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EXPERIMENTAL ARTICLES

The Aeration-Dependent Effect of Vitamin B_{12} on DNA Biosynthesis in *Methylobacterium dichloromethanicum*

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Abstract—The effect of vitamin B_{12} (cobalamin) on DNA biosynthesis in *Methylobacterium dichloromethanicum* was studied. When cultivated in media with methanol or dichloromethane, the bacterium produced approximately 10 μg corrinoids per gram dry biomass, compared to about 7 μg/g when cultivated on ethanol or succinate. Exogenous adenosylcobalamin (AdoCbl) stimulated DNA biosynthesis in *M. dichloromethanicum* cells grown under poor aeration, the effect being mediated by AdoCbl-dependent ribonucleotide reductase. In vitro studies showed that *M. dichloromethanicum* also has AdoCbl-independent ribonucleotide reductase. Under good aeration, exogenous AdoCbl had no effect on DNA biosynthesis, while hydroxyurea suppressed it. These data suggest that AdoCbl-independent ribonucleotide reductase, which is likely to be activated by oxygen, plays an important part in DNA biosynthesis when *M. dichloromethanicum* is cultured with good aeration, whereas AdoCbl-dependent ribonucleotide reductase is active under conditions of poor aeration.

Key words: *Methylobacterium dichloromethanicum*, ribonucleotide reductase, vitamin B_{12}, adenosylcobalamin, DNA biosynthesis.

Methylcobalamin and adenosylcobalamin (AdoCbl), the coenzyme forms of cobalamin (vitamin B_{12}), are involved in many biochemical reactions [1]. Of great significance is AdoCbl-dependent ribonucleotide reductase, an enzyme which is responsible for the conversion of four ribonucleotides into the respective deoxyribonucleotides (the first rate-limiting step of DNA synthesis). In organisms with AdoCbl-dependent ribonucleotide reductase, this enzyme mediates the action of vitamin B_{12} on the formation rate of DNA and its content in cells (the copy number of bacterial chromosomes) [2].

AdoCbl-dependent ribonucleotide reductase is widespread in prokaryotes, which actually may contain several forms of ribonucleotide reductase differing in the coenzyme, mechanisms of free-radical catalysis, and reaction to oxygen [1–5]. An organism may contain either one or two and more forms of ribonucleotide reductase [4, 6–9].

The restricted facultative methylotroph *Methylobacterium dichloromethanicum* is able to degrade the highly toxic solvent dichloromethane, which often contaminates the environment [10]. However, the methylotrophic transformants that express the structural dichloromethane dehalogenase gene *dcmA* are unable to grow on dichloromethane because of the DNA-alkylating action of the intermediates of dichloromethane oxidation. Kayser and Vuilleumier suggested that the DNA of dichloromethane-utilizing bacteria is protected from alkylation by enzymes involved in the synthesis and repair of DNA [11]. Some species of the genus *Methylobacterium* are active producers of vitamin B_{12} [12]. The strain *Methylobacterium extorquens* AM1 has a cobalamin-linked enzyme that is involved in the isocitrate lyase–negative variant of the serine pathway of C_1-assimilation [13, 14]. Some members of the genus *Methylobacterium* are phytosymbionts and can grow under microaerobic conditions [12]. For this reason, it is reasonable to suggest that *M. dichloromethanicum* may have an AdoCbl-dependent ribonucleotide reductase. In the propionibacteria that have this enzyme, vitamin B_{12} controls DNA biosynthesis [15, 16]. It remains so far unclear whether or not this is also characteristic of methylotrophic bacteria.

The aim of this work was to study the synthesis of vitamin B_{12} in *M. dichloromethanicum*, the involvement of this vitamin (through AdoCbl-dependent ribonucleotide reductase) in DNA biosynthesis, and the ability of this bacterium to synthesize DNA without cobalamin.

