JOURNAL OF BACTERIOLOGY, Aug. 1995, p. 4481–4487 Vol. 177, No. 15
0021-9193/95/$04.00+0

Identification and Sequence Analysis of Genes Involved in Late Steps of Cobalamin (Vitamin B_{12}) Synthesis in *Rhodobacter capsulatus*

MICHAEL POLLICH AND GABRIELE KLUG*

Institut für Mikro- und Molekularbiologie, D-35392 Giessen, Germany

Received 21 March 1995/Accepted 24 May 1995

A 6.4-kb region of a 6.8-kb *Bam*HI fragment carrying *Rhodobacter capsulatus* genes involved in late steps of cobalamin synthesis has been sequenced. The nucleotide sequence and genetic analysis revealed that this fragment contains eight genes arranged in at least three operons. Five of these eight genes show homology to genes involved in the cobalamin synthesis of *Pseudomonas denitrificans* and *Salmonella typhimurium*. The arrangement of these homologous genes differs considerably in the three genera. Upstream of five overlapping genes (named *bluFEDCB*), a promoter activity could be detected by using *lacZ* fusions. This promoter shows no regulation by oxygen, vitamin B_{12} (cobalamin), or cobinamide. Disruption of the *bluE* gene by a Tn*5* insertion (strain AH2) results in reduced expression of the *puf* and *puc* operons, which encode pigment-binding proteins of the photosynthetic apparatus. The mutant strain AH2 can be corrected to a wild-type-like phenotype by addition of vitamin B_{12} or cobinamide dicyanide. Disruption of the *bluB* gene by an interposon (strain BB1) also disturbs the formation of the photosynthetic apparatus. The mutation of strain BB1 can be corrected by vitamin B_{12} but not by cobinamide. We propose that a lack of cobalamin results in deregulation and a decreased formation of the photosynthetic apparatus.

Microorganisms synthesize various macrocyclic tetrapyrroles containing different metals for diverse cellular processes, including heme (containing Fe^{2+}) for electron transport, siroheme (Fe^{2+}) as a cofactor of sulfite and nitrite reductases (20), bacteriochlorophyll (Bchl) (Mg^{2+}) and bacteriopheophytin (containing two H^+ ions) as pigments for photosynthesis, F430 (Ni^+) as a cofactor for methanogenesis (17), and the corrinoid cobalamin (Co^{3+}) as a cofactor for various enzymes (e.g., in acetogenesis and methanogenesis). The greatest diversity of naturally occurring tetrapyrroles is found in phototrophic bacteria, since they need heme-containing cytochromes and oxidases and use Bchl and bacteriopheophytin for light energy conversion in photosynthesis. The phototrophic purple nonsulfur bacterium *Rhodobacter capsulatus* synthesizes all of the compounds mentioned above except siroheme and F430, which is synthesized only by methanogenic archaebacteria. Cobalamin and other corrinoids are not synthesized by all bacteria. Eukaryotes have to take up corrinoids with their diet. Cobalamin synthesis in several prokaryotes has been studied recently (5, 9, 10, 40). As a result, the genes required for the synthesis of this macromolecule have been identified and the functions of some of their products have been established. In the last few years, the complete pathway of cobalamin biosynthesis in *Pseudomonas denitrificans* was revealed (2). Little is known about cobalamin synthesis in phototrophic bacteria, although Bchl synthesis in such strains is well understood (3). For the phototrophic, vitamin B_{12} (cobalamin)-auxotrophic, green sulfur bacterium *Chlorobium limicola* 1230, it was shown that cobalamin deficiency results in a decrease of Bchl content. Furthermore, this strain is unable to form chlorosomes without addition of vitamin B_{12} (18). Earlier work showed that 10 of 19 isolates of green sulfur bacteria were vitamin B_{12} auxotrophs and that the Bchl contents of the cells were strongly reduced in the presence of growth-limiting vitamin B_{12} concentrations. The same effect of vitamin B_{12} on Bchl synthesis was also observed in four vitamin B_{12}-auxotrophic strains of purple sulfur bacteria (37). In contrast to the case for green and purple sulfur bacteria, vitamin B_{12} auxotrophy is rare among the purple nonsulfur bacteria (members of the family *Rhodospirillaceae*). Vitamin B_{12} concentrations of about 0.1 μg/liter were sufficient to promote growth of three vitamin B_{12}-auxotrophic strains (43). In the purple nonsulfur bacterium *Rhodobacter sphaeroides*, vitamin B_{12} was found at concentrations of 9 to 11 μg/g of dry cells (8, 24). The specific involvement of cobalamin in the metabolism of the purple nonsulfur bacteria is not yet known. Here, we report the identification and sequence analysis of cobalamin synthesis genes of a phototrophic bacterium. The central five of these genes were named *blu* genes because the *bluE* and *bluB* genes are required to make an aerobic culture blush after reduction of the partial O_2 pressure (pO_2). Disruptions of the *bluE* and *bluB* genes in the Tn*5* mutant strain AH2 and the Ω-interposon mutant strain BB1 of *R. capsulatus*, respectively, lead to a decrease in the formation of the photosynthetic apparatus under semiaerobic growth conditions (pO_2, 1 to 2%).

