

Production of tetrapyrrole compounds and vitamin B₁₂ using genetically engineering of *Propionibacterium freudenreichii*. An overview

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Abstract – *Propionibacterium freudenreichii* is a commercially important bacterium that is used in the production of cheeses, cobalamin (vitamin B₁₂) and propionic acid. Metabolic engineering using genetically improved strains will make the fermentation process more economical and also enhance the quality of the products. Host-vector systems and expression vectors using strong promoters from *P. freudenreichii* were developed in propionibacteria. By using these expression vectors and amplification of various genes, productions of 5-aminolevulinic acid, tetrapyrrole compounds and vitamin B₁₂ were reported. Here, we review the advancement of genetic engineering in *P. freudenreichii* in recent years, covering the molecular aspects of the formation of tetrapyrrole compounds and vitamin B₁₂.

***Propionibacterium* / tetrapyrrole / vitamin B₁₂ / expression vector**

Résumé – Production de composés tetrapyrrole et de vitamine B₁₂ par *Propionibacterium freudenreichii* génétiquement modifié. *Propionibacterium freudenreichii* est une bactérie d'importance commerciale, car elle intervient dans la production de fromages, de cobalamine (vitamine B₁₂) et d'acide propionique. Le procédé de fermentation peut être amélioré sur le plan économique et qualitatif grâce au génie métabolique et l'utilisation de souches améliorées. Des systèmes vecteur-hôtes et des vecteurs d'expression utilisant des promoteurs de *P. freudenreichii* ont été développés pour les bactéries propioniques. Des productions d'acide 5-aminolévulinique, de composés tetrapyrrole et de vitamine B₁₂ ont été réalisées en utilisant ces vecteurs d'expression et l'amplification de différents gènes. Les avancées du génie génétique de ces dernières années, couvrant les aspects moléculaires de la formation des composés tetrapyrrole et de la vitamine B₁₂ chez *P. freudenreichii*, sont passées en revue.

***Propionibacterium* / tetrapyrrole / vitamine B₁₂ / vecteur d'expression**

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1. INTRODUCTION

Propionibacterium species are of interest for their functions as probiotics and their nutraceutical properties as well as for their role as a starter in the cheese-making process. Propionibacteria are also known for their high production of vitamin B₁₂ and this has led to the development of commercially interesting production processes [72]. Since some *Propionibacterium* sp. have been granted GRAS (generally recognized as safe) status by the United States Food and Drug Administration and are not known to produce either endo- or exotoxins [61], *Propionibacterium* sp. are the preferred species for the production of vitamin B₁₂ and other food additives. The genes that were involved in biosynthesis of vitamin B₁₂ were consecutively isolated in this bacterium [11, 12, 37, 58, 63]. The clarification of the genetic organization and the gene products showed more information about tetrapyrrole and vitamin B₁₂ biosynthesis. In this review, we focus on the productivity of these useful compounds in propionibacteria using these gene manipulations.

2. GENETIC MANUPULATION SYSTEMS IN PROPIONIBACTERIA

Researchers in the genetics and molecular biology of propionibacteria are currently making much progress. In order to develop efficient DNA transfer systems for the genus *Propionibacterium*, dairy and environmental propionibacteria were screened for the presence of suitable plasmids. Following nucleotide sequence analysis, potential replication functions were identified on several *Propionibacterium* plasmids such as pLME106/pRGO1, p545 and pLME108. Murooka's group [28, 29] first described the development of an *Escherichia coli* - *Propionibacterium* shuttle vector pPK705, based on a part of the pRGO1 plasmid, containing the replication region of this plasmid, and the *E. coli* cloning vector pUC18. A hygromycin B (*hygB*) gene from *Streptomyces hygroscopicus* [80] was used as a selective marker. Since plasmid pRGO1 has been detected in all four dairy propioni-

bacterial species, a broader host range might be expected for pPK705. Jore et al. [24] also described another efficient transformation system for *Propionibacterium*. Reproducible transformation of *Propionibacterium freudenreichii* was achieved with shuttle vectors based on the plasmid p545 from *P. freudenreichii*. The erythromycin resistance gene (*ermE*) from *Saccharopolyspora erythraea* and the chloramphenicol resistance gene (*cml*) from *Corynebacterium striatum* [69] were used as the selection markers. DNA restriction/modification systems observed in propionibacteria have to be taken into account since successful DNA transformation at high rates (up to 10⁸ transformants·μg⁻¹ DNA) succeeds only with plasmid DNA originating from propionibacteria with the same restriction/modification system(s) as the strain to be transformed, and not from *E. coli* hosts. Furthermore, the basis for an integrating vector has been set up after identification of a potential *attP* site and an adjacent integrase gene from a *Propionibacterium* phage/prophage system [16]. Kiatpapan et al. [30] succeeded in overexpression of heterologous genes in propionibacteria, such as *choA* encoding cholesterol oxidase from *Streptomyces* [39] and *hemA* encoding 5-aminolevulinic acid (ALA) synthase from *Rhodobacter sphaeroides* [41] based on pPK705 and screened endogenous promoters. These successes resulted in the overproduction of ALA [27] and cholesterol oxidase [30]. However, only a few attempts have been made to study the genetics of propionibacteria [28]. The development of genetic tools will facilitate an increase in fundamental and application-oriented knowledge of the genus *Propionibacterium*.

