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Levels of Water-Soluble Vitamins in Methanogenic and Non-Methanogenic Bacteria

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The levels of seven water-soluble vitamins in *Methanobacterium thermoautotrophicum, Methanococcus voltae, Escherichia coli, Bacillus subtilis, Pseudomonas fluorescens*, and *Bacteroides thetaiotaomicron* were compared by using a vitamin-requiring *Leuconostoc* strain. Both methanogens contained levels of folic acid and pantothenic acid which were approximately two orders of magnitude lower than levels in the nonmethanogens. *Methanobacterium thermoautotrophicum* contained levels of thiamine, biotin, nicotinic acid, and pyridoxine which were approximately one order of magnitude lower than levels in the nonmethanogens. The thiamine level in *Methanococcus voltae* was approximately one order of magnitude lower than levels in the nonmethanogens. Only the levels of riboflavin (and nicotinic acid and pyridoxine in *Methanococcus voltae*) were approximately equal in the methanogens and nonmethanogens. Folic acid may have been present in extracts of methanogens merely as a precursor, by-product, or hydrolysis product of methanopterin.

Methanogenic bacteria, members of the phylogenetically distinct archaebacteria (1, 9, 10, 24, 25), possess a novel biochemistry which is characterized partly by the presence of several novel vitamins and coenzymes which, with one exception (7), have not yet been observed in other groups of organisms (12, 26). Among these coenzymes are coenzyme M (3), the nickel tetrapyrrole F_{430} (16), the deazaflavin F_{420} (5, 6), and methanopterin (12), all of which are probably involved in the pathway of methanogenesis. The mechanism of CO_2 fixation into cell carbon in methanogens is different from any known pathway (1, 26) and could, like the pathway of methane production, involve novel coenzymes rather than (or in addition to) familiar ones. Because of the biochemical distinctiveness of methanogens, it seems possible that novel coenzymes function throughout the metabolic pathways and that some coenzymes traditionally considered ubiquitous among organisms may be completely absent from, or at least less important in, methanogens. For example, methanopterin may function in the metabolism of methanogens to the exclusion of folic acid. In light of their phylogenetic distinctiveness, it would not be surprising if methanogens had evolved novel coenzyme forms besides those already discovered. Evidence that methanogens do contain some conventional vitamins has already accumulated. Thiamine, pyridoxine, and *p*-aminobenzoic acid are required for the growth of *Methanomicrobium mobile* (R. S. Tanner and R. S. Wolfe, Abstr. Annu. Meet. Am. Soc. Microbiol. 1982, I90, p. 109); pantothenic acid stimulates *Methanococcus voltae* (23); and riboflavin stimulates *Methanosarcina barkeri* (18). High levels of corrinoids have been observed in methanogenic bacteria (13), and flavin adenine dinucleotide (FAD) has been detected in *Methanobacterium bryantii* (14). Enzymatic reactions in methanogens have been shown to depend on coenzyme A (15, 28), NADP (5, 8, 11, 20, 21, 28), NAD (28), flavin mononucleotide (FMN) (5, 20, 21), and FAD (5, 20, 21); however, F_{420} has been shown to serve as a substrate with a higher affinity than that of FMN or FAD (5, 20). Nevertheless, the above evidence does not exclude the possibility that some familiar vitamins and coenzymes are lacking in methanogens. In this report, I extend these observations by comparing the levels of vitamins in representatives of two orders of methanogens (1) and in a variety of nonmethanogens.

MATERIALS AND METHODS

A heterolactic coccus isolated from raw sewage by F. Nano (*Leuconostoc* sp. strain L-1 [4]) was found to require seven vitamins for growth in a defined medium. It was maintained on a medium consisting of 1% Trypticase (BBL Microbiology Systems, Cockeysville, Md.), 0.5% yeast extract, 0.3% glucose, and 0.2% K_2HPO_4.

Media for cell growth and vitamin activity assays were prepared in acid-washed glassware. *Methanobacterium thermoautotrophicum* ΔH was grown on H_2 and CO_2 by the method of Balch et al. (1, 2), except

TABLE 1. Growth responses of *Leuconostoc* sp. strain L-1 to vitamin additions

Biotin		Folic acid		Riboflavin		Thiamine		Pantothenic acid		Nicotinic acid		Pyridoxine	
μg/liter	A_{660}	μg/liter	A_{660}	μg/liter	A_{660}	μg/liter	A_{660}	μg/liter	A_{660}	μg/liter	A_{660}	μg/liter	A_{660}
0	0.19	0	0.04	0	0.03	0	0	0	0	0	0.01	0	0
0.0005	0.23	0.025	0.18	0.062	0.05	0.062	0.02	6.25	0	6.25	0.08	12.5	0
0.005	0.33	0.25	0.67	0.62	0.13	0.62	0.16	62.5	0.96	62.5	0.38	125	0.08
0.05	0.86	2.5	0.87	6.2	0.94	6.2	1.0	625	1.2	625	1.1	1250	1.0
0.5	0.94[a]	25	1.0[a]	62	1.0[a]	62	1.0[a]	6250	1.0[a]	6250	1.0[a]	12500	1.0[a]

[a] Average of three tubes.

that vitamins were omitted. *Methanococcus voltae* PS was grown by the same method on H_2 and CO_2 with the defined medium of Whitman et al. (23) without pantothenic acid.