MATERIALS AND METHODS

Bacterial strains, plasmids, phage, and growth conditions. The strains and plasmids used and their relevant characteristics are listed in Table 1. *R. capsulatus* strains were grown in a minimal malate medium (12) at 32°C. Kanamycin and tetracycline were added to final concentrations of 10 and 1.5 μg/ml, respectively. *Escherichia coli* strains were grown in Luria-Bertani medium at 37°C. Antibiotics were added to the following final concentrations (in micrograms per milliliter): ampicillin, 50; tetracycline, 10; and kanamycin, 10.

Mating of *R. capsulatus* with *E. coli* S17-1. Since *R. capsulatus* cannot be transformed, plasmids have to be introduced into *R. capsulatus* cells by conjugation with *E. coli* cells carrying a broad-host-range plasmid. Biparental matings between the *R. capsulatus* recipient strains and the *E. coli* donor S17-1 (Table 1) were done with 100 μl of *E. coli* culture and 1,000 μl of *R. capsulatus* culture. Cells were sedimented, resuspended in 50 μl of malate minimal medium, and mixed. The cells were then poured onto nitrocellulose filters (BA85; Schleicher

* Corresponding author. Mailing address: Institut für Mikro-und Molekularbiologie, Frankfurter Str. 107, D-35392 Giessen, Germany. Phone: (641) 7029652. Fax: (641) 7029659. Electronic mail address: Gabriele.Klug@mikro.bio.uni-giessen.de.

TABLE 1. Bacterial strains and plasmids

Strain, plasmid, or phage	Description[a]	Source or reference
Strains		
E. coli		
MC1061	Sp^r Str^r Δ*lacX74*	7
XL1-Blue	Tc^r *recA1 endA1 relA1 lac* [F′ *proAB lacI*^{q} *lacZ*ΔM15 Tn*10*]	6
S17-1	Str^r Tp^r *pro res*$^+$ *mod*$^+$	44
R. capsulatus		
37b4	Wild-type DSM strain 938	19
B10	Wild type	32
AH2	Km^r 37b4 (*bluE*::Tn*5*)	38
BB1	Km^r 37b4 (*bluB*::Ω-Km)	This work
Plasmids and phage		
pGEM-3Zf(−)	Ap^r	39
pHP45Ω-Km	Km^r	14
pPHU235	Tc^r	26
pPHU281	Tc^r suicide vector	25
pRK415	Tc^r pRK404 derivative	27
pAHU4	Tc^r pPHU235 derivative with 1.2-kb *Pst*I fragment	This work
pAHUΔ11	Tc^r deletion clone of pAHU4	This work
pAWB	Ap^r pGEM-3Zf(−) derivative with 6.8-kb *Bam*HI fragment	This work
pAHW25	Tc^r pRK415 derivative with 1.6-kb fragment of pAWB	38
pBBM1	Tc^r Km^r pPHU281 derivative	This work
pBBW1	Tc^r Km^r pRK415 derivative with 6.8-kb *Bam*HI fragment	This work
pBBW2	Tc^r Km^r pRK415 derivative with 5.2 kb of the 6.8-kb *Bam*HI fragment	This work
M13mp18		46

[a] Resistance to the following drugs is indicated: ampicillin (Ap^r), kanamycin (Km^r), spectinomycin (Sp^r), streptomycin (Str^r), tetracycline (Tc), and trimethoprim (Tp).

& Schuell) which were placed on a PY agar (1% tryptone, 0.05% yeast extract, 2 mM $CaCl_2$, 2 mM $MgCl_2$, 0.1 mM $FeSO_4$; pH 7) plate and incubated for 4 h at 32°C. After conjugation, the cells were resuspended and different dilutions were plated on minimal malate medium agar plates.

β-Galactosidase assay. Twenty-milliliter *R. capsulatus* cultures were grown aerobically in 50-ml flasks. Seventy-milliliter *R. capsulatus* cultures were grown semiaerobically in 100-ml flasks. A 1.5-ml sample of each culture was assayed for β-galactosidase (LacZ) activity as described previously (33).

Extraction and measurement of Bchl. A 1.5-ml volume of each culture was centrifuged, the cell pellet was resuspended in 50 μl of H_2O, and Bchl was extracted after addition of 500 μl of acetone-methanol (7:2) by vortexing for 10 s. After centrifugation for 5 min, the A_{770} of the supernatant was measured. The relative amount of Bchl per cell was calculated by division of this Bchl-specific absorption by the optical density at 660 nm of the culture.