3. MOLECULAR ANALYSIS OF PROMOTER ELEMENTS FROM *P. FREUDENREICHII*

The improvement and molecular study of an economically important group of bacterial strains would be greatly facilitated by genetic modification. The efficiency of gene transcription has gained attention in Gram-positive bacteria that are important industrially such as *Bacillus* [14], *Corynebacterium* [44], *Streptomyces* [67] and lactic

acid bacteria [34]. However, little information on transcription, including the genes encoding sigma factor and promoter consensus sequences in propionibacteria, is available [28]. Recently, active promoter sequences from *P. freudenreichii* have been characterized [47]. In order to screen promoter regions in *P. freudenreichii*, Piao et al. [47] tried to screen the promoter library directly in *P. freudenreichii*. However, since the efficiency of transformation in *P. freudenreichii* was not sufficient to make the library, *E. coli* was substituted as a host for *P. freudenreichii* at the first screening using a promoter probe vector, pCVE1, which harbors the modified *choA* gene from *Streptomyces* sp. as a reporter gene [43], and assayed for cholesterol oxidase activity by the filter paper method [39]. Finally, 17 transformants were selected. To confirm if all of the inserted DNA fragments from the 17 transformants were active in *P. freudenreichii*, all of the inserted DNA fragments and the *choA* gene in pCVE1 were subcloned into pPK705 and transferred into *P. freudenreichii* [47]. As a result of the second screening, 12 transformants exhibited some cholesterol oxidase activity in the *P. freudenreichii* cells, but no activity was found in five of the transformants. The initiation sites of these transcripts were determined by primer extension analysis. The putative consensus sequences corresponding to a -35 and -10 hexamer were found to be specific for *P. freudenreichii*. Moreover, a new consensus heptamerous sequence between the -35 and -10 regions, termed the -16 region (ACGCGCA), was also found [47]. It is possible that the putative consensus heptamer is functional and essential to promoter activity in *P. freudenreichii*. Several 10 to 16 nucleotide-length inverted repeats in the promoter regions examined were found. The inverted repeats may form the potential stem-loop in the promoter element. The consensus sequence of the promoter of *P. freudenreichii* found in the study was very different from that of *E. coli* [10], *Bacillus subtilis* [13], or other bacteria including GC-rich Gram-positive bacteria such as *Corynebacterium* [44] and *Brevibacterium* species [70]. The whole consen-

sus sequence of the promoter region of *P. freudenreichii* was also different from that of *Streptomyces* [67]. These results should provide new opportunities for controlled gene expression in *P. freudenreichii*.

4. BIOSYNTHESIS OF TETRAPYRROLE COMPOUNDS

Tetrapyrrole synthesis is initiated by the synthesis of ALA, a comparatively stable amino ketone. ALA is synthesized by one of two routes (Fig. 1), either from the condensation of succinyl-CoA and glycine (C4 pathway) or, more commonly, from the intact carbon skeleton of glutamic acid (C5 pathway). Since Murakami et al. isolated the gene encoding glutamate 1-semialdehyde 2,1-aminomutase (HemL) [37] and no gene involved in the C4 pathway has been found in the genomic sequence of *P. freudenreichii* [45], *Propionibacterium* sp. use the C5 pathway to synthesize ALA. The transformation of succinyl-CoA and glycine into ALA is mediated by ALA synthase (EC 2.3.1.37), a pyridoxal-phosphate-dependent enzyme [5, 21]. The synthesis from glutamate is a more complex process, and requires three separate enzymes [25]. The first step is the changing of a glutamate accepting tRNA (tRNA^{Glu}) with glutamate catalyzed by glutamyl-tRNA synthase (EC 6.1.1.17). The next step is a unique reaction, the reduction of the aminoacylated-tRNA^{Glu} to glutamate-1-semialdehyde (GSA) catalyzed by glutamate-tRNA dehydrogenase and NADPH as a coenzyme [36]. The final step in the synthesis of ALA is a transformation reaction catalyzed by the enzyme GSA aminotransferase (EC 5.4.3.8). The structure of this enzyme has recently been through X-ray crystallography and was found to have a high degree of similarity with amino acid transferase [15]. The conversion of ALA into the first macrocyclic tetrapyrrole structure is mediated by three enzymes common to all organisms that are able to synthesize this type of compound (Fig. 1) [21]. The first of these enzymes is porphobilinogen (PBG) synthase or ALA dehydratase (ALAD; EC 4.2.1.24), which

