  | NDVCPGPLPDS | EVRAIANSI    | WRWITTKSRI | WADGIVVYEA     | TLRARQSAIS  | RKGAAARTAA    |    |       |    |      |     |       |   |
|          |             | *            |            |                |             |               |    |       |    |      |     |       |   |
| pRGO1    | RARRAASI    | TDWEA*       |            |                |             |               |    |       |    |      |     |       |   |
| pRBL1    | SGQVRRQKAL  | ERVSMLEAGKL* |            |                |             |               |    |       |    |      |     |       |   |
| pXZ10142 | TNAERIRRF   | IND*         |            |                |             |               |    |       |    |      |     |       |   |
| pAL5000  | STVARRAKSA  | SAMEALL*     |            |                |             |               |    |       |    |      |     |       |   |
| B        |             |              | * * *      | * * *          | * * *       |               | *  | *     | *  | **   | *** | ****  |   |
| pRGO1    | MADVQHRVCR  | RGTAREAAER   | VGASIRTAQR | WTSIPREEWI     | TQKAVEREEI  | RAYKYDEGH     |    |       |    |      |     |       |   |
| pAL5000  | NRQPTVRKKR  | RVTAAGARI    | TGLSERHVVR | LVAQERSEWL     | AEQAARRERI  | RAYHDDEGH     |    |       |    |      |     |       |   |
| pMB1     | MVRTTLRKKR  | PVSARELAEA   | YGVSTRTIQS | WVAMKREDWI     | DEQAAMREAV  | RSYHDDEGH     |    |       |    |      |     |       |   |
|          |             | * * *        | ***        | *              | **          | **            |    |       |    |      |     |       |   |
| pRGO1    | TWGETSRHFG  | IAKTTAQERA   | RRARRERAA  | EAEKAEAE       | AALR PTLF   | EGQEQGS*      |    |       |    |      |     |       |   |
| pAL5000  | SWPQTAKHFG  | LHLDTVKRLG   | YRARKERAA  | EQ AAQKAH      | NEADN PPLF* |               |    |       |    |      |     |       |   |
| pMB1     | TWPQTAEHFN  | MSQGAVRQRC   | YRARKER    | EDE AAEKSK     | HLPGEIP LF  | D*            |    |       |    |      |     |       |   |

FIG. 2. Comparison of the amino acid sequences of pRGO1 Orf1 (A) and Orf2 (B) with the corresponding amino acid sequences deduced from pRBL1 (accession no. U39878), pXZ10142 (accession no. X72691), pAL5000 (accession no. M23557), and pMB1 (accession no. X84655). Amino acids common to all replication proteins are indicated by asterisks. Gaps have been introduced to optimize the homology. The overline in panel B indicates the HTH motif.

prepared from each of these strains was used for transformation to strain IFO12426. We found  $10^3$ -fold more transformants when plasmid DNA prepared from *Propionibacterium* cells was used than when plasmid DNA prepared from *E. coli* was used. When pPK705 prepared from *E. coli* JM110 (*dam dcm*) was used, no increase in the transformation efficiency was observed. Since both *dam* and *dcm* are associated with methylation of DNA, another methylation modification system may exist in *Propionibacterium*. In light of this result, plasmid pPK705 prepared from strain IFO12426 was used to transform

other propionibacteria. High transformation efficiencies, ranging from about  $10^6$  to  $10^7$  CFU/ $\mu$ g of DNA, were obtained with *P. pentosaceum* HUT8606, *P. freudenreichii* subsp. *shermanii* IFO12426 and HUT8612, and *P. freudenreichii* ATCC 4915. *P. freudenreichii* subsp. *freudenreichii* IFO12424 and *P. freudenreichii* subsp. *shermanii* P93 were also transformed but showed  $10^2$ - to  $10^3$ -fold lower efficiency. These results suggest that the restriction-modification systems of strains HUT8606, HUT8612, and ATCC 4915 are more similar to that of strain IFO12426 than to those of strains IFO12424 and P93.

