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Genetic Engineering of *Escherichia coli* for the Production of Precorrin-3 in Vivo and in Vitro

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Abstract—The construction of a new recombinant strain of *Escherichia coli* in which two vitamin B_{12} biosynthetic genes, *cobA* and *cobI*, from *Pseudomonas denitrificans* are simultaneously overexpressed has resulted in the in vivo synthesis and accumulation of Factor III, an isobacteriochlorin not normally synthesized in *E. coli*. A lysate of the new strain can take the place of two lysates normally required to provide uroporphyrinogen III methyltransferase (*cobA*) and precorrin-2 methyltransferase (*cobI*) in an anaerobic five-enzyme synthesis of the early B_{12} intermediate, precorrin-3 (the reduced form of Factor III) from δ-aminolevulinic acid.

Introduction

In aerobic bacteria such as *Pseudomonas denitrificans* that produce corrins *via* an oxygen-dependent pathway,[1,2] precorrin-3 is the first of the tetrapyrrolic intermediates committed uniquely to vitamin B_{12} biosynthesis (Fig. 1). Earlier tetrapyrrolic intermediates can be shunted down competing pathways (Fig. 1) by decarboxylation of uroporphyrinogen III to afford coproporphyrinogen (heme biosynthesis) and by the oxidation of precorrin-2 to Factor II (siroheme biosynthesis). Precorrin-3 is also an early intermediate in the strictly anaerobic pathway to vitamin B_{12} in organisms such as *Salmonella typhimurium*, although the true intermediate in this organism may be as its cobalt complex.[3] Thus an adequate supply of precorrin-3 is paramount to the success of in vivo and in vitro studies designed to synthesize and identify intermediates of the B_{12} pathways. We have previously described[4] overexpression, in five different recombinant strains of *E. coli*, of the five enzymes required for the in vitro multienzyme synthesis of precorrin-3 from δ-aminolevulinic acid (ALA) (Fig. 1). This early system used the *E. coli cysG* gene product for the source of urogen III methyltransferase[5] to convert urogen III to precorrin-2, Unfortunately, *cysG* has the problem of 'overmethylating' not only precorrin-2[6] but also precorrin-3[7] to afford the undesirable derailment products, trimethylpyrrocorphin and tetramethylpyrrocorphin. The system was substantially improved[8] by the substitution of the *cobA* gene product from *P. denitrificans* for *cysG* to provide a source of urogen III methyltransferase that does not exhibit the overmethylation property. We describe here a further modification of the system in which the *P. denitrificans cobA* and *cobI* genes are simultaneously overexpressed in an engineered strain of *E. coli* thus providing a convenient source not only of urogen III methyltransferase but also of precorrin-2 methyltransferase (Fig. 1). An additional benefit arising from the construction of the strain is that it accumulates substantial quantities of Factor III (Fig. 1) when grown on ordinary culture medium and provides a convenient source of this isobacteriochlorin.

Results and Discussion

Construction of an *E. coli* expression vector for the simultaneous production of urogen III methyltransferase (*cobA*) and precorrin-2 methyltransferase (*cobI*)

In the past we have constructed various vectors for the production of urogen III methyltransferase from the *E. coli cysG* gene,[5] the *P. denitrificans cobA* gene[8] and the *Propionibacterium freudenreichii cobA* gene.[9,10] Urogen III methyltransferase lacking the property of overmethylation has usually been obtained from strain CR395 ≡ BL21:DE3(pLM1:*cobA*) in which the *P. denitrificans cobA* gene is expressed, on induction with

