Construction of a Reporter Vector System for In Vivo Analysis of Promoter Activity in *Propionibacterium freudenreichii*[†]

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A β -galactosidase reporter system for the analysis of promoter elements in *Propionibacterium freudenreichii* was designed. The pTD210 in vivo reporter vector was constructed using a promoterless *lacZ* gene from *Bifidobacterium longum* cloned into the pAMT1 plasmid. The utility of the pTD210 reporter vector was demonstrated by an investigation of six predicted promoters in *P. freudenreichii*. The system produced accurate and reproducible measurements that facilitated both promoter identification and the quantification of promoter activities.

The classical (dairy) propionibacteria (PAB) are nonpathogenic food industry bacteria that play a vital role in the texture and flavor development of Swiss-type cheeses (8). In addition, there is an emerging interest in new biotechnological applications of PAB as cell factories for the production of nutraceuticals (6). In order to investigate and exploit this potential, molecular tools specific for these bacteria have to develop further.

PAB genetics is currently entering the genomics era with the publication of the *Propionibacterium acnes* genome (4). Furthermore, at least two *P. freudenreichii* genomes have been sequenced, although these sequences are not publicly available (9). According to Meurice et al. (10), the sequencing of the genome of *P. freudenreichii* subsp. *shermanii* DSMZ 9614 is nearly complete. The preliminary analysis of this genome has revealed a GC content of 67% and identified 2,611 large putative coding sequences. Based on the 3' termini of the 16S rRNA gene sequences of several PAB species, a consensus Shine-Dalgarno sequence (or ribosome binding site [RBS]; AAAGGAGG) has been deduced. However, typical RBS sequences in only half of the coding sequences in the DSMZ 9614 strain have been identified. In many cases, there appears to be no significant base pairing between the upstream region of the start codon and the 3' terminus of the 16S rRNA (10).

Most of the studies conducted on PAB genes and promoters have concerned vitamin B₁₂ production in *P. freudenreichii* (7, 12). Thus far, nine promoters have been structurally analyzed (12). These results are of utmost interest, indicating that *P. freudenreichii* –10 and –35 promoter elements differ quite extensively from the *Escherichia coli* consensus sequences (12). Sequence alignments indicate poorly conserved –10 (RTSTTG) and –35 (CRNMCA) motifs and a more conserved –16

(ACGCGCA) motif in *P. freudenreichii* promoter structures (12).

These studies used a cholesterol oxidase gene (*choA*) from a *Streptomyces* species as a reporter, a method that requires a quite laborious assay (7, 12).

The aim of the present work was to develop a convenient reporter system for the analysis of promoter activity and gene expression in *P. freudenreichii*. Initial experiments using conventional reporter genes like *gfp* (encoding green fluorescent protein) and *E. coli lacZ* (encoding β -galactosidase) in *P. freudenreichii* failed (results not shown), most probably due to different patterns of codon usage in PAB. Thus, we decided to investigate whether the *lacZ* gene from the closely related species *Bifidobacterium longum* (13), which like PAB has a high GC content in its DNA, could serve as a reporter gene in *P. freudenreichii*.

DNA manipulation, plasmid isolation, and the transformation of *E. coli* or *P. freudenreichii* were performed according to procedures described previously (2). A 3.2-kb fragment containing the *lacZ* gene and its promoter was amplified from DNA isolated from *B. longum* LMGT 3500 by using primers Blo-lacZ-f and Blo-lacZ-r (see Table S1 in the supplemental material), cloned into the pCR2.1-TOPO cloning vector (Invitrogen), and introduced into *E. coli* CC118 ($\Delta lacZ$) (14). The resulting plasmid (pTD208) was digested with XbaI and fused with the PAB replicating vector pTD10 (3) to produce pTD209. To test the expression of the *B. longum lacZ* gene in *P. freudenreichii*, we transformed the IFO12426 strain, which exhibits modest levels of β -galactosidase activity, with pTD209 (Table 1). This transformation resulted in an ~40-fold increase in β -galactosidase activity in early log phase (Table 1). In order to design a useful reporter system, the *lacZ* gene was amplified using primers Blo-ppb-f and Blo-lacZ-r (see Table S1 in the supplemental material) and cloned into the XbaI site of the pAMT1 plasmid. The resulting pTD210 reporter vector was designed to contain a promoterless *lacZ* gene preceded by an RBS signal (AGGAGG) and a multiple cloning site (Fig. 1). The introduction of this vector into IFO12426 revealed only low levels of transcription of the *lacZ* reporter gene (Table 1). In order to test the utility of the pTD210 vector, nine clones