Isolation, cloning, modification, analysis, and sequencing of nucleic acids. Restriction endonucleases were purchased from New England Biolabs (Beverly, Mass.). T4 DNA ligase and calf intestine alkaline phosphatase were purchased from Boehringer, Mannheim, Germany. All recombinant DNA procedures were carried out by standard protocols. All *Pst*I fragments of plasmid pAWB were subcloned in bacteriophage M13mp18. Single-stranded DNA of infected *E. coli* XL1-Blue cultures was prepared by the method of Sambrook et al. (41). DNA deletions were carried out with a Pharmacia double-stranded nested deletion kit according to the instructions provided with the kit. DNA was sequenced by the dideoxy chain termination method (42) using the U.S. Biochemicals Sequenase kit. RNA was isolated from 40 ml of an aerobic *R. capsulatus* culture. Isolation, transfer to nylon membranes (Biodyne B; Pall), and Northern (RNA) blot hybridization have been described elsewhere (45).

Nucleotide sequence accession number. All sequence analyses were performed with programs of the Genetics Computer Group program package (11) installed on the Convex 12 of the German Center for Cancer Research in Heidelberg, Germany. The nucleotide sequence is available in the EMBL/GenBank/DDBJ databases under accession no. Z46611.

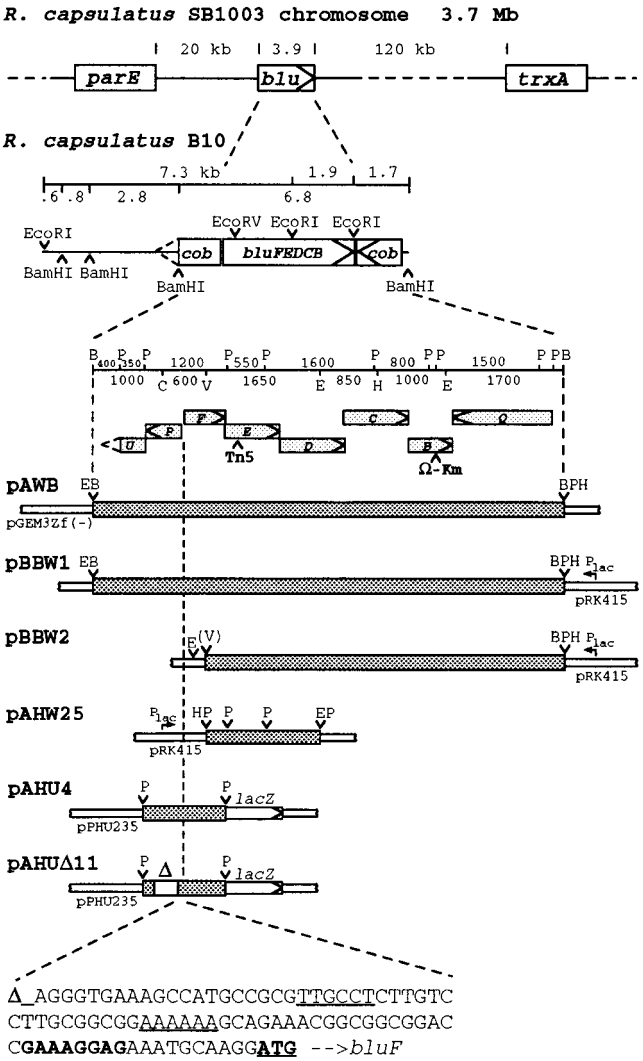

FIG. 1. The *R. capsulatus blu* genes and adjacent *cob* genes: localization on the chromosome, restriction map, gene map, plasmid maps, and sequence of the promoter region. Putative −35 and −10 boxes (underlined) and the putative ribosome binding site (boldface) are indicated. Restriction enzymes: B, *Bam*HI; C, *Cla*I; E, *Eco*RI; H, *Hin*dIII; P, *Pst*I; and V, *Eco*RV.

RESULTS

Nucleotide sequence of the 6.4-kb fragment and identification of eight genes. We previously identified a Tn*5* insertion in an open reading frame of 798 bp (ORF798) in the mutant strain AH2 (38). Strain AH2 forms only small amounts of photosynthetic complexes and low levels of *puf* and *puc* mRNAs, even during growth under low-oxygen tension (38). Since we had evidence that ORF798 was located between two other open reading frames, we cloned a chromosomal 6.8-kb *Bam*HI fragment of the *R. capsulatus* wild-type strain B10, which was able to complement the mutant strain AH2 in *trans*, into the vector pGEM-3Zf(−), resulting in plasmid pAWB (Fig. 1). All *Pst*I fragments of plasmid pAWB were subcloned in bacteriophage M13mp18, and both strands were sequenced. Eight open reading frames could be identified by using the codon preference program (23) of the Genetics Computer Group program package with a codon usage table obtained from sequenced *R. capsulatus* photosynthesis genes. The five central open reading frames were named *bluFEDCB* genes

TABLE 2. Characteristics of the sequenced *R. capsulatus* genes[a]