MATERIALS AND METHODS

The bacterium *Methylobacterium dichloromethanicum* DM4 (=VKM B-2191 = DSM 6343) [12] was

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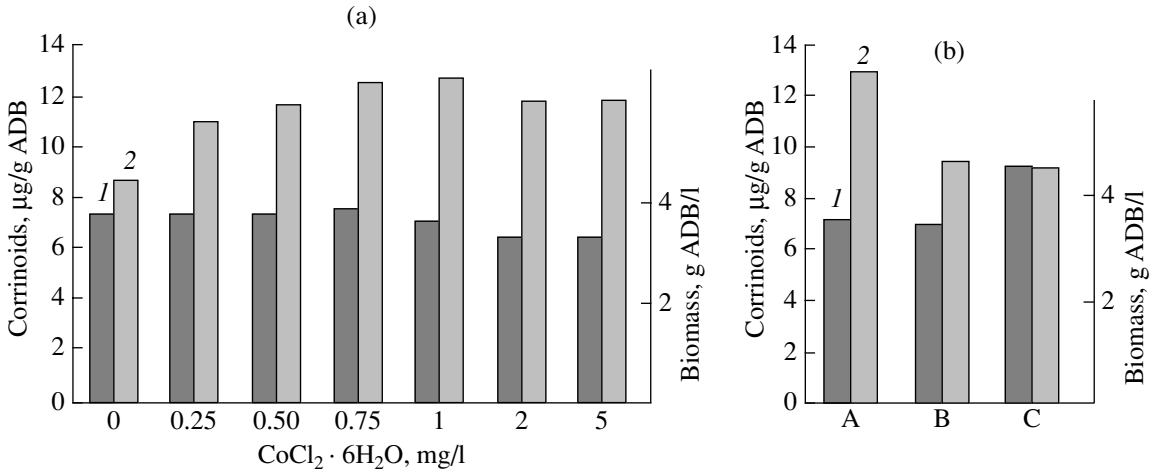

Fig. 1. Biomass yield and corrinoid synthesis in *M. dichloromethanicum* DM4 grown in (a) the medium with 50 mM methanol and different concentrations of cobalt ions and in (b) the medium with 1 mg/ml $CoCl_2 \cdot 6H_2O$ and different carbon sources (A, 50 mM methanol; B, 50 mM ethanol; and C, 50 mM sodium succinate): (*1*) biomass yield, g ADB/l; (*2*) corrinoid content in actively growing cells, μg/g ADB.

grown on a shaker (300 rpm) at 28°C for 48 h in 750-ml flasks with 200 ml of a medium containing (g/l) KH_2PO_4, 2.0; $(NH_4)_2SO_4$, 2.0; NaCl, 0.5; $MgSO_4 \cdot 7H_2O$, 0.125; $FeSO_4 \cdot 7H_2O$, 0.002; and methanol, 50 mM (pH 7.3). In experiments on DNA biosynthesis, cultivation flasks were 250 ml in volume, contained 100 ml of the medium, and were shaken either at 300 rpm (high aeration rate) or at 125 rpm (low aeration rate). Cultivation on dichloromethane was carried out as described earlier [10]. Bacterial growth was evaluated by absolutely dry biomass (ADB, the mass of bacterial cells dried to constant weight at 105°C). The total content of corrinoids was estimated microbiologically with *Escherichia coli* 113-3 as the test culture [16].

