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Alternate Requirement for Vitamin B_{12} or Methionine in Mutants of *Pseudomonas denitrificans*, a Vitamin B_{12}-producing Bacterium

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Experiments are described which indicate that *Pseudomonas denitrificans*, an organism that overproduces vitamin B_{12}, uses the B_{12} pathway exclusively for methionine synthesis.

Extensive research on the formation of methionine has implicated vitamin B_{12} as a cofactor in the final reaction of the biosynthetic sequence. Since plants and fungi do not produce or require B_{12}, an alternate reaction must also exist. It is thus not surprising that *Escherichia coli* has been found to possess two mechanisms for the methylation of homocysteine to methionine (9). These reactions are mediated by two enzymes which differ with respect to the folate derivative required for activity. Only one of these enzymes requires vitamin B_{12} for activity. *E. coli* cannot make significant levels of vitamin B_{12} and, therefore, uses the non-B_{12} reaction when grown in the absence of the vitamin. When B_{12} is added, the holoenzyme (cobamide-methionine synthetase) is formed from the apoenzyme and methionine can be produced by the B_{12}-dependent reaction. The isolation of *E. coli* mutants with an alternative requirement for B_{12} or methionine is explained by a genetic block of the non-B_{12} reaction, which results in a total dependence on exogenous B_{12} for methionine synthesis. Similar mutants have been obtained from the related organism, *Salmonella tryphimurium* (8). Although wild-type *S. typhimurium* produces detectable levels of B_{12}, it is thought that the amount synthesized is too low to allow growth of the mutants in the absence of exogenous B_{12} or methionine (3). Another related organism, *Aerobacter aerogenes*, has been found to possess both pathways of methionine synthesis (7). To our knowledge, no B_{12}/methionine auxotrophs have been isolated; this correlates with the known ability of *A. aerogenes* to make B_{12} (9).

Although mammalian cells appear to use exclusively a B_{12} pathway for methionine synthesis (6), the existence of bacteria that produce methionine by this system *alone* is a possibility left unanswered by the above studies. A mutation to the alternate methionine or B_{12} requirement in a species which normally produces B_{12} would indicate that the organism possesses only the B_{12} pathway for methionine production. Although

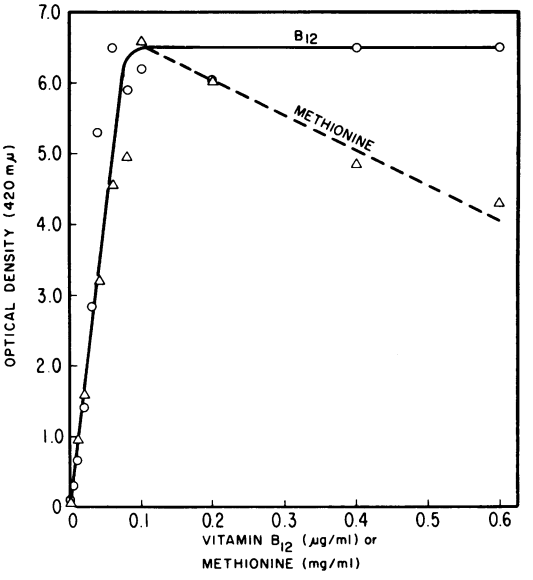

FIG. 1. *Growth response of Pseudomonas denitrificans MB-2196 to increasing concentrations of* L-*methionine and vitamin* B_{12}. *The defined medium used at 20 ml per 250-ml Erlenmeyer flask contained 2% sucrose, 0.2% sodium-*L-*glutamate, 0.2% sodium citrate, 0.2%* $(NH_4)_2HPO_4$, *0.1%* $MgSO_4 \cdot 7H_2O$, *0.08% KCl, 0.02%* $MnSO_4 \cdot H_2O$, *0.005%* $Co(NO_3)_2 \cdot 6H_2O$, *0.005%* $Na_2MoO_4 \cdot 2H_2O$, *0.003%* $ZnSO_4 \cdot 7H_2O$, *and 0.002%* $FeSO_4 \cdot 7H_2O$ *(pH 6.6 before autoclaving). Growth was determined in a Klett-Summerson colorimeter with a no. 42 filter after 88 hr of incubation at 28 C on a rotary shaker (250 rev/min).*

B_{12}/methionine mutants have been reported in *Proteus mirabilis* (5) and in *Actinomyces spheroides* (2), no information is available on the capacity for B_{12} synthesis in the prototrophic parents. Similarly, strains of *Bacillus stearothermophilus* and *B. coagulans* require methionine or B_{12} (1) but, again, no information is available on the ability of closely related strains lacking this requirement to produce B_{12}. The experiments described in the present paper indicate that *Pseudomonas denitrificans*, an organism that overproduces vitamin B_{12} (R. A. Long, U.S. Patent 3,018,225, 1962), uses the B_{12} pathway exclusively for methionine synthesis. We have found that certain auxotrophs that fail to grow on homocysteine respond to either methionine or B_{12}. Accompanying this auxotrophic mutation is loss of ability to produce the vitamin.

Mutants of *P. denitrificans* were obtained after treatment with *N*-methyl-*N*-nitroso-*N'*-nitroguanidine. Two types of methionine-requiring mutants unable to grow on homocysteine were obtained. Nine (type I) mutants respond only to methionine and presumably contain a defective methionine synthetase. Twelve (type II) mutants respond to vitamin B_{12} or to methionine; they are apparently unable to provide the B_{12} required for methionine synthetase activity. Not all of these mutants were stable; many reverted to the original prototrophic state too frequently for accurate physiological determinations. Two stable mutants, one of type I (MB-2202) and one of type II (MB-2196), were selected for further study.