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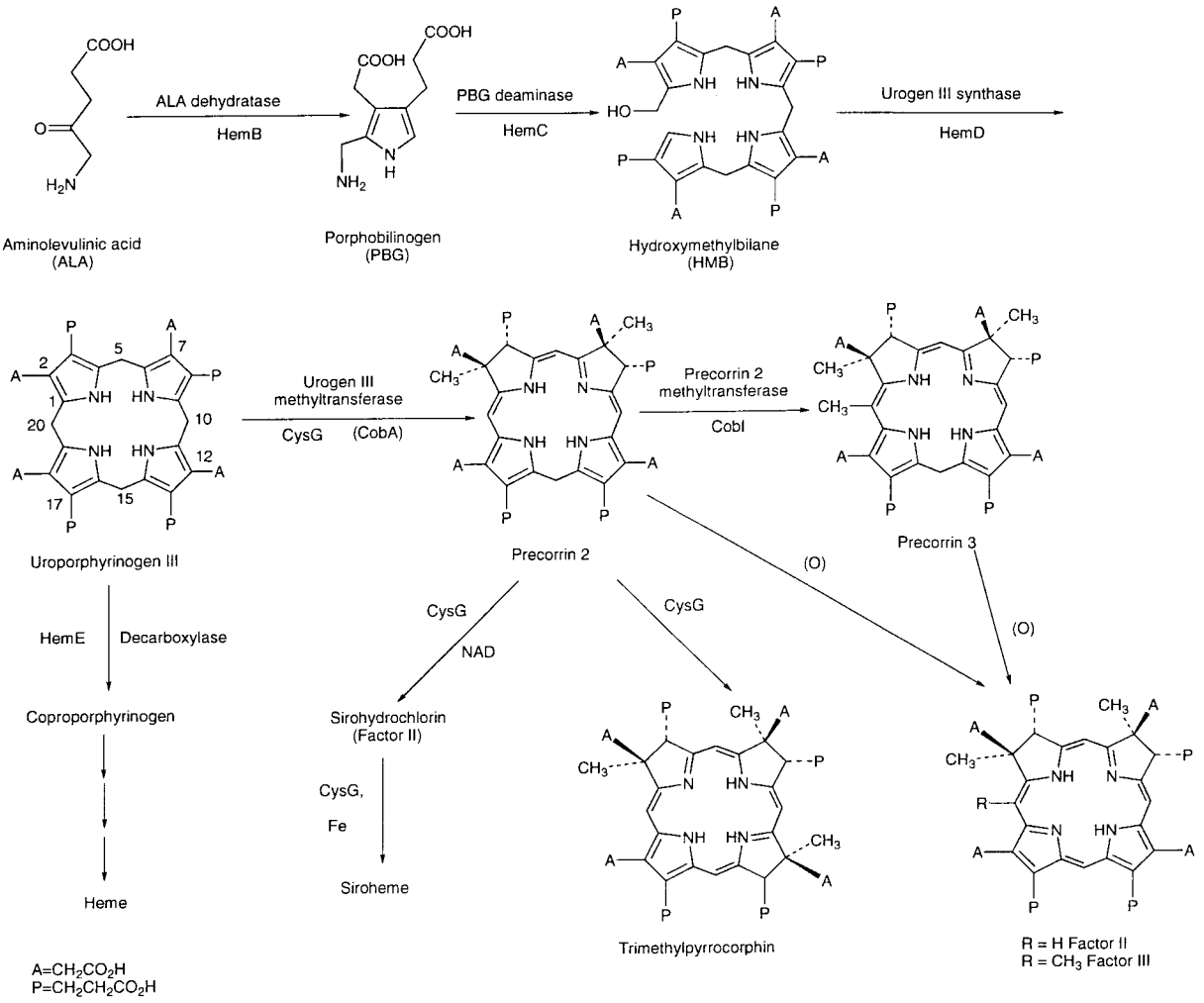

Figure 1. The biosynthetic pathway from aminolevulinic acid to precorrin-3 and competing pathways that shunt intermediates away from precorrin-3.

IPTG, from the T7 polymerase promoter.[8] In *P. denitrificans*, the stop codon for the *cobA* gene overlaps the start codon for the *cobB* gene (encoding hydrogenobyrinic acid a,c-diamide synthase). In one attempt to express the *cobB* gene, a DNA fragment of *P. denitrificans* containing *cobA* and *cobB* was amplified by pcr providing a 5′ *Bam*HI site, a 3′ *Pst*I site, and optimal *E. coli* translational signals (ribosome binding site and spacer) to initiate translation of *cobA*.[11] The pcr product was ligated into *Bam*HI-*Pst*I cleaved pUC18 (Fig. 2) to provide pJH103 (pUC18:*cobAB*) which was transformed into TB1. Although no expression of *cobB* was observed from the vector, the *cobA* gene was expressed at a level equivalent to that seen in CR396, and the new strain now replaces CR396 as a source of urogen III methyltransferase since induction with IPTG is not necessary, thus making it a less expensive and more convenient source of the enzyme.

Plasmid pJH103 was used as the starting material to construct a plasmid containing both *cobA* and *cobI* (Fig. 2). The *cobB* gene has an *Eco*RV site 416 base pairs downstream from the start codon and a *Sal*I site 194 base pairs upstream from the stop codon. This fragment was replaced in pJH103 by a pcr product in which the *P. denitrificans cobI* gene was amplified with a 5′ *Eco*RV site and optimal *E. coli* translation signals and a 3′ stop codon and *Sal*I site. The resultant plasmid was transformed into TB1 to give strain CR462 ≡ TB1(pUC18:*cobAI*).