Gene	Length (bp)	Start codon	Stop codon	Overlap with following gene	GC content (%)	No. of amino acids	Homology[b]
cobU	?	ATG	?	?	?	?	*cobU*/*cobT*
cobP	504	ATG	TAG	TAGGTATG	65.5	167	*cobP*/*cobU*
bluF	588	ATG	TGA	ATGA	71.9	195	
bluE	798	ATG	TGA	ATGA	75.8	265	
bluD	945	ATG	TGA	TGATG	69.1	314	*cobD*/*cbiB*
bluC	963	ATG	TGA	ATGAACTTTGA	72.2	320	*cobC*/*cobD*
bluB	621	ATG	TAA		68.7	206	
cobQ	1,452	ATG	TAG		69.0	483	*cobQ*/*cbiP*

[a] ?, unknown.
[b] Homologous *P. denitrificans* gene/homologous *S. typhimurium* gene.

because the *bluE* (ORF798) and *bluB* genes are required to make an aerobic culture blush after reduction of the pO_2. The other three genes were named *cobP*, *cobU*, and *cobQ* for their strong sequence homology to genes of *P. denitrificans* and *Salmonella typhimurium*. These genes are involved in the final steps of cobalamin synthesis and in cobinamide synthesis. *cobU* is only partially present on the 6.8-kb *Bam*HI fragment, and only 350 bp of the 5′ end was sequenced. The five *blu* genes overlap by 1 to 11 bases and therefore seem to be translationally coupled (Table 2). A comparison of the locations of these genes with the locations of the homologous genes of *S. typhimurium* and *P. denitrificans* showed that the arrangement differs considerably between these bacteria. The homologs of the *cobU* and *cobP* genes, which are coupled in *R. capsulatus*, are separated in *S. typhimurium* by the *cobS* gene and are located on two different operons in *P. denitrificans* (Fig. 2). The homologs of the *bluD* and *bluC* genes are distant on the *S. typhimurium* chromosome: *cbiB* is located at 41 min in a 17-kb operon, whereas *cobD* is located at 14 min (22). In *P. denitrificans*, genes *cobC* and *cobD* are coupled but in inverse order in comparison with the *R. capsulatus bluC* and *bluD* genes (Fig. 2). M. Fonstein mapped the *blu* operon on the *R. capsulatus* SB1003 chromosome (15, 16) between genes *parE* and *trxA* (Fig. 1).

Putative functions of the proteins encoded by the identified genes. The nucleotide sequences were translated into amino acid sequences, and homology searches in the Swiss-Prot and NBRF/PIR protein databases were performed by using the FASTA program (11). The protein sequences and alignments with the Multalign program (11) are shown in Fig. 3. All homologies are summarized in Table 3. For the proteins BluF, -E, and -B and their genes, no homologies could be found in either protein or DNA databases. Since the mutation of strain AH2 (*bluE*::Tn*5*) can be corrected by addition of cobinamide dicyanide, the BluE protein seems to be involved in the synthesis of this compound. The mutation of strain BB1 (*bluB*::Ω-Km) can be corrected by addition of vitamin B_{12} but not cobinamide dicyanide. This indicates that the BluB protein is involved in the conversion of cobinamide to cobalamin (Fig. 4). The CobP protein has 58.3% amino acid identity with CobP of *P. denitrificans* and 52.1% identity with CobU of *S. typhimurium*. CobP of *P. denitrificans* is a bifunctional enzyme with cobinamide kinase and cobinamide phosphate guanylyltransferase activities (10). The BluD protein shows homology to CobD of *P. denitrificans* (46.9% identity) and CbiB of *S. typhimurium* (36.9%), which are involved in the transformation of cobyric acid into cobinamide (9). BluC shares 43.6% identity with the *P. denitrificans* CobC protein, which is homologous to the *S. typhimurium* CobD protein (35). CobQ shows homology to the cobyric acid synthase CobQ of *P. denitrificans* (58.9% identity) and CbiP of *S. typhimurium* (45.1%), which catalyze amidation reactions in cobinamide synthesis (10). All of the proteins with homologs in other bacterial species show highly conserved regions (Fig. 3). Mutant strain AH2 was obtained by Tn*5* mutagenesis of wild-type strain 37b4. We also sequenced the *bluFEDC* genes and *cobP* in *R. capsulatus* 37b4. The proteins of *R. capsulatus* B10 and 37b4 are 91 to 98% identical (Table 3). The *R. capsulatus* proteins share more identical residues with the *P. denitrificans* proteins (43 to 59%) than with the *S. typhimurium* proteins (37 to 52%).

Localization and characterization of a promoter upstream of the *bluF* gene. In order to find out whether the *bluF* gene is preceded by a promoter, we cloned the 1.2-kb *Pst*I fragment of plasmid pAWB into vector pPHU235 (26). This resulted in a

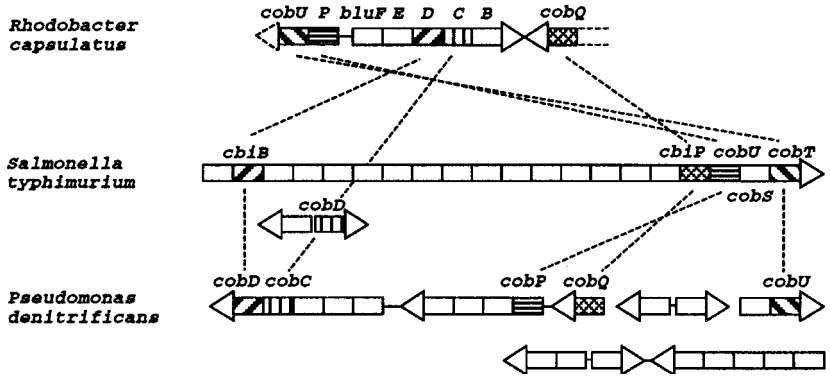

FIG. 2. Arrangements of cobalamin synthesis genes in *R. capsulatus*, *S. typhimurium*, and *P. denitrificans*.