To assay ribonucleotide reductase, cells were washed and resuspended in 50 mM potassium phosphate buffer (pH 7.0; 0°C) containing 1 mM dithiothreitol (DTT) and 1 mM Na_4EDTA in a proportion of 1 : 2 (wet weight of cells to buffer volume). The cell suspension was sonicated at 0°C with a UZDN-2T ultrasonic disintegrator (22 kHz; 40 mA) for a total of 5 min in 30-s bursts with 2-min breaks. The cell homogenate was centrifuged at 30000 *g* for 30 min (6°C) to remove cell debris and dialyzed against the phosphate buffer. Ribonucleotide reductase was assayed in a reaction mixture (0.2 ml) containing 0.5–4 mM ADP (Reanal), 25 mM DTT, and cell extract in an amount of 4–6 mg protein/ml. The concentrations of other components in the reaction mixture were varied in the particular experiments. The reaction mixture was incubated at 37°C for 15 min. The reaction product deoxyribose was determined with the diphenylamine reagent [17] or by the radiochromatographic method with a (4.6 × 250 mm) HPLC column packed with Altex Ultrasphere ODS C18 (5 μm). The mobile phase was 50 mM K_2HPO_4 (pH 4.5) with 10 vol % methanol [4].

The DNA synthesis rate was evaluated by the incorporation of [8-^{14}C]adenine (Isotop, Russia) into the alkali-stable acid-insoluble fraction of cells [15]. The specific radioactivity of labeled adenine was 10.2×10^9 Bq/g. Labeled and unlabeled adenines were added to a cell suspension at concentrations of 74×10^3 Bq/ml and 5 μg/ml, respectively. The suspension was prepared by suspending the washed exponential-phase cells (1.5 to 2 mg ADB/ml) in the 0.05 M phosphate buffer with 0.125 vol % methanol. The incubation temperature was 30°C.

Protein was quantified by the method of Lowry *et al.*

RESULTS AND DISCUSSION

The presence of cobalt, which is a constituent of corrinoids, in the medium is a necessary condition of corrinoid biosynthesis. In the medium with methanol or dichloromethane that was not enriched in cobalt ions intentionally, *M. dichloromethanicum* produced approximately 10 μg corrinoids/g ADB. The addition of cobalt ions to the methanol-containing medium at concentrations up to 1 mg/l led to an increase in the content of corrinoids in cells (Fig. 1a), although the growth of *M. dichloromethanicum* remained almost unchanged. In the media with ethanol and succinate, the amount of synthesized corrinoids was about 30% smaller than in the media with methanol (Fig. 1b). Dichloromethane provided for approximately the same synthesis of corrinoids as methanol. In general, the level of corrinoid synthesis in *M. dichloromethanicum* is not high as compared to other prokaryotes (such as *Propionibacterium freudenreichii, Streptomyces olivaceus, Methanosarcina barkeri*, and cyanobacteria), which synthesize one to two orders more corrinoids and for which a stim-

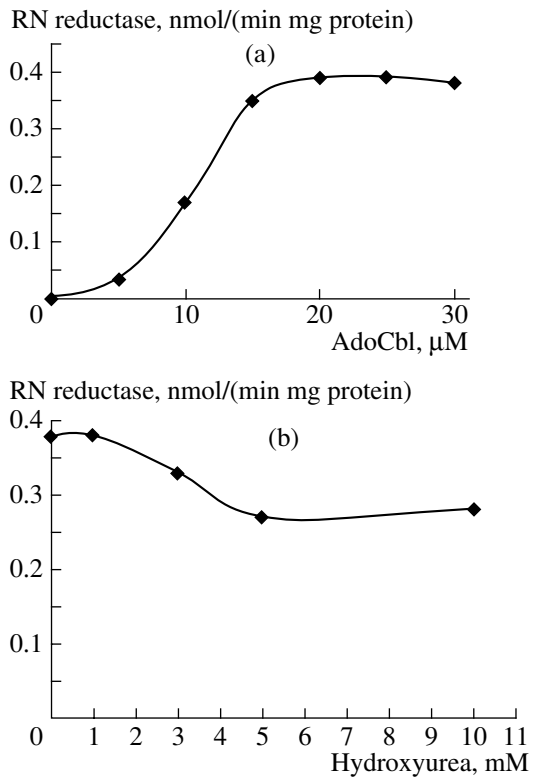

Fig. 2. The effect of (a) AdoCbl and (b) hydroxyurea on the activity of ribonucleotide reductase (RN reductase) in *M. dichloromethanicum* cells. The reaction mixture had pH 7.0 and contained 2 mM ADP. The concentration of AdoCbl in the experiments on the effect of hydroxyurea (panel b) was 20 μM.

ulating action of cobalt ions on corrinoid synthesis and sometimes on growth is typical [1].