Characterization of the products formed by CR462. When grown in LB-ampicillin, cells of CR462 develop an uncharacteristic yellow–red color and exhibit a bright red fluorescence at 301 nm. All of the intracellular fluorescent material was isolated, esterified, and analyzed by TLC as described in Materials and Methods. Based on UV–VIS spectroscopy, the DEAE-adsorbant compounds were found to consist mostly of Factor III (>90%) with only trace amounts of porphyrins. A 1 L culture (4 g of cells, wet weight) produced 125 μg of Factor III.

Control cells of TB1(pUC18) are not fluorescent and do not accumulate substantial amount of fluorescent materials, suggesting that the amount of these compounds is regulated at some level. Indeed, heme is a feedback inhibitor of ALA synthesis in *E. coli*[12] and has

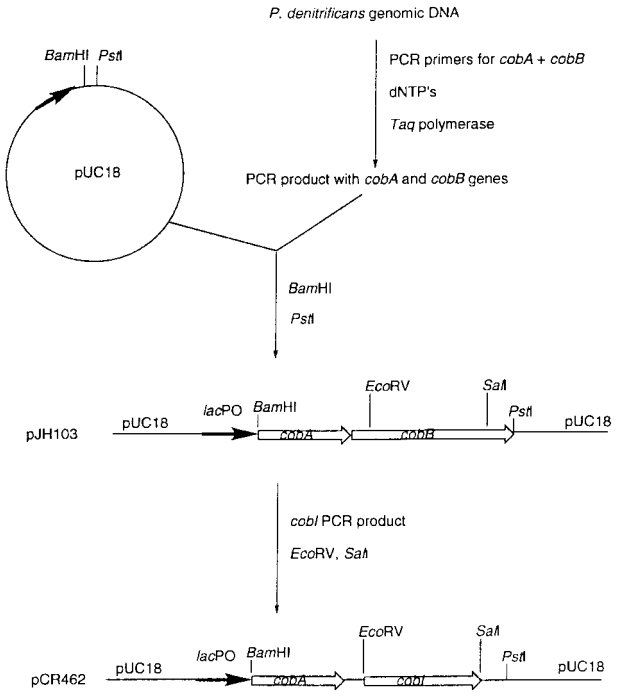

Figure 2. The scheme for the construction of pCR462 for the simultaneous expression of the *P. denitrificans cobA* and *cobI* genes.

been shown to operate by reducing the level of the activity of the enzyme, glutamyl-tRNA reductase, encoded by the *hem*A gene[13] required for the first step of ALA biosynthesis (Fig. 3). The level of heme increases in an *E. coli* strain in which rat cytochrome b5 is overexpressed and serves as a "heme sink".[12] Similarly, in our strains that overexpress urogen III methyltransferase, the methylation of urogen III provides a "sink" for this heme intermediate, effectively reducing the levels of the regulatory agent. In addition, since *E. coli* does not synthesize corrins, precorrin-3 is not a substrate for any enzyme thus accounting for the accumulation of relatively high levels of the trimethylisobacteriochlorin (Factor III) in CR462.

Synthesis of precorrin-3 with lysates of CR462. In earlier multienzyme reactions used for the biosynthesis of precorrin-3, the two enzymes needed to convert urogen III to precorrin-3, urogen III methyltransferase and precorrin-2 methyltransferase, were supplied from lysates derived from two different recombinant strains of *E. coli*, CR395 and CR333, that overexpress the *cobA* and *cobI* genes, respectively.[8] When a lysate derived from a 1 L culture of CR462 was used in a precorrin-3 synthesizing system in which the substrate was [4-^{13}C]-ALA, the ^{13}C-NMR spectrum of the product (Fig. 4) was indistinguishable from that of precorrin-3 synthesized using one liter cultures of CR395 and CR333 as the source of the two enzymes. An important feature of the spectrum shown in Figure 4 is the complete lack of signals corresponding to urogen III or precorrin-2. This result indicates that both *cobA* and *cobI* are overexpressed in CR462 at a level equivalent to that obtained in the strains expressing the individual genes, and that this single strain can be used to produce the two enzymes, leading to an almost quantitative yield of precorrin-3.

