JOURNAL OF BACTERIOLOGY, Dec. 1996, p. 7308–7310 Vol. 178, No. 24
0021-9193/96/$04.00+0


# The *bluF* Gene of *Rhodobacter capsulatus* Is Involved in Conversion of Cobinamide to Cobalamin (Vitamin $B_{12}$)

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Received 5 July 1996/Accepted 4 October 1996

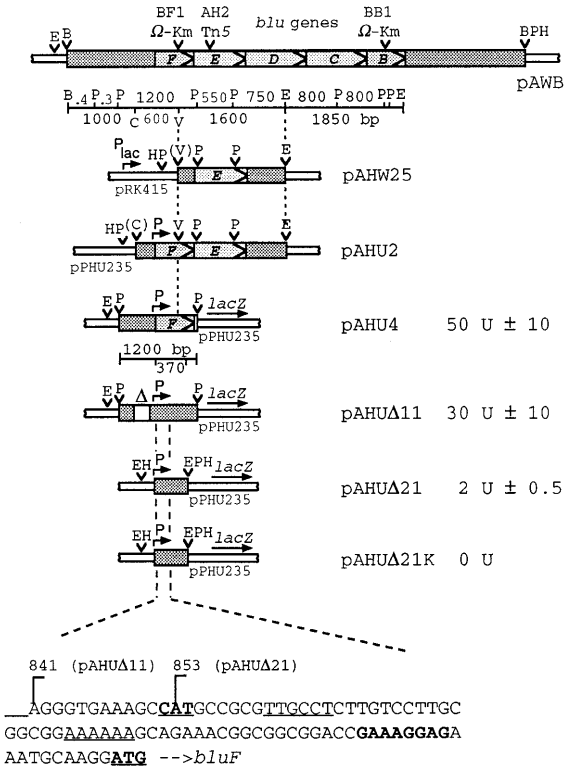
**The *bluF* gene of *Rhodobacter capsulatus* is the first gene of the *bluFEDCB* operon which is involved in late steps of the cobalamin synthesis. To determine the function of the *bluF* gene product, a *bluF*::Ω-Km mutant strain was constructed and characterized. This vitamin $B_{12}$ auxotrophic mutant strain shows a 3.5-times higher vitamin $B_{12}$ requirement under phototrophic growth conditions than under chemotrophic growth conditions. Surprisingly, the *bluF* promoter activity does not respond to alterations to the oxygen tension or vitamin $B_{12}$ concentration.**

a

| medium / strain | RÄ | +Cbi | +Cbl | +DMB | +DMB +Cbi |
|---|---|---|---|---|---|
| BF1 | - | - | + | - | - |
| BF1+pAHW25 | - | - | + | - | - |
| BF1+pAHU4 | - | - | + | | |
| BF1+pAHU2 | + | | | | |
| AH2 | +/-(1) | + | + | | |
| AH2+pAHW25 | + | | | | |

(1) growth but no formation of the photosynthetic apparatus

b




Recently we reported the identification of eight genes involved in late steps of the cobalamin synthesis of the purple nonsulfur bacterium *Rhodobacter capsulatus*. Since five of those genes show strong homology to cobalamin synthesis genes of *Pseudomonas denitrificans* and *Salmonella typhimurium*, the function of their encoded proteins is probably the same as in these bacteria. By correction assays with the mutant strains AH2 and BB1, the functions of two of the remaining three genes (*bluE* and *bluB*) could be assigned to late steps in the cobalamin synthesis (8). The function of the third gene (*bluF*) was not known until now. Here we report the construction and characterization of a *bluF*::Ω-Km mutant strain.

**Construction, correction, and complementation assays of the mutant strain BF1.** The Ω-Km interposon (4) was inserted into the *Eco*RV site of the *bluF* gene. The mutated gene was introduced into the wild-type strain 37b4 (3) by recombination following the strategy already described (6).

Correction assays with the cobalamin precursor cobinamide showed that in contrast to the *bluE*::Tn*5* mutant strain AH2, which can be corrected with this compound (8), the mutant strain BF1 cannot. Complementation assays with different plasmids showed that the complete *bluF* gene transcribed from its own *bluF* promoter is not sufficient for complementation of the mutant strain BF1. Complementation is only achieved by plasmid pAHU2 carrying the *bluF* and *-E* genes (Fig. 1). This indicates that the insertion of the Ω-Km interposon into the *bluF* gene also interferes with the *bluE* gene function, suggesting that both genes form a transcriptional unit which is transcribed from the *bluF* promoter. Mutant strain BF1 must therefore be regarded as a *bluF* and *-E* double mutant. Since the *bluE*::Tn*5* mutant strain AH2 can be corrected by cobinamide whereas the mutant strain BF1 can only be corrected by cobalamin (Fig. 1), the function of the *bluF* gene product can be assigned to a step in the conversion of cobinamide to cobalamin. No correction of the *R. capsulatus bluF* mutant phenotype could be achieved when high levels of 5,6-dimethylben-