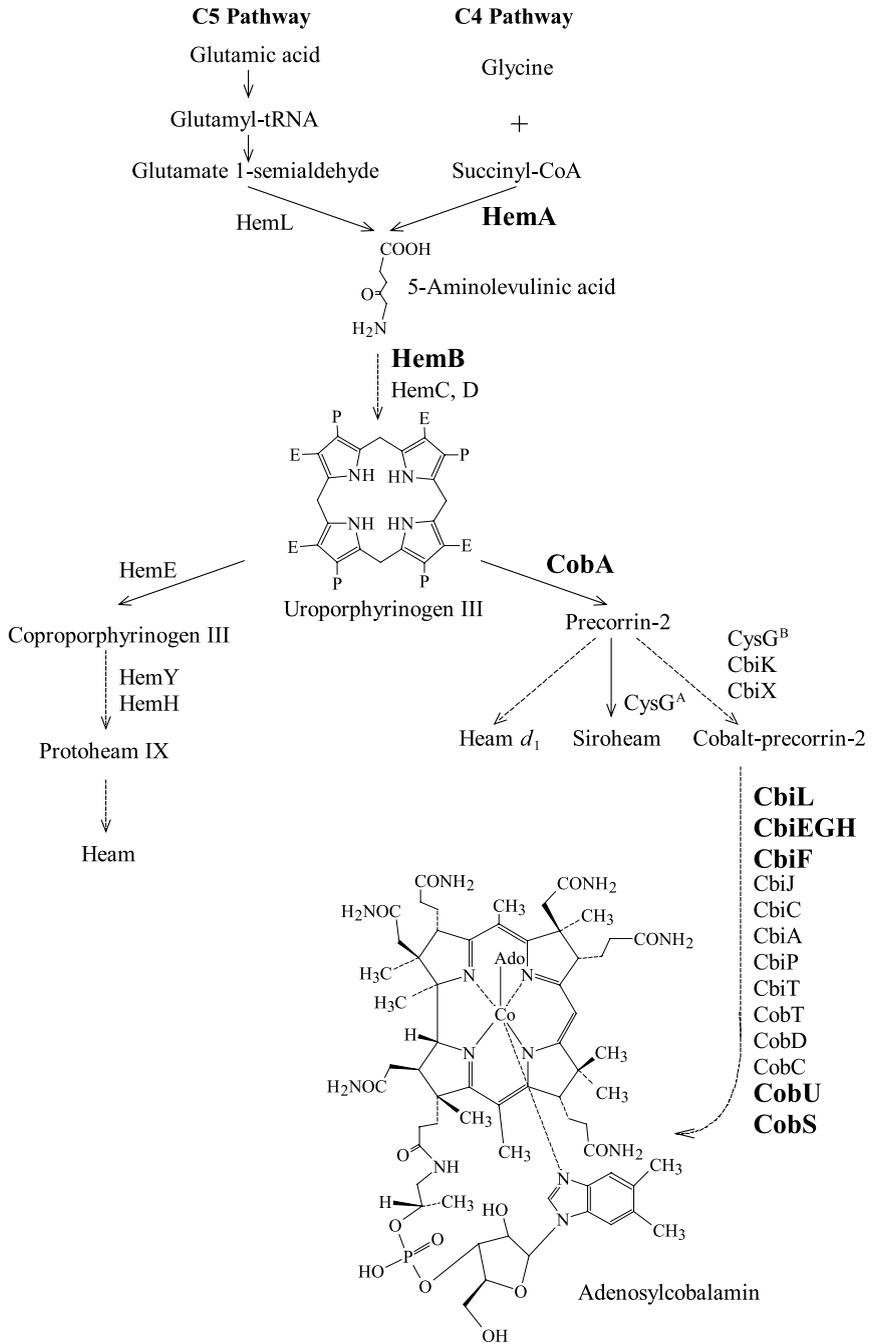


Figure 1.