Conclusion

The use of CR462 as a source of the biosynthetic enzymes *cobA* and *cobI* has greatly simplified the in vitro synthesis of precorrin-3 from ALA using a five-enzyme system.

Experimental

Bacteria, plasmids, and molecular biology

E. coli strain TB1[14] was used as the host strain for transformation and expression of foreign genes. The expression vector, pUC18,[15] was from New England Biolabs. TB1 was grown in LB medium which was supplemented with 50 μg/mL ampicillin when transformed with pUC18-based plasmids. Restriction enzymes and T4 DNA ligase were purchased from New England Biolabs and used as directed by the supplier following standard protocols.[16] *Taq* polymerase was purchased from Promega and used for polymerase chain reactions following standard protocols in which the primers were supplied by the Gene Technologies Laboratory, Texas A&M University.

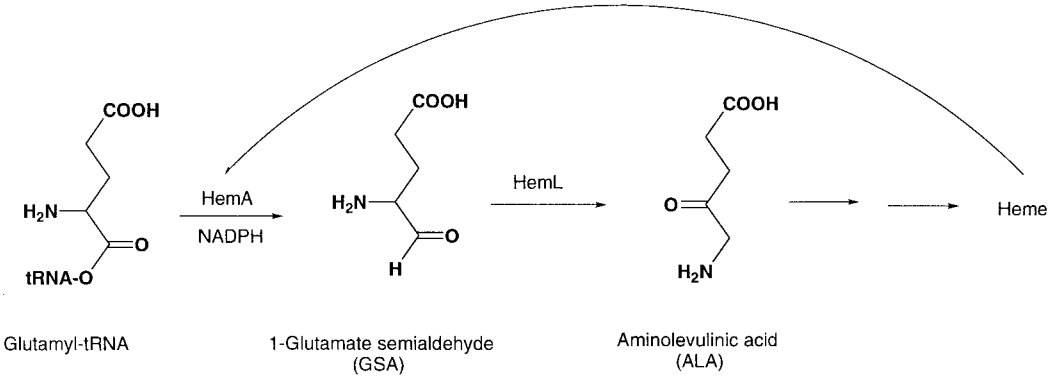

Figure 3. The biosynthesis of ALA in *E. coli* with feedback inhibition of glutamyl *t*-RNA reductase by heme, the normal end product derived from ALA. HemA, glutamyl *t*-RNA reductase; HemL, glutamate semi-aldehyde aminomutase.