Thus, like other methylobacteria, *M. dichloromethanicum* synthesizes corrinoids. In many prokaryotes, cobalamin is involved in DNA biosynthesis at the level of ribonucleotide reductase, which produces the deoxyribosyl precursors of DNA. The wide distribution of the class II AdoCbl-dependent ribonucleotide reductase in prokaryotes is due to the oxygen tolerance of this enzyme. The cobalamin synthesis or auxotrophy of an organism is a necessary, but not a sufficient, condition of the synthesis of AdoCbl-dependent ribonucleotide reductase. Irrespective of whether obligate aerobes can synthesize corrinoids or not, they may contain a ribonucleotide reductase from class I, II, or IV. The ribonucleotide reductases of classes I and IV are activated by molecular oxygen (hence, they are also called aerobic-type ribonucleotide reductases) and contain a protein subunit with nonheme Fe or Mn [1, 3, 5]. The occurrence of AdoCbl-dependent ribonucleotide reductase in an organism can easily be demonstrated by the enhancement of ribonucleotide reductase activity with AdoCbl.

Experiments showed that exogenous AdoCbl at concentrations of 20 μM or lower stimulated ribonucleotide reductase activity in the cell extract of *M. dichloromethanicum* (Fig. 2a). Such an effect of exogenous AdoCbl is typical of most of the cobalamin-dependent ribonucleotide reductases studied earlier [1, 3].

The cobalamin-linked ribonucleotide reductase was relatively tolerant to hydroxyurea at concentrations up to 10 mM (Fig. 2b). It should be noted that hydroxyurea acts as a radical scavenger and, hence, inhibits the aerobic-type ribonucleotide reductases of classes I and IV but that it has no effect on AdoCbl-dependent ribonucleotide reductases [3].

The activity of AdoCbl-dependent ribonucleotide reductase in *M. dichloromethanicum*, estimated by the rate of ADP reduction to deoxy-ADP, was proportional to the incubation time within the interval of 5–20 min and to the protein concentration within the range of 4–8 mg protein/ml. The optimum values of pH, temperature, and ADP concentration for the AdoCbl-dependent ribonucleotide reductase were 7.0, 37°C, and 2 mM, respectively. The positive effector deoxy-GTP at a concentration of 2 mM stimulated this enzyme by about 1.5 times. Mg^{2+} ions at concentrations higher than 2 mM inhibited ribonucleotide reductase activity.

Further studies showed that *M. dichloromethanicum* might also possess an alternative, AdoCbl-independent, ribonucleotide reductase, as is evident from the 70% inhibition of the ribonucleotide reductase activity of the cell extract by hydroxyurea. The optimum pH for this activity was 8.0. The activity was stimulated by Ca^{2+} and Mg^{2+} ions (Fig. 3).

Thus, like *P. freudenreichii* [6–8], *M. dichloromethanicum* DM4 probably contains two, AdoCbl-dependent and AdoCbl-independent, ribonucleotide reductases. The AdoCbl-independent ribonucleotide reductase seems to be activated by oxygen and to contain metal ions.

The presence of several ribonucleotide reductases in cells was also shown for bacteria of the genera *Pseudomonas* [9], *Streptomyces* [18], and others, which contain a class I metal-activated aerobic-type ribonucleotide reductase and a class II AdoCbl-dependent anaerobic-type (specifically, independent of oxygen) ribonucleotide reductase.

Further experiments were aimed at determining the contribution of each of the enzymes to DNA biosynthesis, whose intensity was estimated by the rate of incorporation of labeled adenine into DNA. Bearing in mind that the most significant difference between the ribonucleotide reductases of classes I and IV and the enzymes of class II is their reaction to oxygen, DNA synthesis was studied in *M. dichloromethanicum* cells grown under different aeration conditions.