catalyzes a Knorr-type condensation reaction between two molecules of ALA to generate PBG, and the enzyme requires a metal ion for full activity, such as zinc, magnesium, etc. [8, 22, 65]. The next enzyme in the pathway, PBG deaminase (PBGD; EC 4.3.1.8) [21], polymerizes four molecules of PBG into 1-hydroxymethylbilane (HMBL; also called preuroporphyrinogen) [23]. The final enzyme of tetrapyrrole synthesis is uroporphyrinogen III (urogen III) synthase (EC 4.2.1.75) [21], which is known as cosynthetase. In the presence of the cosynthetase, the enzyme is responsible for inverting the final pyrrole unit (ring D) of the newly synthesized linear tetrapyrrole and for linking it to the first pyrrole unit (ring A), thereby synthesizing a large macrocyclic structure called urogen III (Fig. 1). For heme and chlorophyll syntheses, urogen III is metabolized by three successive enzymic steps that modify the side groups of the macrocycle to yield protoporphyrin. Urogen III represents the first branch point of the pathway. In efforts to clarify details of the biosynthesis of cobalamin and tetrapyrrole derivatives in *Propionibacterium* sp., several genes for enzymes involved in these cobalamin biosynthetic pathways have been identified [12, 37, 63]. Porphyrinogens are colorless, but the oxidation of porphyrinogens yields porphyrins, which are photosensitizing moieties. Porphyrin compounds are strong absorbers of light from 400 to 405 nm and from 600 to 650 nm (the blue portion of the visible spectrum) [57]. Transfer of the energy absorbed by porphyrins to vital cellular components or to molecular oxygen can lead to the destruction of cells. Exploitation of this property has led to the use of porphyrin derivatives in clinical phototherapy directed against tumor tissues [42].

In order to improve production of tetrapyrrole compounds, Kiatpapan and Murooka [27] and Piao et al. [49] constructed a series of expression vectors to express the *hemA* gene, which encodes ALA synthase from *Rhodobacter sphaeroides*, and the *hemB* gene, which encodes PBG synthase from *P. freudenreichii* subsp. *shermanii* IFO12424, under the control of the P138 and P4 promoters isolated from *P. freudenreichii* [47], using the shuttle vector pPK705. The activities of ALA synthase and PBG synthase, respectively, in recombinant strains that harbored one or both genes were higher than those in strain IFO12426. The recombinant strains accumulated larger amounts of ALA and PBG, with a resultant ten- to twenty-two-fold higher production of porphyrinogens, such as uroporphyrinogen and coproporphyrinogen, than that observed in the control strain [49] (Fig. 2). However, levels of protoporphyrinogen were unaffected. More than 98% of the porphyrins produced by *P. freudenreichii* IFO12426 were present in the culture supernatant. Addition of ALA also stimulated the production of total porphyrin in *P. freudenreichii* IFO12426, causing an increase of 2.3 times during the course of incubation [49]. These results suggest that the synthesis of ALA might be the rate-limiting step in the biosynthesis of PBG or, at least, an important step in the ALA-metabolic pathway.

5. BIOSYNTHETIC PATHWAY OF VITAMIN B₁₂

After 10 years of work involving more than 100 researchers, the complete chemical synthesis of vitamin B₁₂ was achieved by Woodward and Eschenmoser [7].



Figure 1. Proposal overview of the pathway of tetrapyrrole compounds and vitamin B₁₂ biosyntheses in *P. freudenreichii* with the gene products indicated. Dashed lines denote multistep pathways. An intermediate is called a “precorrin” or “cobalt-precorrin” if it precedes the formation of the corrin ring present in cobyrinic acid. The number after “precorrin” or “cobalt-precorrin” gives the number of methyl groups that have been introduced from S-adenosyl-L-methionine to form that substance during the steps going forward from uroporphyrinogen III. The interrelated genes used in this study are indicated by large bold letters.

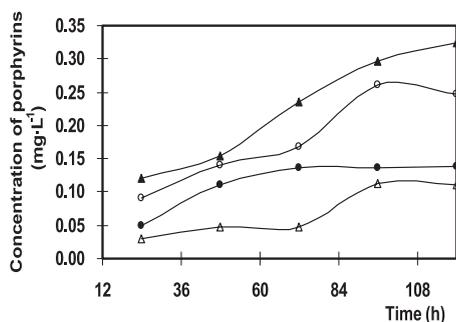


Figure 2. The time-course of porphyrin production and effect of ALA on porphyrin production in *P. freudenreichii* IFO 12426 carrying pPK705. Symbols: open circle, Uroporphyrinogen III; open triangle, Coproporphyrinogen III; solid circle, Uroporphyrinogen III+ALA; and solid triangle, Coproporphyrinogen III+ALA.