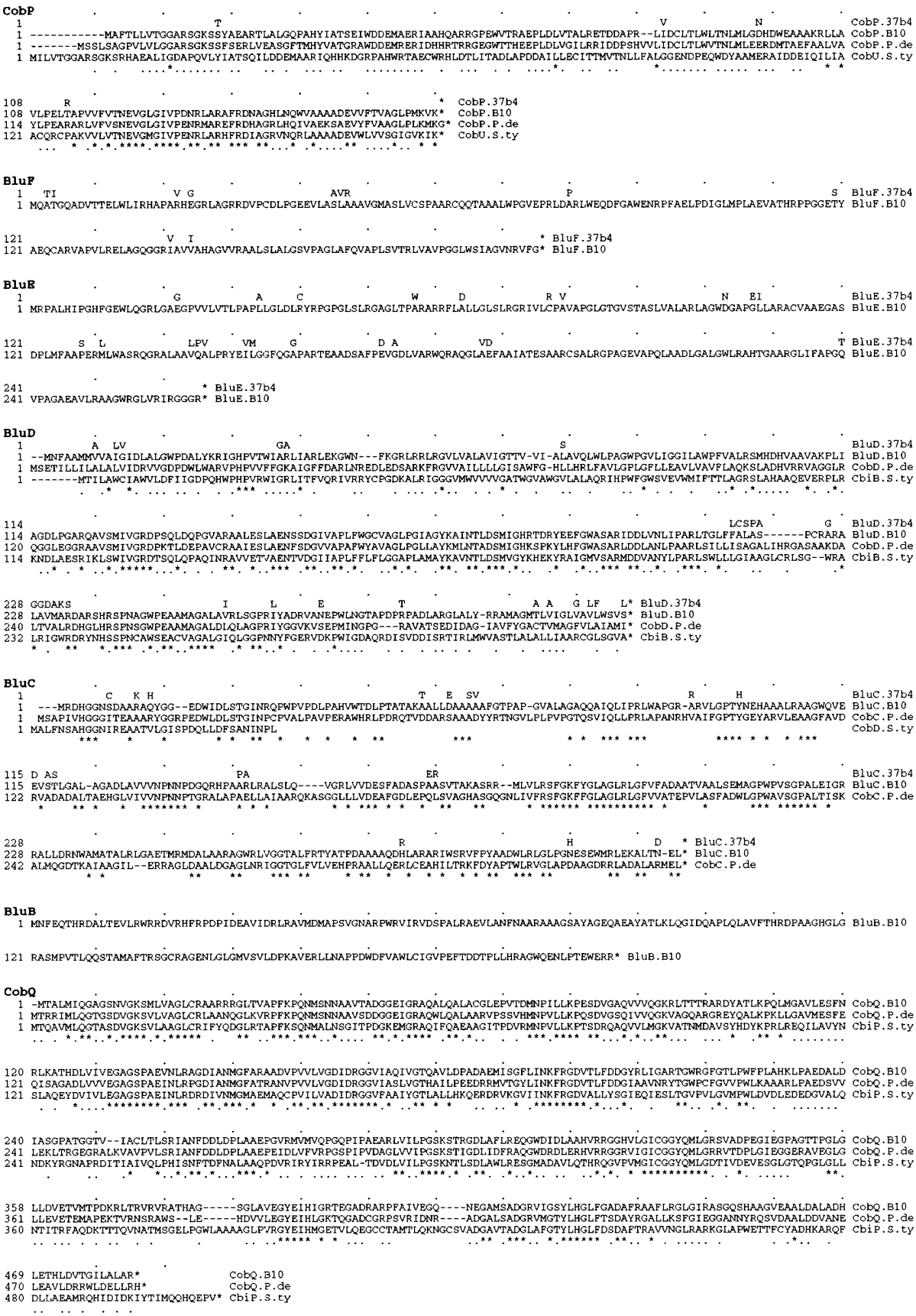

FIG. 3. Amino acid sequences of proteins CobP, BluF, BluE, BluD, BluC, BluB, and CobQ. Differences between *R. capsulatus* strains B10 and 37b4 and alignments with homologous proteins of *P. denitrificans* and *S. typhimurium* are shown. Amino acids that are identical in all strains (asterisks), in two of three strains (dots), and gaps introduced (dashes) are indicated.