The incorporation of [^{14}C]adenine into the DNA of bacterial cells grown under poor aeration was relatively slow but greatly increased when AdoCbl was added to the cell suspension at concentrations of 0.5–1.5 μg/ml (Fig. 4a). The same concentrations of AdoCbl exerted no effect on the incorporation of labeled adenine into

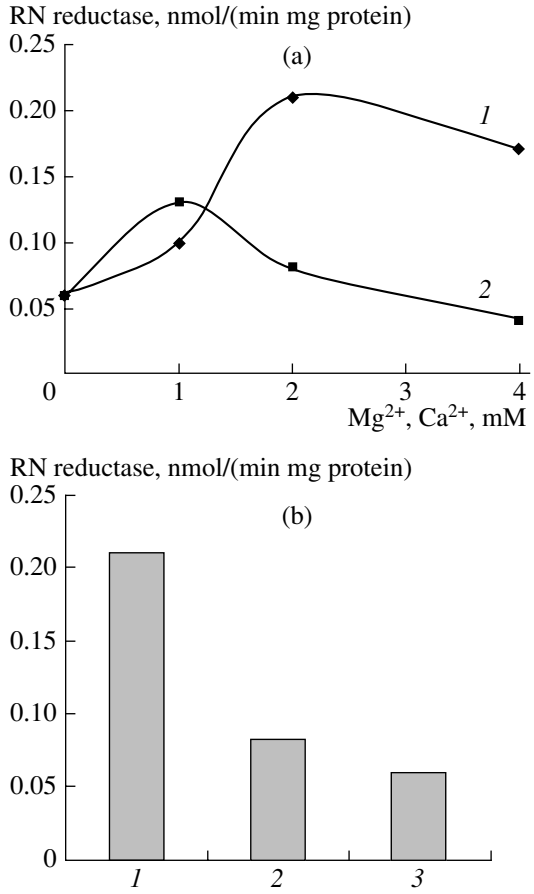

Fig. 3. The effect of (a) bivalent cations (curve *1*, Mg^{2+}; curve *2*, Ca^{2+}) and (b) hydroxyurea on the activity of ribonucleotide reductase (RN reductase) in *M. dichloromethanicum* cells. The reaction mixture had pH 8.0 and contained 2 mM ADP. The effect of hydroxyurea (panel b) was studied in the presence of 2 mM Ca^{2+} ions in the medium. The concentrations of hydroxyurea were 0, 5, and 10 mM (bars *1*, *2*, and *3*, respectively).