Bacteroides thetaiotaomicron 5428A (a gift from A. Salyers, University of Illinois, Urbana) was grown on glucose with the medium described by Varel and Bryant (22), except that the buffer was 0.1 M potassium phosphate and hemin was added after autoclaving as a filter-sterilized complex with histidine. The histidine-hemin complex was assayed and contained no significant vitamin levels. *Escherichia coli* UB1005 (a gift from J. Cronan, University of Illinois, Urbana), *Bacillus subtilis*, and *Pseudomonas fluorescens* were grown on medium consisting of 1% glucose, 0.1% NH_4Cl, 0.013% $MgSO_4$, 0.3% KH_2PO_4, 0.6% Na_2HPO_4, 1% vitamin-free Casamino Acids (Difco Laboratories, Detroit, Mich.), 0.02% tryptophan, 0.01% cystine, 0.02% methionine, and 20 ml of trace mineral solution per liter (27). Basal assay medium consisted of 0.5% KH_2PO_4, 0.5% K_2HPO_4, 1% vitamin-free Casamino Acids, 0.02% tryptophan, 0.01% cystine, 0.1% sodium acetate, 1% glucose, and 10 ml of a trace mineral solution (0.05% NaCl, 0.05% $FeSO_4$, 0.05% $MnSO_4$, 0.005% $MgSO_4$) per liter.

To prepare vitamin assays, 8-ml portions of basal assay medium were dispensed in Pyrex tubes (18 by 150 mm). To assay for a vitamin, each sample was added to a tube lacking that vitamin; all other vitamins were added in excess (highest concentrations listed in Table 1). A duplicate sample was added to a tube containing an excess of all vitamins as a test for possible inhibition of growth by factors in the sample. Positive control tubes contained an excess of all vitamins. Tubes were covered with closures and sterilized. An overnight culture of *Leuconostoc* sp. strain L-1 grown in maintenance medium was centrifuged, rinsed twice with 10-ml portions of sterile water, and diluted 100-fold with sterile water. One drop of inoculum was added to each assay tube. Tubes were incubated at 30°C for 48 h, and the absorbance at 660 nm (A_{660}) was measured in a spectrophotometer (Spectronic 20; Bausch & Lomb, Inc., Rochester, N.Y.).

Three types of cell extracts were prepared. (i) Cells were suspended in water (1 g of wet cells per ml) and incubated at 100°C for 20 min. Insoluble material was removed by centrifugation. (ii) To obtain acid-hydrolyzed extracts, cells were suspended in 6 N HCl (1 g of wet cells per ml) and incubated at 100°C for 2 h in sealed vials. Samples were evaporated to dryness, resuspended in the same volume of water, and centrifuged. (iii) Anaerobic alkaline hydrolysis of cell extracts was performed in 1 N NaOH at 100°C for 20 min (19). Vitamin hydrolysis was performed in 1 N (F_{420} and methanopterin) and 6 N (methanopterin) HCl at 100°C in sealed tubes for 30 min and 24 h (6). Samples were evaporated to dryness and dissolved in water.

Vitamins and coenzymes were obtained from Sigma Chemical Co., St. Louis, Mo.

RESULTS

Table 1 shows representative growth responses of *Leuconostoc* sp. strain L-1 to vitamins. Four vitamin concentrations spanning three orders of magnitude served to calibrate the assays. The vitamin activities of coenzymes (the active form of vitamins likely to be found in cell extracts) are shown in Table 2. NAD and NADP had no detectable activities; therefore, acid-hydrolyzed extracts were used for all nicotinic acid assays. All other coenzymes were approximately as active as their corresponding vitamin forms. Apparent differences between coenzyme and vitamin forms of FAD and coenzyme A can be attributed to the addition of equal microgram amounts rather than equimolar amounts.