TABLE 3. Homologies of proteins encoded by the sequenced genes with proteins of other strains

R. capsulatus B10 protein	% Identity (similarity) to the indicated protein of:		
	R. capsulatus 37b4	*P. denitrificans*	*S. typhimurium*
CobP	97.6 (98.2) CobP	58.3 (70.8) CobP	52.1 (69.9) CobU
BluF	93.4 (95.4) BluF		
BluE	91.0 (95.1) BluE		
BluD	91.1 (94.3) BluD	46.9 (67.8) CobD	36.9 (60.5) CbiB
BluC	94.1 (96.6) BluC	43.6 (61.1) CobC	?[a] CobD
BluB	? ? BluB		
CobQ	? ? CobQ	58.9 (73.9) CobQ	45.1 (62.8) CbiP

[a] ?, percentage unknown.

translational fusion of the *bluE* gene to the *lacZ* gene of the vector in plasmid pAHU4 (Fig. 1). Nevertheless, *E. coli* cells carrying plasmid pAHU4 showed no detectable β-galactosidase activity. This indicated that transcription was not initiated in *E. coli*. When we transferred plasmid pAHU4 conjugatively in *R. capsulatus* 37b4 and grew the cells aerobically, we measured a β-galactosidase activity of 55 ± 5 Miller units (mean ± standard deviation). The negative control (plasmid pPHU235) showed no activity (1 Miller unit). By exonuclease III deletion of 308 bases upstream of the *bluF* gene, we constructed plasmid pAHUΔ11 (Fig. 1), which still showed β-galactosidase activity of 25 ± 5 Miller units under aerobic conditions. The 86-bp region between the deletion and the translational start of the *bluF* gene is shown in Fig. 1. A ribosome binding site with good homology to the 16S rRNA binding sequence is located upstream of the start codon of *bluF*. The other sequenced genes are preceded by ribosome binding sites with much weaker homology. Sequences similar to −35 and −10 regions of known *R. capsulatus* promoters from genes involved in pigment synthesis (28, 31) were located 40 and 65 nucleotides upstream of the translational start (Fig. 1). Using plasmid pAHU4 in *R. capsulatus* 37b4, we measured the β-galactosidase activity under aerobic and semiaerobic growth conditions, with addition of 100 μg of vitamin B_{12} per liter and with 10 and 100 μg of cobinamide dicyanide per liter. The assays showed identical β-galactosidase activities, indicating that the *blu* promoter is not regulated by oxygen, vitamin B_{12}, and cobinamide dicyanide. In order to determine the length of the *blu* mRNA, we tried to detect this mRNA species by Northern blot hybridization. Northern blots of 8 and 20 μg of 37b4 RNA probed with [^{32}P]dCTP-labelled 550-bp *Pst*I and 1,000-bp *Hin*dIII-*Eco*RI fragments of plasmid pAWB (Fig. 1) showed no detectable *blu* mRNA.

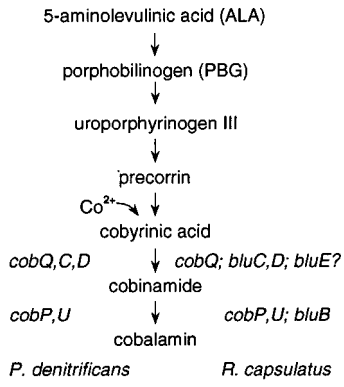

FIG. 4. Simplified pathway of cobalamin synthesis of *P. denitrificans*. The *P. denitrificans* genes which are homologous to the sequenced *R. capsulatus* genes are indicated.

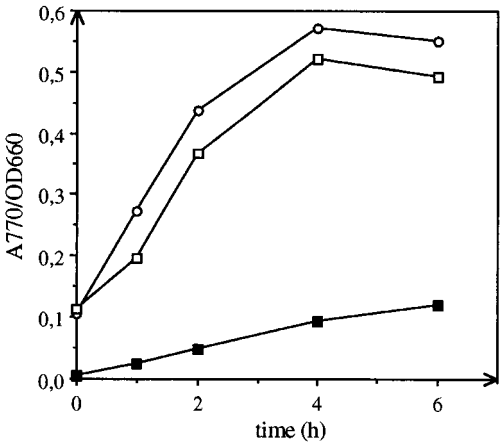

FIG. 5. Relative amounts of Bchl per cell of BB1 and 37b4 cultures after reduction of the pO_2 at time zero. Symbols: open circles, 37b4; open and closed squares, BB1 plus 10 or 1 μg of vitamin B_{12} per liter, respectively. A770, A_{770}; OD660, optical density at 660 nm.