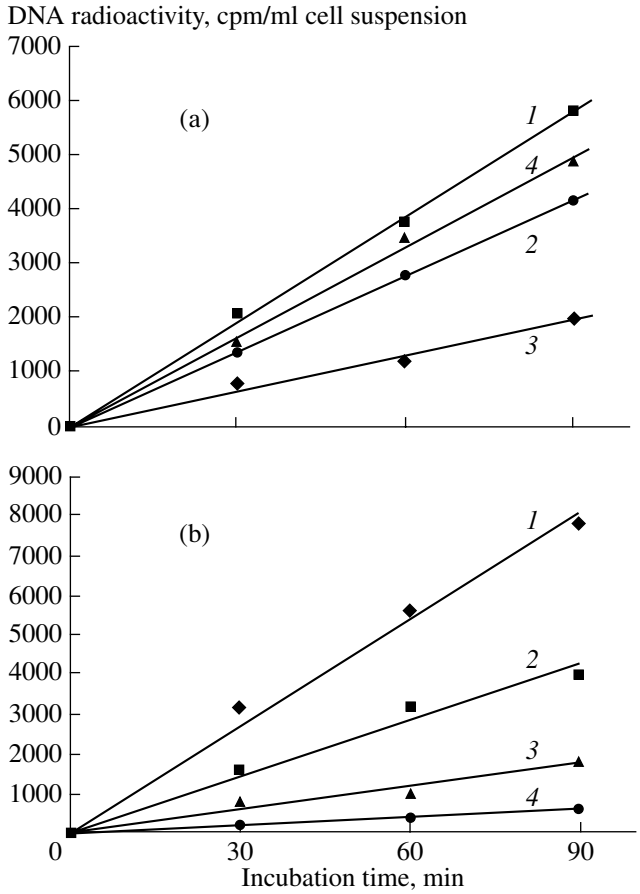

Fig. 4. (a) The effect of AdoCbl on the incorporation of labeled adenine into the DNA of *M. dichloromethanicum* DM4 cells grown under poor aeration. The concentrations of AdoCbl were 0, 0.5, 1, and 1.5 μg/ml (curves *1, 2, 3*, and *4*, respectively). (b) The effect of hydroxyurea on the incorporation of labeled adenine into the DNA of *M. dichloromethanicum* DM4 cells grown under good aeration. The concentrations of hydroxyurea were 0, 25, 50, and 100 mM (curves *1, 2, 3*, and *4*, respectively).

the DNA of cells grown under good aeration. In these cells, hydroxyurea inhibited DNA synthesis (Fig. 4b). Such effects are typical of bacteria possessing an aerobic-type metal-containing ribonucleotide reductase [3].

The data obtained suggest that the AdoCbl-dependent ribonucleotide reductase is involved in DNA biosynthesis in *M. dichloromethanicum* cells grown under oxygen deficiency, whereas the alternative aerobic-type, probably metal-containing, ribonucleotide reductase operates under conditions of good aeration. The presence of two or more alternative ribonucleotide reductases in a bacterium improves its adaptive capability, which also depends on the availability of cobalamin (necessary for AdoCbl-dependent ribonucleotide reductases) and oxygen (necessary for aerobic-type ribonucleotide reductases). For instance, *P. freudenreichii* cells grown in a cobalt-free medium under cobalamin deficiency do not contain AdoCbl-dependent ribonucleotide reductase and synthesize DNA at the expense of Mg-containing ribonucleotide reductase [8, 16]. The facultative anaerobe *Paracoccus denitrificans* synthesizes DNA due to the activity of a class II ribonucleotide reductase under anaerobic conditions and due to the activity of an AdoCbl-independent enzyme under aerobic conditions [19]. The anaerobic bacterium *Bacteroides fragilis* is able to synthesize an aerobic-type ribonucleotide reductase of class I, which enhances bacterial survival in the presence of air [20].

To conclude, the methylotrophic bacterium *M. dichloromethanicum* DM4 synthesizes cobalamin, which is involved in DNA biosynthesis as a constituent of AdoCbl-dependent ribonucleotide reductase. Under certain conditions, DNA biosynthesis in the methylotroph is limited by cobalamin. The bacterium also contains cobalamin-independent ribonucleotide reductase, which is involved in DNA biosynthesis under the conditions of intense aeration of the culture.

M. dichloromethanicum is an active degrader of the toxic compound dichloromethane. The putative intermediate of dichloromethane oxidation (*S*-chloromethylglutathione) alkylates DNA bases, breaking DNA molecules [11]. It can be suggested that the presence of two different ribonucleotide reductases enhances the genome stability in *M. dichloromethanicum.* On the other hand, many methylobacteria are believed to be able to form associations with plants, as they have been detected not only on the plant surface but also inside plant tissues [12], where conditions are microaerobic. It is tempting to suggest that the AdoCbl-dependent ribonucleotide reductase may provide for the life of endophytosymbiotic methylobacteria under oxygen deficiency. The enhanced synthesis of cobalamin during the methylotrophic growth of *M. dichloromethanicum* is of interest from the standpoint of its involvement in dichloromethane metabolism.

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