This highly complicated synthesis, with about 70 synthesis steps, makes any industrial production of vitamin B₁₂ by chemical methods far too technically challenging and expensive. Therefore, today vitamin B₁₂ is exclusively produced by biosynthetic fermentation processes using selected and genetically optimized microorganisms [17, 60, 71]. Two different biosynthesis routes for vitamin B₁₂ exist in nature: (a) an aerobic, or more precisely an oxygen-dependent pathway that is found in *Pseudomonas denitrificans*, and (b) an anaerobic, oxygen-independent pathway investigated in organisms like *B. megaterium*, *Propionibacterium shermanii* and *Salmonella typhimurium* [53, 62]. Biosynthesis of vitamin B₁₂ can be divided into three sections: the first part is the synthesis of the corrin ring component, the second is the construction of the lower axial ligand and the third is the piecing together of the components to yield the final coenzyme. The genes required for the synthesis of vitamin B₁₂ are also divided into three sections, which are defined as *cobI*, *cobII* and *cobIII* [59]. In general, genes encoding enzymes contributing to the oxygen-dependent vitamin B₁₂ biosynthesis are recognized by the prefix *cob*, while genes involved in the oxygen-independent pathway are usually named

using the prefix *cbi*. Methylation of urogen III at C-2 and C-7 results in the synthesis of precorrin-2, a dimethylated dipyrrocorphin, which is also the last common intermediate in the synthesis of coenzyme F430 and siroheme. The methyl groups are added by the activation of a single methyltransferase that is able to catalyze the addition to C-2 and C-7 positions, and the methyl groups are derived from (S)-adenosyl-L-methionine (SAM) [59, 74]. At precorrin-2 the two pathways for vitamin B₁₂ biosynthesis are diverged [55]. The oxygen-dependent and independent pathways for vitamin B₁₂ biosynthesis are quite distinct: the oxygen-independent part of the pathway starts with the insertion of cobalt into precorrin-2, while this chelation reaction in the oxygen-dependent part occurs only after nine further reaction steps. Viz.: in the anaerobic pathway, precorrin-2 is chelated with cobalt to yield cobalt-precorrin-2, a reaction that is catalyzed in *S. typhimurium* by CbiK [54], while in the aerobic pathway, precorrin-2 is methylated at C-20 by a further methyltransferase to give precorrin-3A. Due to the early cobalt insertion of the oxygen-independent pathway, the majority of the intermediates are cobalt-complexes. Therefore, they require enzymes with different substrate specificities, compared with the metal-free intermediates of the oxygen-dependent pathway. A further difference between the two routes is the method employed to promote the ring-contraction process, with the removal of C-20 from the ring. Under aerobic conditions, the C-20 atom of precorrin-3A is oxidized by molecular oxygen, sustained by a Fe₄S₄ cluster-containing protein (CobG), with the subsequent release of C-20 as acetate. Under anaerobic conditions, the ring contraction process is likely to be mediated via the complexed cobalt ion with its ability to assume different valence states (+1 to +3) to assist in the oxidation, resulting in the release of C-20 as acetaldehyde. Indeed, Scott's group has identified a number of ring-contracted cobalt-corrinoid compounds, some of which are incorporated into cobyrinic acid [64]. While the B₁₂ biosynthetic pathways diverged at precorrin-2, they do join again at the step of adenosyl-cobyric

acid, which is converted into cobinamide by the attachment of an aminopropanol arm to the propionic acid side-chain of ring D. The lower nucleotide loop is attached by transferring the phosphoribosyl residue of nicotinic acid mononucleotide to dimethylbenzimidazole (DMB). The resulting α -ribazole is finally covalently linked to GDP-activated adenosylcobinamide, thereby releasing GMP and giving rise to the completely manufactured coenzyme B₁₂ molecule.

6. FERMENTATION OF COBALAMIN

Although vitamin B₁₂ is present in small amounts in almost every animal tissue, e.g., 1 mg·kg⁻¹ in beef liver, it originates from microorganisms. Depending on the nature of their nutritional habits and digestive physiology, animals obtain the vitamin from their own intestinal flora or from other animals through their meat diet. An exogenous supply is mandatory for man. Vitamin B₁₂ derived from cultures of microorganism soon supplanted beef liver as a practical source of the vitamin for therapeutic purposes. Around 1950, materials rich in biomasses, such as activated sludges or broths of antibiotic-producing *Streptomyces*, were used for isolating vitamin B₁₂ either in a crude form for animal feeding or in a pure state for a medical use. Later, bacterial strains that produced a lot of vitamin B₁₂ were specially selected for commercial production. Among the B₁₂-producing species are the following genera: *Aerobacter*, *Agrobacterium*, *Alcaligenes*, *Arthrobacter*, *Azotobacter*, *Bacillus*, *Butyrivacterium*, *Citrobacter*, *Clostridium*, *Corynebacterium*, *Escherichia*, *Flavobacterium*, *Klebsiella*, *Lactobacillus*, *Micromonospora*, *Mycobacterium*, *Nocardia*, *Propionibacterium*, *Protaminobacter*, *Proteus*, *Pseudomonas*, *Rhizobium*, *Rhodopseudomonas*, *Salmonella*, *Serratia*, *Streptomyces*, *Streptococcus* and *Xanthomonas* [26, 33, 46, 68]. Now two genera, *Propionibacterium* and *Pseudomonas*, are mainly used for industrial production of vitamin B₁₂ [17, 19, 60, 71]. All *Propionibacterium* strains employed for vitamin B₁₂ production are microaerophilic and produce vitamin B₁₂ in high yields only under