Cobalamin is required for Bchl synthesis in mutant strains AH2 and BB1. Mutant strain AH2 was isolated after Tn*5* mutagenesis of the wild-type strain 37b4 because of its lighter red color indicating an altered composition of the photosynthetic apparatus. Cell extracts of strain AH2 showed strongly reduced absorbances of the photosynthetic reaction center and light-harvesting complexes (LHI and LHII) (38). Since the gene following the *bluE* gene (ORF798) with the transposon insertion shows homology to a cobalamin synthesis gene, we tried to correct the mutation in AH2 with different amounts of vitamin B_{12} (1, 10, and 100 μg/liter). Vitamin B_{12} at a concentration of 10 μg/liter was sufficient for a wild-type-like formation of the photosynthetic apparatus after reduction of the pO_2 to 1 to 2%. In order to determine the function of the BluB protein, we constructed mutant strain BB1 by insertion of the Ω-interposon into the *Stu*I site of the *bluB* gene in plasmid pBBM1. The suicide plasmid pBBM1 was then transferred by conjugation in the wild-type strain 37b4, and transconjugants were screened for homologous recombination of the Ω-interposon into the *bluB* gene of the chromosome (Fig. 1). Mutant strains BB1 and AH2 have similar phenotypes. Figure 5 shows the increase of the Bchl content of aerobic BB1 cultures after reduction of the pO_2 (semiaerobic growth). Addition of 10 μg of vitamin B_{12} per liter resulted in almost wild-type-like amounts of Bchl, whereas 1 μg of vitamin B_{12} per liter was not sufficient for normal synthesis of Bchl.

The *blu* genes are transcribed from at least two promoters. To determine whether the *bluB* gene is transcribed from the *bluF* promoter, we constructed plasmids pBBW1, containing the 6.8-kb *Bam*HI fragment, and pBBW2, containing 5.2 kb of this fragment, for complementation assays with mutants AH2 and BB1 (Fig. 1). Plasmid pBBW1 complements both mutants, whereas plasmid pBBW2, which lacks the *bluF* promoter and parts of the *bluF* gene, complements only strain BB1 and not strain AH2. It was shown in a control experiment that plasmid pAHW25 containing the *bluE* gene transcribed from the *lac* promoter of the vector (Fig. 1) was sufficient to complement strain AH2. This indicates that the *bluE* gene of plasmid pBBW2 is not transcribed. Since plasmid pBBW2 is able to complement strain BB1, its *bluB* gene must be transcribed, indicating that the transcription of the *bluB* gene is initiated by a second promoter within gene *bluE* or downstream.

DISCUSSION

In this report, we describe the nucleotide sequences and arrangement of eight *R. capsulatus* genes involved in late steps of cobalamin synthesis. Five of the eight proteins encoded by the sequenced genes show homology to *P. denitrificans* and *S. typhimurium* proteins involved in cobalamin synthesis (CobU, CobP, BluD, BluC, and CobQ; Fig. 3). Three proteins (BluF, BluE, and BluB) share no homology with known proteins. The *R. capsulatus* proteins share more identical or similar amino acids with the *P. denitrificans* proteins than with the *S. typhimurium* proteins (Table 3). This may be due to a closer evolutionary relationship of *R. capsulatus* to *P. denitrificans* than to *S. typhimurium*. *R. capsulatus* belongs to the alpha subgroup of the proteobacteria, whereas *S. typhimurium* and other enteric bacteria such as *E. coli* are placed in the gamma subgroup. The species *P. denitrificans* cannot be placed definitely in one of the four subgroups, since different strains of *P. denitrificans* have been shown to belong to the alpha, beta, and gamma subgroups (47). The proteins of *R. capsulatus* B10 and 37b4 differ in 2 to 9 amino acid residues per 100, mostly in nonconserved regions. There are highly conserved regions in the proteins of all three species (Fig. 3, asterisks) which could represent binding sites or catalytic sites. The functional regions of the *P. denitrificans* and *S. typhimurium* proteins are not known yet.

The cobalamin biosynthetic pathway is divided into three parts. In part I of the pathway, precorrin-3 is converted into cobinamide. Part II reactions lead to the synthesis of dimethyl benzimidazole, probably from flavin precursors. In part III of the pathway, cobinamide is finally converted into cobalamin (40). From comparison with the homologous genes in *P. denitrificans* and *S. typhimurium*, the *bluC*, *bluD*, and *cobQ* gene products should be involved in amidations and the addition of aminopropanol to cobyrinic acid to yield cobinamide (part I reactions). The *cobP* and *cobU* gene products catalyze the final conversion of cobinamide to cobalamin (part III reactions). In *S. typhimurium*, all three genes involved in part III reactions (*cobUST*) are located at the end of the cobalamin gene cluster (Fig. 2). Therefore, it is possible that the *R. capsulatus cobPU* genes are followed by the third part III gene (homologous to *cobV* and *cobS*) to form an operon containing all three part III genes.

The correction assays of the *bluE* and *bluB* mutant strains AH2 and BB1, respectively, indicate that the BluE protein is involved in the synthesis of cobinamide and that the BluB protein is involved in the conversion of cobinamide to cobalamin (Fig. 4). The function of the BluF protein is not yet known. In mutant strain AH2, *bluE* expression is prevented by insertion of Tn*5* into the *bluE* gene. In *trans*-complementation assays with different plasmids containing *blu* genes showed that the *bluE* gene alone is sufficient for complementation. This suggests that the *bluDCB* genes are expressed in mutant strain AH2 and that transcription of these genes is initiated within the coding sequence or within the transposon, as suggested previously (4). In plasmid pAHW25, which does not contain the *bluF* promoter (located upstream of the *bluF* gene) but complements strain AH2, the *bluE* gene is transcribed from the *lac* promoter of the vector (Fig. 1). Complementation of strain BB1 by plasmid pBBW2 indicates that the transcription of the *bluB* gene is initiated within the *bluE* gene or downstream.