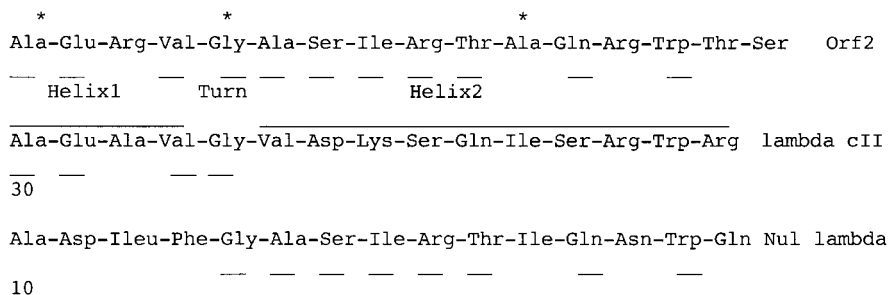


FIG. 3. HTH motif of Orf2. Asterisks indicate the conserved amino acids which are crucial in the structure. Numbers indicate amino acid positions in the protein sequence. Helix 1 and helix 2 are overlined. Amino acids found at the same position in the motif in Orf2 and cII or in Orf2 and Nul (6) are underlined.

**Stability of pPK705 in propionibacteria.** To assess plasmid stability, *Propionibacterium* strains carrying plasmid pPK705 were grown in a nonselective medium. After 10 serial transfers and cultivation in the nonselective medium, 400 colonies were randomly picked and replicated on the selective and nonselective media, and percentages of stability were calculated. Twenty colonies grown on the selective medium were analyzed for plasmid DNA by gel electrophoresis. All of the hygromycin B-resistant colonies tested possessed plasmid pPK705. As shown in Table 2, plasmid pPK705, which has the pRGO1 replicon, was stably maintained in all of the *Propionibacterium* strains except strain P93 (percentage of stability, 48%). These results indicate that pPK705 can be replicated and maintained with good stability in propionibacteria.

DISCUSSION

To develop a host-vector system in propionibacteria, a preferred replication origin from a multicopy propionibacterial plasmid and an appropriate selective marker are required. In order to locate the region where foreign DNA can be inserted without impairing the replication properties, the nucleotide sequence of the plasmid whose replication origin is used also needs to be determined. pRGO1 is the first plasmid from propionibacteria whose DNA has been completely sequenced. Comparison of the nucleotide sequence with the restriction endonuclease cleavage map previously described (24) revealed two more unique restriction sites, *ScaI* and *PstI*, and one more cleavage site for *SalI*. Analysis of the DNA sequence of pRGO1 showed that the putative proteins Orf1 and Orf2 are very similar to replication proteins RepA and RepB, respectively, in a number of plasmids from *B. linens*, *B. longum*, *C. glutamicum*, and *M. fortuitum*. These bacteria are gram positive and belong to the subdivision whose members have G+C contents greater than 55% (36). The base composition corresponds to the range of G+C values determined for propionibacteria (65 to 68%) (14). The significant similarity of Orf1 of pRGO1 to the Rep proteins of ColE type plasmids strongly suggests that Orf1 is involved in plasmid replication via a theta type of replication. The existence in the plasmid from *P. acidipropionici* of tandem genes encoding Orf1 and Orf2 which are highly homologous to genes in plasmids from *B. longum*, *C. glutamicum*, and *M. fortuitum* suggests that these two genes are essential for plasmid replication. On the basis of this information concerning pRGO1, we succeeded in constructing a useful shuttle vector for shuttling between *Propionibacterium* cells and *E. coli* using regions including *orf1*, *orf2*, *orf5*, and *orf6* of pRGO1 and pUC18. Although several attempts have been made to develop a transformation system in propionibacteria, a successful transformation system suitable for practical use has not been reported previously (35). This

may be due to the selection marker and the existence of a strong restriction-modification system in propionibacteria. These problems were overcome by using the *hygB* gene as a selective marker and a plasmid prepared from a *Propionibacterium* strain. The result of transformation of *Propionibacterium* cells by the pPK705 vector suggests that the replicon of pRGO1, which consists of Orf1 (RepA), Orf2 (RepB), Orf5, and Orf6, is functional in propionibacteria, although the functions of Orf5 and Orf6 remain unknown. The characteristic HTH motif of DNA-binding regulatory proteins (20) suggests that Orf2 (RepB) may be involved in initiation of plasmid replication, since RepB in pAL5000 was shown to be involved in initiation of replication by binding to the *ori* region (33). Similarly, a shuttle vector constructed to shuttle between *Bifidobacterium animalis* and *E. coli* by using pMB1 from *B. longum* showed that both *orf1* and *orf2* are necessary for plasmid replication in *Bifidobacterium* cells (27). Usually, the plasmid *ori* region is located in the AT-rich region. In pRGO1, there are two AT-rich regions, which probably contain the *ori* sequence and are located upstream of *orf1* and downstream of *orf2*. Deletion of the region downstream of *orf2* had no effect