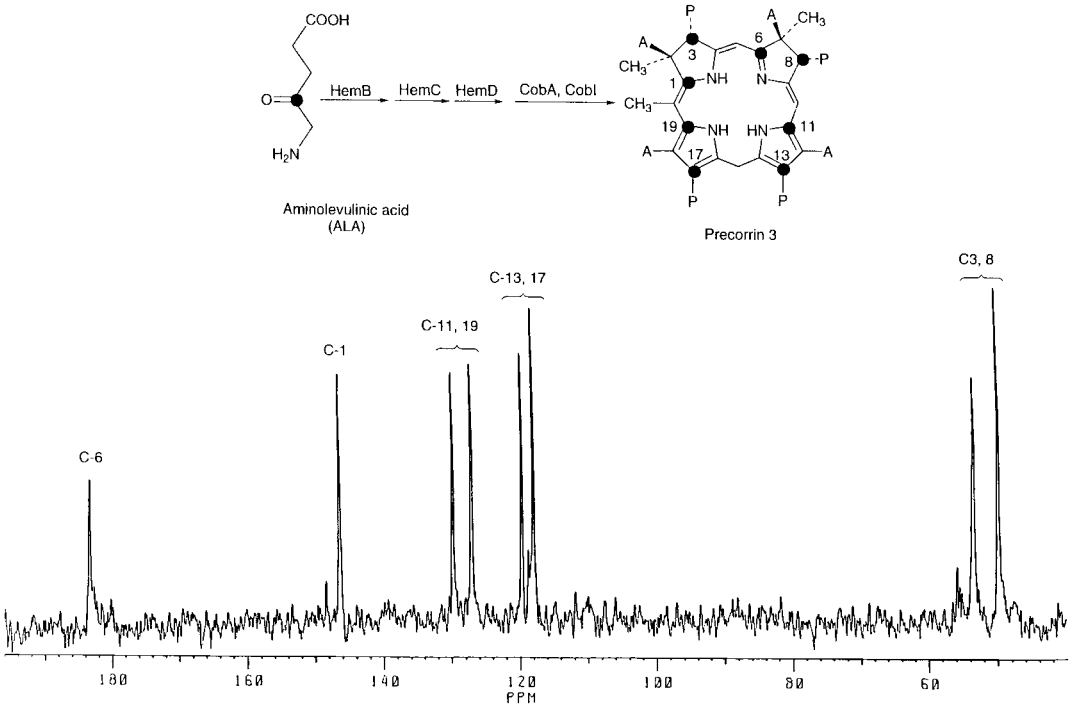

Figure 4. The ^{13}C-NMR spectrum of precorrin-3 synthesized in vitro from [4-^{13}C]-ALA in a five enzyme system in which *cobA* and *cobI* were provided in a lysate derived from a one liter culture of CR462. The sample in 2 M KCl in 10% D_2O was prepared as described in Materials and Methods.

The sequence of the 5′ primer for amplifying the contiguous *P. denitrificans cobA* and *cobB* genes was GGTACCCGGGGATCC**AGGAGGA**ATTTAAA**ATG** ATCGACGACCTCTTTGCCGGATTGCCGGCG and provided a *Bam*HI restriction site (GGATCC), an optimal *E. coli* ribosome binding site (**AGGAGGA**) and spacer (A–T rich), a start codon (**ATG**), and codons for the first ten amino acids of the *cobA* gene. The sequence of the 3′ primer was AGGCTTGCATGC CTGCAG**TTA**TGCAGAACCTGCGACGTCGATCA GATGCAT and provided a *Pst*I restriction site (CTGCAG), a stop codon (**TTA**), and codons for the last ten amino acids of the *cobB* gene.

The sequence of the 5′ primer for amplifying the *P. denitrificans* cobI gene was GGCCTCAACAAGGGA-TATCCCGCGGAGGAGGAATTTAAAATGAGCG GCGTCGGCGTGGGG and provided an *Eco*RV restriction site (GATATC), optimal *E. coli* translational signals, and the codons for the first seven amino acids of the *cobI* gene. The sequence of the 3′ primer was CTGCAGGTCGACTTATGGTCGGTCCTTCCAGC CGGGA ACGAGCAC and provided a SalI restriction site (GTCGAC), a stop codon and codons for the last ten amino acids of *cobI*.

In vivo biosynthesis, isolation and characterization of tetrapyrroles produced by TB1 bearing cob genes

TB1, bearing plasmids for the expression of *cob* genes, was grown 16–20 h in LB-ampicillin. The cells from 1 L of culture were collected by centrifugation, resuspended in 20–40 mL of buffer (50 mM Tris–HCl, pH 8, 5 mM EDTA), and incubated at room temperature with 50 μg/mL lysozyme. Cell lysis was achieved by sonication and the lysate was centrifuged at 10,000×*g* for 10 min to pellet unlysed cells and cell debris. DEAE-Sephadex 25 (1 g) was added to the supernatant and stirred for 15 min to adsorb the tetrapyrroles. The Sephadex was then collected on a small column, washed with 0.3 M KCl and water, dried, and suspended under argon for 16 h in methanol:sulfuric acid (95:5) to esterify the tetrapyrroles. The solution was neutralized with saturated sodium bicarbonate and the esters extracted into CH_2Cl_2. The products were analyzed by thin layer chromatography on silica gel plates using CH_2Cl_2:MeOH (98:2) as solvent.

In vitro biosynthesis of ^{13}C-precorrin-3

ALA dehydratase, porphobilinogen (PBG) deaminase, and uroporphyrinogen (urogen) III synthase were isolated from recombinant strains of *E. coli* as previously described.[8] PBG was generated in a reaction containing 5 mg [4-^{13}C]-ALA[17] and 3 mg ALA dehydratase in 100 mL of buffer (100 mM Tris–HCl, pH 8, 10 mM $MgCl_2$, 1 mM dithiothreitol). A lysate, derived as above from a 1 L culture of TB1 bearing a plasmid expressing both the *cobA* and *cobI* genes, was added and the solution was degassed by three cycles of freeze-thaw under vacuum. The degassed solution was transfered to an argon-purged glove box, and 2 mg each of lyophilized PBG deaminase and urogen III synthase were added. The reaction mixture was incubated at ambient temperature for 16–20 h and the product adsorbed to DEAE-Sephadex, washed with water and 0.3 M KCl, and then eluted from the Sephadex with 2 M KCl. The product was adjusted to 10% D_2O and analyzed by NMR as previously described.[4]

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