very low oxygen concentrations. However, the biosynthesis of DMB requires oxygen. Therefore, the bioprocess of vitamin B₁₂ production using *Propionibacterium* strains is divided into two stages. In the first 3 days of fermentation, the bacteria are grown anaerobically to produce vitamin B₁₂ precursor cobamide, an intermediate vitamin B₁₂ in the absence of DMB moiety. Subsequently, vitamin B₁₂ formation is completed by gentle aeration of the whole culture for 1–3 days, allowing the bacteria to undertake the oxygen-dependent synthesis of the DMB and to link it to cobamide [6]. In contrast to the *Propionibacterium* fermentation process, *Ps. denitrificans* exhibit oxygen-dependent growth and high vitamin B₁₂ production rates. The culture is aerated during the whole fermentation process and maintained at 30 °C, pH 6–7 for 3–4 days [6]. Usually, the whole broth or an aqueous suspension of harvested cells is heated with cyanide or thiocyanate at 80–120 °C at pH 6.5–8.5 and the conversion to cyanocobalamin (vitamin B₁₂) is obtained [66]. After clarification of the whole solution, via e.g. filtration or treatment with zinc hydroxide, vitamin B₁₂ is precipitated by the addition of auxiliaries such as tannic acid or cresol. This procedure leads to a product of about 80% purity, which is used as animal feed additive. Further purification via different extraction steps, using organic solvents such as cresol, carbon tetrachloride and water/butanol, is often supplemented by adsorption to ion exchangers or activated carbon. Finally, vitamin B₁₂ is crystallized by the addition of organic solvents, leading to a product of recommended quality for food and pharmaceutical applications [6]. Since some *Propionibacterium* species do not produce either endo- or exotoxins [61], *Propionibacterium* species are the preferred species for the production of food additives or medicines. Thus, processes of vitamin B₁₂ production using *Propionibacterium* species have the advantage; *Propionibacterium* species allow the production of vitamin B₁₂ together with the biomass in which vitamin B₁₂ is produced, as described in a patent [2].

Many fermentative processes usually focus on bacteria growth to high cell densities

[20, 56, 78]. For example, there are fermentation with cross-flow filtration, fermentation coupled with an activated charcoal adsorption column [40], extractive fermentation [31], electro dialysis culture [81], and immobilized culture [75, 79]. The nutrient composition of the culture medium, such as amino acid or mineral composition including cobalt ions, affects production of vitamin B₁₂. Two experimental findings led to major improvements in the production of vitamin B₁₂: (a) addition of the precursor dimethylbenzimidazole, and (b) aerobic incubation in the latter phase of fermentation [32, 51, 52, 60, 76, 77]. Moreover, an increase in these precursors or intermediary metabolites in the cells of the producer strain using genetic recombinant DNA technology will achieve overproduction of vitamin B₁₂. However, until recently, genetic engineering has so far led to only limited improvement of vitamin B₁₂ production by microorganisms. For enhancement of production of vitamin B₁₂, common strategies such as random mutagenesis have been used to generate mutant strains to produce vitamin B₁₂ in high yield. Generally, this has been achieved by treating the microorganisms with UV-light or chemical reagents and selecting mutant strains with practical advantages, such as productivity, genetic stability, reasonable growth rates and resistance to high concentrations of toxic intermediates present in the medium [1, 9].

7. GENETIC ENGINEERING OF *PROPIONIBACTERIUM* SP. FOR VITAMIN B₁₂ PRODUCTION

Advances in the molecular biology and biochemistry of vitamin B₁₂ biosynthesis have led to the isolation of several enzymes responsible for the synthesis of vitamin B₁₂. In addition, most of the steps to biosynthesize vitamin B₁₂ have been characterized recently in *Ps. denitrificans* [4], *S. typhimurium* [53, 59] and *P. freudenreichii* [58, 63]. Vitamin B₁₂ biosynthesis genes of both the aerobic and anaerobic pathways have been revealed in several other Eubacteria and Archaea as the result of genomic sequencing projects and have been anno-

tated on the basis of similarities [50, 62]. Recently, two groups [38, 45] have reported the genomic sequence of *P. shermanii* and *P. freudenreichii*, respectively, and found genes involved in vitamin B₁₂ synthesis. Twenty-two *cob* genes involved in vitamin B₁₂ biosynthesis have been isolated and most of the functions of the majority of the polypeptides encoded by these genes have been identified (Fig. 1). The biosynthesis of uroporphyrinogen (urogen) III, a precursor of vitamin B₁₂, involves a multistep pathway from the ALA via porphobilinogen [35, 37]. The synthesis of DMB has not been completely elucidated. DMB is derived from riboflavin with five reactions, one of which, interestingly enough, seems to require oxygen [18].