FIG. 1. (a) Chemotrophic growth and formation of the photosynthetic apparatus of the mutant strain BF1 with different plasmids on RÄ minimal malate medium. Cbi, cobinamide dicyanide (30 nM); Cbl, cyanocobalamin (7 nM); DMB: 5,6-dimethylbenzimidazole (140 μM). (b) Restriction maps of plasmids used for complementation experiments and *lacZ*-fusion plasmids (β-galactosidase activities in Miller units). Sequence of the *bluF* promoter region. Putative −35 and −10 boxes (underlined), the putative ribosome binding site (bold), and the *cobP* and *bluF* start codons (underlined, bold) are indicated. The nucleotide numbers above the sequence refer to the published sequence of the vitamin $B_{12}$ gene cluster (EMBL/GenBank/DDBJ databases, accession number Z46611). B, *Bam*HI; C, *Cla*I; E, *Eco*RI; H, *Hin*dIII; P, *Pst*I; V, *Eco*RV.

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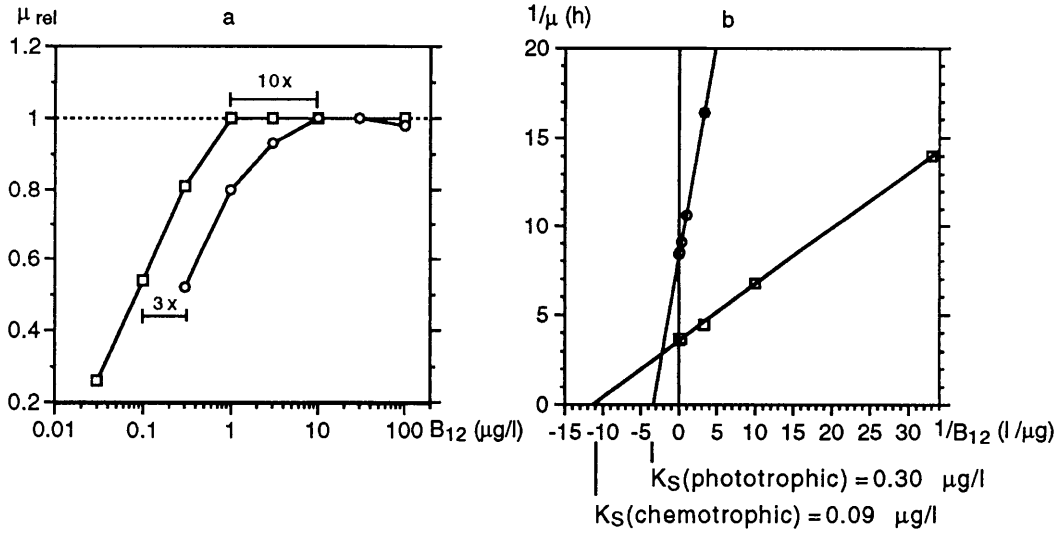


FIG. 2. (a) Relative growth rates ($\mu_{rel}$) of mutant strain BF1 at different vitamin $B_{12}$ concentrations in relation to the wild-type 37b4 ($\mu_{rel}=1$, $t_d$(chemotrophic)=150 min, $t_d$(phototrophic)=350 min). Squares: chemotrophic (aerobic) growth (20-ml cultures in 100-ml flasks at 33°C). Circles: phototrophic (anaerobic) growth (50-ml cultures in 50-ml screw-cap flasks at 23°C in 35-cm distance from a 60-W tungsten bulb). (b) Reciprocal plot of growth rates versus vitamin $B_{12}$ concentration and calculation of the half-saturation constants. Squares: chemotrophic (aerobic) growth rates. Circles: phototrophic (anaerobic) growth rates.

zimidazole (DMB) were provided together with cobinamide or in the presence of the *bluE* expression plasmid pAHW25 (Fig. 1), suggesting that *bluF* is not a phosphoribosyltransferase.

The fact that the genes *bluF* and *-E* carried by plasmid pAHU2 are sufficient for complementation of the mutant BF1 also indicates that the genes *bluD*, *-C*, and *-B* are expressed in the mutant and therefore must be transcribed by a second promoter (*bluD* promoter) which can be localized in the *bluE* gene by combining these results with earlier data (8). The *bluF* promoter, which transcribes at least the *bluF* and *-E* genes (8), was localized more exactly to a region of 74 bp upstream of the *bluF* gene by using PCR and *lacZ* fusions (Fig. 1b).