|         |             |            |            |             |             |             |   |   |   |   |   |   |   |
|---------|-------------|------------|------------|-------------|-------------|-------------|---|---|---|---|---|---|---|
|         |             | *          | *          | *           | *           | *           | * | * | * | * | * | * | * |
| pRGO1   | MS          | ITGYARV    | STAEQHEAAQ | IDALKKAAGAD | PIYIDKASGA  | TMARPQWRAC  |   |   |   |   |   |   |   |
| PinTF   | MA          | LIGYARV    | STAEQDTALQ | TDAYARRDAS  | AFSRTLPLGL  | SQTRHALAAA  |   |   |   |   |   |   |   |
| paeR7IN | MGKRLGYARV  | STDDQRLDLQ | RDALTRAGCA | DIYEKVSQK   | SADRPALDYC  |             |   |   |   |   |   |   |   |
| PinEc   | M           | LIGYVRV    | STNDQNTDLQ | RNALNCAGCE  | LIFEDKISGT  | KSERPGLKKL  |   |   |   |   |   |   |   |
| CinP7   | M           | LIGYVRV    | STNEQNTALQ | RNALESAGCE  | LIFEDKASQG  | T ERPGLKKV  |   |   |   |   |   |   |   |
|         |             | *          | *          | *           | *           | *           | * | * | * | * | * | * | * |
| pRGO1   | LAGLPGGDTL  | MVTRIDRLGR | SLADLVATLD | ALGRGVGFR   | SLAEQ       | LDTT        |   |   |   |   |   |   |   |
| PinTF   | LAYLRDGDVIL | AVWRLDRFGR | SLPHLIETIS | ALEARGVG    | SVADRSIDTT  |             |   |   |   |   |   |   |   |
| paeR7IN | LRALSAGDTL  | VVWRLDRLGR | SLPDLVRIVG | ELEQRGVGFE  | SVTER       | IETG        |   |   |   |   |   |   |   |
| PinEc   | LRTLSAGDTL  | VVWKLDRLGR | SMRHLVVLVE | ELRERGINFR  | SLTD        | SIDTS       |   |   |   |   |   |   |   |
| CinP7   | LRMLSRGDTL  | VVWKLDRLGR | SMRHLVVLVE | ELDRDRGINFR | SLTD        | SIDTS       |   |   |   |   |   |   |   |
|         |             | *          | *          | *           | *           | *           | * | * | * | * | * | * | * |
| pRGO1   | SPGGLALFQM  | AGVFAEFERQ | LIAARTDGL  | AAARARGHRP  | GRPAALTAEQ  |             |   |   |   |   |   |   |   |
| PinTF   | TPGGRLIFHV  | FGALGQFERD | LIRERTKAGV | ERCRARGRKG  | GRKPVITADK  |             |   |   |   |   |   |   |   |
| paeR7IN | SATGKLFTHL  | FSALAEFERN | LIRERTQAGL | AAARARGRKG  | GRKPALDAKQ  |             |   |   |   |   |   |   |   |
| PinEc   | TPMGRFFPHV  | MGALAEEMER | LIVERTKAGL | ETARAQGRIG  | GRRPKLTPEQ  |             |   |   |   |   |   |   |   |
| CinP7   | TPMGRFFPHV  | MGALAEEMER | LIVERTRAGL | DAARAEGRIG  | GRRPKYQEET  |             |   |   |   |   |   |   |   |
|         |             |            | *          | *           |             |             |   |   |   |   |   |   |   |
| pRGO1   | QASARDMRAA  | ASLSGSI    | AR         | ILGVSTRIT   | RVTT*       |             |   |   |   |   |   |   |   |
| PinTF   | LQRAREHIAN  | GLNVREAAAR | LK         | VGKTALL     | PCRRIVSAVP  | STPDFRAIAF* |   |   |   |   |   |   |   |
| paeR7IN | VREIKALLRD  | PDIQVADVAK | RYGVSRTIY  | KHVGAVRPER  | GASPMI*     |             |   |   |   |   |   |   |   |
| PinEc   | WAQGR       | LIAA       | S          | TPRQKVAI    | IYDVGVSTLY  | KTFPAGDK*   |   |   |   |   |   |   |   |
| CinP7   | WQOMRRLLEN  | S          | IPRQKVAI   | IYDVAVSTLY  | KKFPASSFQS* |             |   |   |   |   |   |   |   |