The *blu* genes overlap by 1 to 11 nucleotides and seem to be translationally coupled. Upstream of the first *blu* gene (*bluF*), a ribosome binding site with homology to the 16S rRNA binding sequence in eight nucleotides (underlined) (5′-AGAAAG GAGGTGAT-3′) is located. The other *blu* genes are preceded by ribosome binding sites with much weaker homology to the consensus sequence. For these *blu* genes, an efficient ribosome binding site would not be necessary because the translational coupling of the genes leads to an easier recognition of these internal ribosome binding sites by ribosomes just terminating translation of the preceding gene. We suppose that the *blu* genes are translated from at least two mRNAs, but we were not able to detect these mRNAs by Northern blot analysis. Since the cells need only very low concentrations of vitamin B_{12}, the *blu* mRNA amounts in the cell might not be sufficient to be detected by Northern blot hybridization. The mRNA of the *R. capsulatus trxA* gene (encoding thioredoxin) gives rise to only very faint signals in Northern blot hybridizations of 20 μg of RNA. Translational fusion of *trxA* to *lacZ* in plasmid pPHU234 results in a β-galactosidase activity of 150 Miller units (36). Since the activity of the translational fusion of *bluE* to *lacZ* in plasmid pPHU235 is only about 55 Miller units, we assume that the *blu* mRNA cannot be detected by Northern blot hybridization.

A promoter activity was localized within a region of 86 bp upstream of the *bluF* gene by using *lacZ* fusions. This region contains sequences with homology to the *E. coli* σ^{70} promoter consensus sequence and promoters of *R. capsulatus* pigment synthesis genes (28). These putative −35 and −10 regions are underlined in Fig. 1. However, this promoter, like many other promoters in *R. capsulatus*, is not active in *E. coli*, indicating that it is not recognized by *E. coli* RNA polymerase. Since the expression of the *cob* operon of *S. typhimurium* is regulated by redox potential and vitamin B_{12} (1, 13), the regulation of the *bluF* promoter was examined by using the translational *bluE-lacZ* fusion in plasmid pAHU4. Neither the pO_2 nor vitamin B_{12} or cobinamide appeared to regulate the activity of the *bluF* promoter.

The formation of the photosynthetic apparatus after reduction of the pO_2 was examined in different assays with various vitamin B_{12} concentrations. Addition of 10 μg of vitamin B_{12} per liter was sufficient for correction of the mutations in strains AH2 (38) and BB1 (Fig. 5). The minimal correcting concentration of 10 μg of vitamin B_{12} per liter is in good accordance with the finding that in the vitamin B_{12}-auxotrophic, green sulfur bacterium *C. limicola* 1230, Bchl synthesis and chlorosome formation can be restored by addition of 20 μg of vitamin B_{12} per liter to the growth medium (18). The influence of vitamin B_{12} on the formation of the photosynthetic apparatus could be due to the fact that the methylation of protoporphyrin IX to Mg-protoporphyrin monomethyl ester in Bchl synthesis requires *S*-adenosylmethionine as a methyl donor (21). Cobalamin is required by homocysteine methyltransferase during the formation of *S*-adenosylmethionine (8). The methylation of protoporphyrin IX in Bchl synthesis is disturbed if the cell lacks cobalamin. A lack of Bchl results in a decrease in the formation of the photosynthetic apparatus. Earlier work showed that *R. capsulatus* mutants with defects in Bchl synthesis show a transcription of photosynthetic genes which is independent of oxygen tension. Bchl mutant strain FM65, with a defect in the *bchNBF* genes involved in chlorophyllid a synthesis, shows almost no increase in *puf* and *puc* mRNA levels after reduction of the pO_2, whereas the levels of both mRNAs increase in the wild type (29, 30). A similar effect was reported for *bluE* mutant strain AH2 (38). In *R. sphaeroides* mutants impaired in 5-aminolevulinic acid synthesis, both transcriptional and posttranscriptional regulations of light-harvesting complex expression are affected (34). Therefore, it seems that the regulation of *puf* and *puc* mRNA synthesis is dependent on the availability of Bchl. Possibly, there are more steps than the methylation of protoporphyrin IX in the formation of the pho-

tosynthetic apparatus which require cobalamin. Vitamin B_{12}-auxotrophic mutants of *R. capsulatus* could be helpful in the elucidation of these steps.

ACKNOWLEDGMENTS

We thank M. Fonstein for mapping the *blu* genes to the *R. capsulatus* chromosome.

This work was supported by the Bundesministerium für Forschung und Technologie, by the Deutsche Forschungsgemeinschaft (SFB272), and by the Fonds der Chemischen Industrie.

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