In *Ps. denitrificans*, the gene dosage effect of the *cobF-cobM* operon, *cobA* and *cobE* resulted in a 20–30% increase in cobalamin production [3]. In *P. freudenreichii*, many genes in the *cob* and *cbi* gene families were cloned, and the DNA sequences have been deposited in the Genebank (accession nos.: AY033236, AB176692, and U13043) or published reports [12, 58, 63]. These are clusters *cobMNQOA* [63], *hemYHBXRL* [37], *cbiLFEGH-cysG-cbiJTCD* [58] and *cobUS* [48] (Fig. 3). For the construction of expression vectors, Piao et al. [48] selected eight genes of the *cob* and *cbi* gene families to be subcloned under the control of the P4 promoter isolated from *P. freudenreichii* [27], and the resultant plasmids were introduced into *P. freudenreichii* IFO12426. CobA catalyzes the SAM-dependent bismethylation of uroporphyrinogen III, resulting in the formation of dihydrosirohydrochlorin (known as precorrin-2), which is also considered to be the last common intermediate for the synthesis of cobalamin, sirohaem and haem d₁. Since the other genes are also known to be involved in the synthesis of cobalamin from precorrin-2 (Fig. 1), Piao et al. examined the effects of these genes on the production of vitamin B₁₂. The expression vectors were constructed to mono- or polycistronically express the *cobA*, *cbiL*, *cbiF*, *cbiEGH*, *cobU*, and *cobS* genes; cobalt precorrin-3 synthase is encoded by *cbiL*, cobalt precorrin-5 synthase by *cbiF*, cobalt precorrin-8

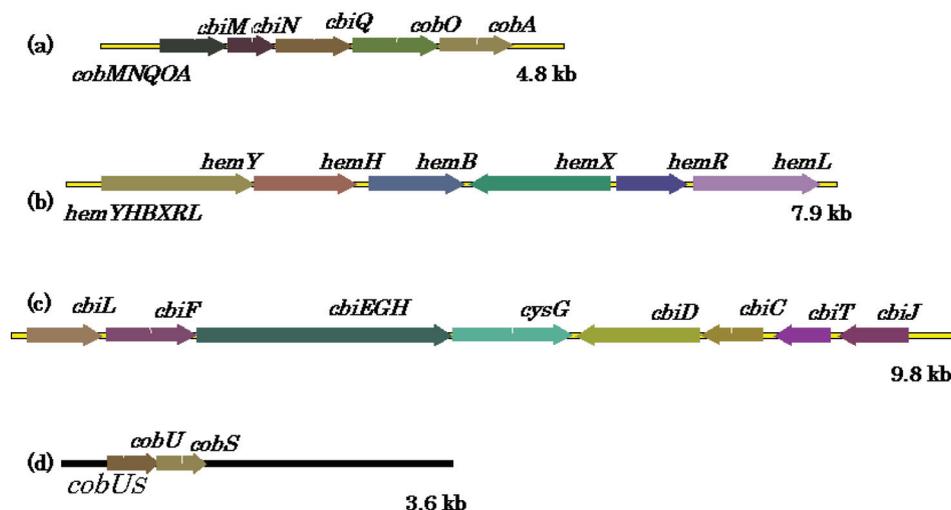


Figure 3. Structure of four clusters in *P. freudenreichii* genomic DNA involved in vitamin B₁₂ biosynthesis. Genbank accession numbers: (a) U13043, (b) AY033236, (c) D85417, and (d) AB176692.

synthase (C-5, C-10 methyltransferase) by *cbiE*, an unknown protein by *cbiG*, cobalt precorrin-4 synthase (C-17 methyltransferase) by *cbiH*, cobinamide kinase/cobinamide phosphate guanylyltransferase by *cobU*, and cobalamin synthase by *cobS*. In the strains carrying these expression vectors, the vitamin B₁₂ produced ranged from 0.96 to 1.46 mg·L⁻¹ (Tab. I). The results suggest that *cobA* and *cbiLF* out of the examined *cob* and *cbi* genes, which are involved in the biosynthesis of vitamin B₁₂ from uroporphyrinogen III, enhance the production of vitamin B₁₂.