FIG. 4. Comparison of the amino acid sequence of Orf3 of pRGO1 with the corresponding deduced amino acid sequences of PinTF from *T. ferrooxidans* (accession no. U73041), *PaeR7IN* from *P. aeruginosa* (accession no. S78872), PinEc from *E. coli* (accession no. K03521 and X01805), and CinP7 from bacteriophage P7 (accession no. X07224). The putative HTH motifs are enclosed in a box. Asterisks indicate the conserved amino acids.

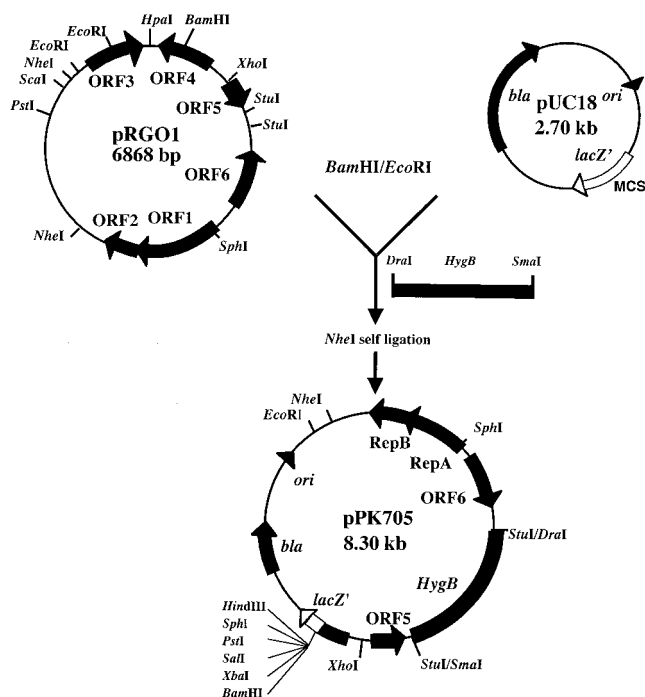


FIG. 5. Scheme for vector pPK705 construction. Abbreviations: *HygB*, hygromycin B resistance gene; RepA, gene coding for RepA protein; RepB, gene coding for RepB protein. Unique cleavage sites of restriction enzymes are indicated. *bla*,  $\beta$ -lactamase; *lacZ'*,  $\beta$ -galactosidase; *ori*, colE1 replication origin; MCS, multicloning site.

on replication of pPK705 in propionibacteria (data not shown). This result suggests that the *ori* region is not located downstream of *orf2*. Since *orf6*, located upstream from *orf1*, contains AT-rich and repeated sequences, *orf6* may play a role in plasmid replication, as reported in the case of a shuttle vector used for shuttling between *Mycobacterium* and *E. coli* cells (33).

*orf4* and Orf4 showed no DNA or amino acid sequence homology to any of the database sequences. The amino acid sequence homology of the predicted protein product of *orf3* to various DNA invertases suggests that *orf3* of pRGO1 encodes a putative recombinase. Since pPK705 does not contain *orf3* or *orf4*, these ORFs are not essential for plasmid replication, but they might improve the process.