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TABLE 1. β -Galactosidase assay measurements of promoter activities in *P. freudenreichii*

Strain or clone	Promoter	Size of promoter (bp)	Activity (MU) ^a in early log phase	Ratio ^b	Activity (MU) ^a in late log phase	Ratio ^b	GenBank accession no.
IFO12426 wild type			51.0 $\pm$ 6.8	NA	272.0 $\pm$ 50.1	NA	
IFO12426(pTD261)	P_{lacZ} (native promoter)		2,051.8 $\pm$ 163.8	59.8 (40.2) ^c	4,934.3 $\pm$ 142	20.7	AJ242596
IFO12426(pTD210)			34.3 $\pm$ 8.4	1.0	238.7 $\pm$ 73.8	1.0	
IFO12426(pTD287)	P_{4E} inverted	300	37.1 $\pm$ 16.6	1.1	142.1 $\pm$ 51.8	0.6	
IFO12426(pTD290)	P_{4E}	300	1,807.3 $\pm$ 275.0	52.7	4,041.2 $\pm$ 582	16.9	AB052791
IFO12426(pTD430)	P_{pctA}	508	272.4 $\pm$ 9.3	7.9	643.5 $\pm$ 152	2.7	EU563950
IFO12426(pTD431)	P_{orfI}	250	97.5 $\pm$ 7.9	2.8	270.8 $\pm$ 22.2	1.1	EU563951
IFO12426(pTD433)	P_{hpk}	274	85.4 $\pm$ 7.9	2.5	339.8 $\pm$ 108.5	1.4	EU563952
IFO12426(pTD435)	P_{tr}	659	376.0 $\pm$ 47.7	11.0	877.8 $\pm$ 41.0	3.7	EU563953
IFO12426(pAA104)	P_{pcfAS}	347	2,946.6 $\pm$ 441.1	85.9	7,251.4 $\pm$ 475.7	30.4	EU563955
IFO12426(pAA105)	P_{pcfAE}	682	945.8 $\pm$ 65.3	27.6	3,089.2 $\pm$ 385.8	12.9	EU563954

^a Promoter activity was measured in Miller units (MU). The experiments were conducted with independent triplicates, and the results shown are the averages with standard deviations.

^b Activity ratio determined using pTD210 as the reference. NA, not applicable.

^c The value in parentheses is the ratio relative to the activity of wild-type IFO12426.

containing fragments of six predicted promoters from different PAB species were investigated (Table 1; also see Table S1 in the supplemental material). The plasmids containing the different promoter elements were assayed for β -galactosidase activity in IFO12426. Each clone was grown anaerobically at 30°C in SLB medium (5), and samples were harvested in early exponential phase (24 h) and late exponential phase (48 h). Bacteria from 10-ml culture aliquots were collected by centrifugation and resuspended in 1 ml of Z-buffer (Na phosphate [pH 7.0], 100 mM; KCl, 10 mM; MgSO₄, 1 mM; β -mercaptoethanol, 50 mM). The samples were further diluted in Z-buffer