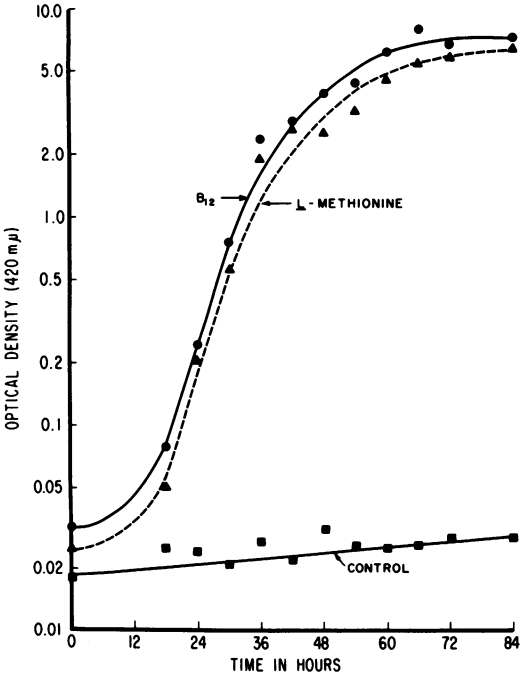

FIG. 2. *Growth of Pseudomonas denitrificans MB-2196 in chemically defined medium alone or with the addition of 0.1 mg of L-methionine per ml or 0.07 μg of vitamin B_{12} per ml. The optical densities of the parent (prototrophic) culture were 4.90, 4.25, and 3.75, respectively. The composition of the medium is described in the legend for Fig. 1.*

Figure 1 shows that 0.04 mg of L-methionine per ml and 0.04 μg of vitamin B_{12} per ml support half-maximal growth of strain MB-2196. Growth on L-methionine cannot be attributed to presence of contaminating vitamin B_{12} in the methionine; no vitamin B_{12} was detected by bioassay with *Lactobacillus lactis* Dorner in a solution of L-methionine (Sigma Chemical Co.) at four times the concentration required for optimal growth of the *P. denitrificans* mutant. Figure 1 also shows that excessive levels of methionine, but not of B_{12}, are inhibitory.

That vitamin B_{12} is a totally effective substitute for L-methionine for growth of type II mutants was shown by measuring the rate of growth of strain MB-2196 at optimal concentrations of these two supplements. The rate of growth of MB-2196 at optimal supplement concentration is identical on L-methionine and vitamin B_{12} (Fig. 2).

TABLE 1. *Vitamin B_{12} production by methionine-requiring mutants and prototrophic Pseudomonas denitrificans*[a]

Strain	Growth requirement	Vitamin B_{12} produced	Dry wt
		μg/ml	*mg/ml*
MB-2196	B_{12}/methionine	0	1.6
(type II)		0	1.6
MB-2202	Methionine	14.7	1.6
(type I)		14.3	1.5
Parent	(Prototrophic)	13.3	1.6
		13.7	1.6

[a] Cultures were grown in duplicate in 250-ml Erlenmeyer flasks containing 40 ml of a medium which contained 3% sucrose, 1% betaine, 0.5% sodium glutamate, 0.5% $(NH_4)_2HPO_4$, 0.1% $MgSO_4 \cdot 7H_2O$, 0.09% KCl, 0.01% L-methionine, 0.003% $FeSO_4 \cdot 7H_2O$, 0.0025% dimethylbenzimidazole, 0.002% $MnSO_4 \cdot H_2O$, 0.002% $ZnSO_4 \cdot 7H_2O$, 0.0016% $Co(NO_3)_2 \cdot 6H_2O$, and 0.0002% $Na_2MoO_4 \cdot 2H_2O$ (*p*H 6.8 before autoclaving). Fermentation was done at 28 C with rotary shaking for 5 days. Vitamin B_{12} was determined by an agar-diffusion assay with *Lactobacillus lactis* Dorner ATCC 10697; vitamin B_{12} was used as standard. Whole broth samples were prepared for assay by boiling for 3 min with 2.25% $NaNO_2$-0.01% KCN at *p*H 3.0 to 4.0 (H_2SO_4). At the end of the fermentation, cultures MB-2196 and MB-2202 contained only 1.9 and 1.8 prototrophs/10^7 cells, respectively.

Vitamin B_{12} production by type I and type II mutants in a defined medium containing methionine is shown in Table 1. Strain MB-2196, as expected, makes no vitamin B_{12}, whereas MB-2202 makes the same amount as the parent strain.

Mutants that show a requirement for methionine as a consequence of a block in vitamin B_{12} synthesis can occur only if the single significant pathway for methionine synthesis in the parent requires vitamin B_{12} as a cofactor. It is clear that our culture of *P. denitrificans*, which was selected for ability to produce high levels of this vitamin, synthesizes methionine only by the B_{12}-mediated mechanism. It cannot be concluded that the species, as it occurs in nature, has only one pathway for methionine synthesis. The abolition of the alternate pathway could have occurred during selection for vitamin production. However, this is rendered unlikely by the recent data of Cauthen et al. (4), suggesting that *Rhodopseudomonas spheroides* also possesses exclusively the B_{12} pathway for methionine biosynthesis.

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