TABLE 1. Effects of resistance, field strength, and time constant on transformation efficiency of *P. freudenreichii* subsp. *shermanii* IFO12426<sup>a</sup>

| Electroporation conditions |                           |                       | Transformation efficiency<br>(CFU/ $\mu$ g of DNA) |
|----------------------------|---------------------------|-----------------------|--|
| Resistance<br>( $\Omega$ ) | Field strength<br>(kV/cm) | Time constant<br>(ms) |  |
| 129                        | 10.0                      | 5.1                   | $1.0 \times 10^6$                                  |
| 246                        | 10.0                      | 5.0                   | $1.0 \times 10^6$                                  |
| 480                        | 6.0                       | 8.8                   | $1.1 \times 10^6$                                  |
| 480                        | 8.0                       | 8.9                   | $1.0 \times 10^6$                                  |
| 480                        | 10.0                      | 8.9                   | $5.0 \times 10^5$                                  |
| 480                        | 12.0                      | 8.8                   | $4.0 \times 10^5$                                  |
| 720                        | 10.0                      | 22.5                  | $5.0 \times 10^5$                                  |

<sup>a</sup> As described in Materials and Methods, 100  $\mu$ l of cells (about  $1.8 \times 10^{10}$  cells) in electroporation solution was mixed with 1  $\mu$ g of pPK705 DNA prepared from *P. freudenreichii* subsp. *shermanii* IFO12426 and electroporated. After pulse treatment, addition of 800  $\mu$ l of NLB, and incubation for 8 h at 32°C, transformants were selected on NLB agar with 250  $\mu$ g of hygromycin B per ml.

TABLE 2. Efficiency of transformation and stability of pPK705 in *Propionibacterium* strains<sup>a</sup>

| Strain  | Transformants/<br>$\mu$ g of DNA | Stability<br>(%) <sup>c</sup> |
|---|----------------------------------|-------------------------------|
| <i>P. freudenreichii</i> subsp. <i>shermanii</i> IFO12426 <sup>b</sup>      | $8 \times 10^3$                  |                               |
| <i>P. freudenreichii</i> subsp. <i>shermanii</i> IFO12426 <sup>c</sup>      | $6 \times 10^3$                  |                               |
| <i>P. freudenreichii</i> subsp. <i>shermanii</i> IFO12426 <sup>d</sup>      | $8 \times 10^6$                  | 83                            |
| <i>P. pentosaceum</i> HUT8606 <sup>d</sup>                                  | $1.8 \times 10^7$                | 80                            |
| <i>P. freudenreichii</i> subsp. <i>shermanii</i> HUT8612 <sup>d</sup>       | $1.4 \times 10^6$                | 85                            |
| <i>P. freudenreichii</i> ATCC 4915 <sup>d</sup>                             | $7 \times 10^7$                  | 73                            |
| <i>P. freudenreichii</i> subsp. <i>freudenreichii</i> IFO12424 <sup>d</sup> | $5 \times 10^5$                  | 83                            |
| <i>P. freudenreichii</i> subsp. <i>shermanii</i> P93 <sup>d</sup>           | $4 \times 10^4$                  | 48                            |

<sup>a</sup> Competent cells were prepared for transformation as described in Materials and Methods. Electroporation was carried out by using an electric field strength of 6.0 kV/cm, a capacitance of 25  $\mu$ F, a resistance of 129  $\Omega$ , and 1.0  $\mu$ g of plasmid DNA in 100  $\mu$ l of competent cells.

<sup>b</sup> Plasmid pPK705 prepared from *E. coli* JM109 (*dam*<sup>+</sup> *dcm*<sup>+</sup>) was used for transformation.

<sup>c</sup> Plasmid pPK705 prepared from *E. coli* JM110 (*dam* *dcm*) was used for transformation.

<sup>d</sup> Plasmid pPK705 prepared from *P. freudenreichii* subsp. *shermanii* IFO12426 was used for transformation.

<sup>e</sup> Stability was calculated as follows: number of colonies grown on NLB with hygromycin B/number of colonies grown on NLB without the antibiotic  $\times$  100.

The high transformation efficiency of pPK705 containing the pRGO1 replicon in various strains of *Propionibacterium* spp. suggests that shuttle vector pPK705 has a broad host range and is fairly stably maintained in *Propionibacterium* cells. Even in *P. freudenreichii* subsp. *shermanii* P93, which was reported to carry two endogenous plasmids, pRG07 and pRG03 (24), transformations were obtained; however, the stability of pPK705 was rather low. Successful manipulation of a host-vector system should facilitate genetic studies and lead to creation of some useful genes in propionibacteria that are likely to be industrially important.

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