to an A_{620} of 0.1. One milliliter of the cell suspension was broken with glass beads in a Fast-Prep bead beater (Bio 101) two times for 10 s each at the maximum speed with a 5-min pause on ice. Subsequently, cellular debris was removed by 5 min of centrifugation at 13,000 $\times$ g (4°C). The β -galactosidase activity was assayed in a microtiter format using a 20- μ l sample added to 160 μ l of Z-buffer mixed with 20 μ l of ONPG (*o*-nitrophenyl- β -D-galactopyranoside; 4 mg ml⁻¹), and the mixture was incubated for 30 min at 30°C before the reaction was stopped with 20 μ l of Na₂CO₃ (1 M). Absorbance at 405 nm was measured in a microtiter plate reader, and promoter activities were expressed as Miller units (11). The results presented are the averages of results from at least three independent assays. Promoter activities were determined during early and late logarithmic growth phase, and highly accurate and reproducible measurements were obtained (Table 1). Total cell protein sodium dodecyl sulfate-polyacrylamide gel electrophoresis (see Fig. S1 in the supplemental material) showed that the amounts of the 116-kDa β -galactosidase enzyme produced were in line with the measured in vivo promoter activities.

As a case study, we selected the P_4 promoter, which has been described previously as a strong *P. freudenreichii* promoter preceding the *orf4* gene (7, 12). In our study, P_{4E} comprised a 300-bp fragment upstream from the ATG initiation codon of the *orf4* gene. P_{4E} was the third most active promoter examined and exhibited ~55 and 61% of the activity of P_{pcfAS} in early log phase and late log phase, respectively. Furthermore, the cloning of P_{4E} in the opposite direction abolished the β -galactosidase activity (Table 1). In a previous study, we described an operon (*pcfABIC*) involved in the biosynthesis of the propionin F bacteriocin (1, 3). In order to identify the position of the promoter directing the transcription of *pcfABIC*, we devised clones pAA104 and pAA105, encompassing P_{pcfAS} and P_{pcfAE} fragments, respectively. The results (Table 1) indicate that the promoter elements controlling the expression of the *pcfABIC* operon most likely are located within the P_{pcfAS} fragment, as the extended promoter P_{pcfAE} showed a considerably lower level of activity than P_{pcfAS} . In terms of activity, P_{pcfA} , P_4 , and P_{lacZ} are efficient while P_{pctA} and P_{tr} are intermediate promoters in *P. freudenreichii*. The predicted pro-

FIG. 1. PAB reporter vector pTD210 for in vivo assessment of promoter activity. The 9.4-kb pTD210 reporter vector contains a promoterless *lacZ* from *B. longum* (GenBank accession no. AJ242596). The *lacZ* gene is preceded by an RBS site (AGGAGG) and a multiple cloning site (MCS). The BlolacZ seq-2 primer was designed to facilitate sequence analyses of inserted promoter fragments. The vector contains the rolling-circle replicon from pLME108 (accession no. AJ006662) and the *cmlA-cmxA* chloramphenicol selection marker for maintenance in PAB. The *bla* gene and ColE1 allow ampicillin selection and replication in *E. coli*, respectively. *, restriction of the XbaI site is blocked by Dam methylation.

moter fragments P_{orf1} and P_{hpk} directed only low-level expression under the conditions tested. None of the promoter fragments analyzed in this study showed significant similarity to the -10 , -16 , or the -35 consensus sequences proposed by Piao et al. (12). This finding suggests that more comprehensive investigations of promoters and gene regulation in PAB are needed. With the forthcoming determination of entire genome sequences, such as that of *P. freudenreichii* DSMZ 9614 (10), the presented reporter system can make an important contribution to facilitate promoter analysis on a larger scale. The metabolic engineering of PAB presents unexploited potential for the production of certain enzymes or metabolites but requires the availability of promoters with low, medium, and high levels of activity, depending on the specific application. We have developed a convenient tool for the quantitative assessment of promoter activities in PAB. The results show a range in promoter strength corresponding to 2- to 85-fold increases in gene expression compared to that in negative controls, thus demonstrating the possibility of choosing promoters of the desired strengths for gene technological purposes involving PAB.

Nucleotide sequence accession numbers. The nucleotide sequences for P_{pctA} , P_{orf1} , P_{hpk} , P_{rr} , P_{pcfAE} , and P_{pcfAS} were submitted to GenBank under accession no. EU563950, EU563951, EU563952, EU563953, EU563954, and EU563955, respectively.

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