Table 3 shows the vitamin levels found in methanogens and nonmethanogens. Although these figures cannot be considered precise, the trends observed were reproducible. None of the extracts inhibited growth. Vitamin levels among the four nonmethanogens were in approximate

TABLE 2. Vitamin activities of coenzymes in the growth assay

FMN		FAD		Thiamine pyrophosphate		Coenzyme A		NAD		NADP		Pyridoxal phosphate	
μg/liter	A_{660}	μg/liter	A_{660}	μg/liter	A_{660}	μg/liter	A_{660}	μg/liter	A_{660}	μg/liter	A_{660}	μg/liter	A_{660}
0	0.08	0	0.08	0	0.03	0	0.01	0	0.03	0	0.03	0	0.01
0.62	0.38	0.62	0.08	0.62	0.10	62	0.12	62	0.03	62	0.05	125	0.70
62	0.83	62	0.82	62	0.78	6,200	0.78	6,200	0.08	6,200	0.09	12,500	0.81

TABLE 3. Vitamin levels in bacterial cells

Organism	Vitamin level (μg/g [dry wt] of cells)[a]						
	Biotin	Folic acid	Riboflavin	Thiamine	Pantothenic acid	Nicotinic acid	Pyridoxine
E. coli	0.13	1.1	310	7.4	170	450	6,300
B. subtilis	0.11	1.1	36	5.8	210	1,530	2,200
P. fluorescens	0.72	3.6	31	7.3	300	220	5,400
B. thetaiotaomicron	ND[b]	0.72	54	3.6	405	270	9,000
M. thermoautotrophicum	0.036	0.0086	37	1.1	8.4	30	900
M. voltae	ND	0.023	40	1.6	1.4	220	6,100

[a] Values were calculated from the growth response information in Table 1 and on the assumption that 1 g of cells (dry weight) yielded 9 ml of extract. Most values are averages for two or more assays.
[b] ND, Not determined.

agreement, differing from one another by a factor of <10 in most cases. The only vitamin found in approximately equal levels in methanogens and nonmethanogens was riboflavin (and nicotinic acid and pyridoxine in *Methanococcus voltae*). In *Methanobacterium thermoautotrophicum*, the nicotinic acid level was lower by factors of 8 to 50 than levels found in the nonmethanogens, and the pyridoxine level was lower by factors of 2.5 to 10. The thiamine levels in both methanogens were lower than levels in the nonmethanogens by a factor of about 5. Pantothenic acid levels in *Methanobacterium thermoautotrophicum* and *Methanococcus voltae* were lower than levels in nonmethanogens by factors of 20 to 50 and 120 to 280, respectively. The biotin content of *Methanobacterium thermoautotrophicum* was lower than those of the nonmethanogens by factors of 3 to 20. Neither pure F_{420} nor hydrolyzed F_{420} (both 30-min and 24-h hydrolysates) possessed riboflavin activity when assayed at 6.2 mg/liter, and neither inhibited growth. Hydrolyzed F_{420} samples were prepared by a method which has been shown to yield a variety of fragments, including the riboflavin analog (6).

The folic acid level in *Methanobacterium thermoautotrophicum* was lower than those in the nonmethanogens by factors of 80 to 400, and the level in *Methanococcus voltae* was lower than those in the nonmethanogens by factors of 30 to 150. *Leuconostoc* sp. strain L-1 grew in response to reduced folates, since N^5-formyl-5,6,7,8-tetrahydrofolic acid (2.5 μg/liter) possessed saturating folic acid activity in the assay. The methanogen extracts did not contain polyglutamyl derivatives of folic acid: anaerobic alkaline hydrolysis did not increase folic acid activities in the extracts (19). Pterin, pterin-6-carboxylic acid, and *p*-aminobenzoic acid (all added at 6.2 mg/liter) did not substitute for folic acid in the growth assay and did not inhibit growth. End products of biosynthetic pathways requiring folic acid did not replace the folic acid requirement: growth was not supported by a combination of serine (0.2 mg/ml), methionine (0.2 mg/ml), thymine (0.05 mg/ml), thymidine (0.05 mg/ml), adenine (0.01 mg/ml), and guanine (0.01 mg/ml) (17). Growth was inhibited 20%. An acid-hydrolyzed extract from 0.1 g (wet weight) of *Methanococcus voltae* cells resulted in a final A_{660} value of 0.21, whereas in the presence of aminopterin (5 μg/liter), the same extract caused no growth (17). Pure methanopterin possessed no folic acid activity when assayed at 6.2 mg/liter and did not inhibit growth. Methanopterin hydrolyzed for 30 min in 1 or 6 N HCl possessed saturating folic acid activity at 6.2 mg/liter.

DISCUSSION

Despite the presence of F_{420} in methanogens, high levels of riboflavin were found, in agreement with an earlier observation of FAD in *Methanobacterium bryantii* (14). Methanopterin, on the other hand, may replace folic acid as the pterin of methanogens; the slight folic acid activity found in hydrolyzed methanopterin suggests that folic acid at the very low levels found in methanogen extracts is merely a precursor, by-product, or hydrolysis product of methanopterin. In any case, a major role for folic acid in methanogenesis seems unlikely. All other vitamins tested were also present in methanogen extracts, although many were present at relatively low levels. It will be interesting to see if those vitamins present at lower levels (e.g., pantothenic acid) have more abundant novel coenzyme counterparts, or if the metabolism of methanogens simply does not require higher concentrations of conventional coenzymes.

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