Kiatpapan and Murooka succeeded in the overproduction of ALA via the C4 pathway in *P. freudenreichii* by bypassing ALA synthase, which catalyzes the condensation of glycine and succinyl coenzyme A into ALA [27]. PBG is formed by the condensation of two molecules of ALA in a reaction catalyzed by δ -aminolevulinic acid dehydratase (HemB). PBG is the immediate precursor of the tetrapyrrole uroporphyrinogen III. Piao et al. subcloned the *hemB* gene from *P. freudenreichii* directly under the control of the P4 promoter and also downstream of the *hemA* gene from *Rhodobacter*

sphaeroides to provide a new multigene expression system in *P. freudenreichii* [48, 49]. The expression vectors are named pKHEM06 and pKHEM05, respectively. The levels of vitamin B₁₂ in *P. freudenreichii* IFO12426 that harbored the cloned *hemA* and *hemB* gene or both *hemA* and *hemB* genes are shown in Table I. The amounts of vitamin B₁₂ were 1.02 mg·L⁻¹, 1.12 mg·L⁻¹, and 0.82 mg·L⁻¹ in the respective recombinant strains carrying pKHEM04, pKEHM05 and pKEHM06, respectively [48]. In the strain harboring only the cloned *hemB*, there was no effect on the production of vitamin B₁₂. Since the *cobA* and *cbiLF* genes caused enhanced production of vitamin B₁₂, Piao et al. constructed a novel heterogenous expression vector containing *hemA*, *hemB* and *cobA* in an effort to overproduce vitamin B₁₂. The *cobA* gene was subcloned downstream of *hemAB* for polycistronic expression and the resultant plasmid was named pKHEM07. The recombinant *P. freudenreichii* that harbored pKHEM07 also produced an amount of vitamin B₁₂: 1.68 mg·L⁻¹ (Tab. I). Finally, they achieved an increase of 2.2 times in the production of vitamin B₁₂ using the novel operon containing

Table I. Effects of the *hemAB*, *cob* and *cbi* genes on production of vitamin B₁₂ by recombinant strains of *P. freudenreichii*.

Plasmid	Cloned genes	Production of vitamin B ₁₂ (mg·L ⁻¹ culture ^a)
pPK705	-	0.77
pCobA	<i>cobA</i>	1.32
pCbiL	<i>cbiL</i>	1.00
pCbiLF	<i>cbiL</i> , <i>cbiF</i>	1.46
pCbiEGH	<i>cbiEGH</i>	1.18
pCobU	<i>cobU</i>	0.96
pCobS	<i>cobS</i>	0.98
pCobUS	<i>cobU</i> , <i>cobS</i>	1.00
pPK705	-	0.77
pKHEM04	<i>hemA</i>	1.02
pKHEM05	<i>hemA</i> , <i>hemB</i>	1.12
pKHEM06	<i>hemB</i>	0.82
pKHEM07	<i>hemA</i> , <i>hemB</i> , <i>cobA</i>	1.68

^a The production of vitamin B₁₂ was measured in triplicate under the conditions described and averaged in agreement to within 15%. The growth condition was described in Materials and Methods [48].

the *hemA* gene from *R. sphaeroides*, and the *hemB* and *cobA* genes from *P. freudenreichii*, compared with that in the strain harboring pPK705. Taken together, these results suggest that an increase in intermediary metabolites in the branched biosynthetic pathway of vitamin B₁₂, such as ALA, PBG, uroporphyrinogen III and precorrin-2, lead to enhanced production of vitamin B₁₂.

8. CONCLUSION

Microorganisms produce coenzyme B₁₂ or deoxyadenosylcobalamin via a complicated pathway involving at least 25 steps from the beginning of urogen III, precursor for heme, F430, cobalamin-dimethylbenzimidazole and adenosyl-moiety [73]. However, neither the complete pathway of vitamin

B₁₂ biosynthesis, nor feedback mechanisms have been clarified in the genus *Propionibacterium* even when the genome sequence of *Propionibacterium* was determined [45]. The desirable limiting step of the *cob* and *cbi* genes remained to be clarified. The experimental data in *Propionibacterium* provides information on the relationship between expression of the *cob* and *cbi* genes and the production of vitamin B₁₂. Furthermore, the multigene expression system seems to improve the productivity of vitamin B₁₂ in metabolically and genetically engineered propionibacteria. Moreover, an increase in the precursors, such as ALA, or intermediary metabolites of vitamin B₁₂ in the cells would be expected to result in the overproduction of vitamin B₁₂ by controlling the metabolic flow from ALA to tetrapyrrole compounds or cobalamins using mutations and amplification of genes.

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