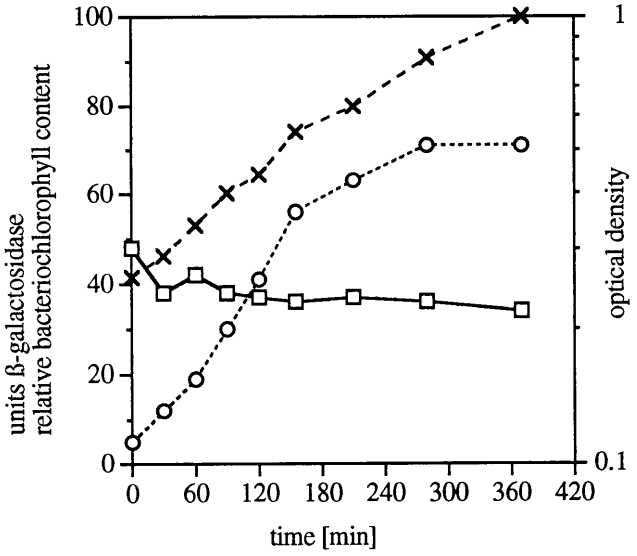


FIG. 3. β-galactosidase activity (squares), relative bacteriochlorophyll content (circles), and optical density (crosses) of wild-type strain 37b4 containing a *bluE:lacZ* fusion in plasmid pAHU4 after reduction of the oxygen partial pressure at time 0. All datum points represent the average values of three independent experiments.

**Vitamin $B_{12}$ requirement of the mutant strain BF1.** The vitamin $B_{12}$ auxotroph mutant strain BF1 was used to determine the vitamin $B_{12}$ requirement under chemotrophic (aerobic) and phototrophic (anaerobic) growth conditions. The doubling times of cultures with different vitamin $B_{12}$ concentrations were calculated from the optical densities measured in the first half of logarithmic growth (optical density at 600 nm = 0.1 to 0.3). From these doubling times the relative growth rates of the mutant strain in relation to the growth rate of the wild-type strain were calculated. The hyperbolic saturation curves which follow the Monod kinetics (7) are shown in Fig. 2a. The half-saturation constant ($K_S$) for vitamin $B_{12}$ is 3.5-fold higher under phototrophic growth conditions than under chemotrophic growth conditions ($K_S$ = 0.30 μg/liter versus 0.09 μg/liter; Fig. 2b). Complementation of the mutant strain BF1 with plasmid pAHU2 (Fig. 1) resulted in doubling times similar to those of the wild-type strain 37b4 (chemotrophic, 150 min; phototrophic, 350 min).

**Regulation of the *bluF* promoter.** In order to determine whether the elevated vitamin $B_{12}$ requirement during phototrophic growth is correlated to that in an increased transcription of the *blu* genes, we measured the β-galactosidase activities of the *bluE:lacZ* reporter plasmid pAHU4 in wild-type strain 37b4 under different growth conditions. The procedures for the β-galactosidase assay and for measurements of bacteriochlorophyll are described elsewhere (8). The β-galactosidase activity during phototrophic growth (50 ± 2 U) was even slightly decreased when compared to that in an aerobically grown culture (58 ± 4 U). Maximal rates of bacteriochlorophyll synthesis are required when aerobic cultures (20% oxygen) of *R. capsulatus* which contain only small amounts of photosynthetic complexes are shifted to growth under low oxygen tension (1 to 2% oxygen). When we followed the β-galactosidase activity during such a transition experiment, we did not find a significant influence of the oxygen tension on the *bluF* expression despite the strong increase in the bacteriochlorophyll content (Fig. 3). The average β-galactosidase activity during growth under low oxygen was 48 ± 3 U, with slightly decreased levels when cultures reached stationary growth phase.

We also measured the β-galactosidase activities of the *bluE:lacZ* reporter plasmid pAHU4 in the mutant strains BF1 (interposon in *bluF*), BB1 (interposon in *bluB*), and AH2 (transposon in *bluE*) and in wild-type strain 37b4 after addition of vitamin $B_{12}$, DMB, or cobinamide. Addition of up to 70 nM vitamin $B_{12}$, up to 290 nM cobinamide, or up to 140 μM DMB to the growth medium resulted in no change in the β-galactosidase activities (under aerobic growth conditions: 60 ± 2 U for cobinamide, 62 ± 3 U for DMB, 62 ± 2 U for vitamin $B_{12}$, 58 ± 2 U for control without addition of any compounds). This shows that the *bluF* promoter is not a target for feedback regulation by any of these compounds. The β-galactosidase activity expressed from plasmid pAHU4 under low oxygen tension showed no significant differences for the wild-type strain (50 ± 2 U) and the mutants BF1 (41 ± 3 U), AH2 (45 ± 2 U), and BB1 (44 ± 4 U), supporting this assumption.

The correlation between cobalamin synthesis and the formation of the photosynthetic apparatus is not yet known. The only known cobalamin-dependent enzyme in *Rhodobacter sphaeroides* is the methionine synthase (2). Methionine is required for the synthesis of *S*-adenosylmethionine, which serves as a methyl donor in the syntheses of bacteriochlorophyll (1, 5) and carotenoids (9, 10) of *R. sphaeroides* and *R. capsulatus*. This indirect involvement of cobalamin in the methylation of photosynthetic pigments could explain the increased vitamin $B_{12}$ requirement under phototrophic conditions.

This work was supported by the Deutsche Forschungsgemeinschaft (SFB272) and by the Fonds der Chemischen Industrie.

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