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Folic Acid and Pteroylpolyglutamate Contents of Archaebacteria

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Cell extracts of methanogens and the thermoacidophile *Sulfolobus solfataricus* contained little or no folic acid (pteroylglutamate) or pteroylpolyglutamate activity (<0.1 nmol/g [dry weight]). However, the halophile *Halobacterium salinarum* contained pteroylmono- or pteroyldiglutamates, and *Halobacterium volcanii* and *Halobacterium halobium* contained pteroyltriglutamates at levels equivalent to those in eubacteria (>1 nmol/g [dry weight]).

The archaebacteria, eubacteria, and eucaryotes differ from each other in numerous biological properties (16–18, 35), including coenzyme biochemistry. The methanogenic archaebacteria employ novel cofactors in the pathway of methane biosynthesis (36), and novel pterins and cofactor-like compounds are present in halophilic and thermoacidophilic archaebacteria (22–24, 30, 34). However, the familiar growth factors thiamine, riboflavin, nicotinic acid, pantothenate, pyridoxine, and biotin are found in methanogens (21); and the first five of these have been detected in halophiles and thermoacidophiles (K. M. Noll, personal communication). Methanogens contain extremely low levels of folic acid activity and tetrahydrofolate-dependent enzymes (9, 21). Traces of folate activity have been attributed to a hydrolytic product of methanopterin (a folate analog) which functions as a one-carbon carrier (8, 12, 20, 21, 33).

Folate is a generic term for forms of pteroylglutamic acid ($PteGlu_n$; $1 \leq n \leq 7$) which are central to one-carbon metabolism in eubacteria and eucaryotes (2, 3, 11, 19, 26, 31). A given organism mainly contains a single chain length (2, 11). The specificities of the bioassay strains and changes in folate content after treatment with conjugase (which releases the PteGlu nucleus from $PteGlu_n$) permit the identification of the folate moieties (10, 26, 31).

Here we provide evidence for pteroylpolyglutamates in halobacteria.

(A preliminary report of this study was presented previously [D. P. Nagle, Jr., V. E. Worrell, C. W. Jones, and R. Teal, Abstr. Annu. Meet. Am. Soc. Microbiol. 1987, I130, p. 194].)

Bacterial strains and media. The bacterial strains used in this study are listed in Table 1. The methanogens were grown essentially as described previously (1) in folate-free media from which vitamins, yeast extract, Casitone (Difco Laboratories, Detroit, Mich.), and Trypticase (BBL Microbiology Systems, Cockeysville, Md.) were omitted. *Methanococcus voltae* was supplemented with 0.5 g of leucine, 1.0 g of isoleucine, and 50 mg of calcium pantothenate per liter; 40 μM $NiCl_2$; and 1 μM sodium selenate. *Methanosarcina barkeri* was grown on methanol (medium 1043 [5]) with 1 g of vitamin-free Casamino Acids (Difco) per liter. *Sulfolobus solfataricus* was grown at 60° C in medium containing, per liter, 1.3 g of $(NH_4)_2SO_4$, 0.2 g of KH_2PO_4, 0.25 g of $MgSO_4 \cdot 7H_2O$, 76 mg of $CaCl_2 \cdot 2H_2O$, and 1.0 g of Casamino Acids (Difco) adjusted to pH 3.0. Halobacteria were grown at 43°C in medium containing, per liter, 7.6 g of vitamin-free Casamino Acids (Difco), 3 g of sodium citrate, 250 g of NaCl, 2 g of KCl, 20 g of $MgCl_2 \cdot 7H_2O$, 0.4 g of $FeSO_4 \cdot 7H_2O$, and 0.2 mg of $MnSO_4 \cdot H_2O$ (from medium 97 [4]). Eubacteria were grown in folic acid assay medium without folate (Difco). Pteroic acid was a gift from L. D'Ari (University of California, Berkeley), and methanopterin was from J. C. Escalante-Semerena and W. J. Jones (University of Illinois, Urbana). Acid hydrolysis of methanopterin was in 6 N HCl (1 h, 100°C).

Bioassay for folic acid. Cells were centrifuged and, except for the halophiles, washed by suspension in 0.9% NaCl and centrifuged. Pellets were heated (20 min, 100°C) in H_2O (1 to 2 ml per g of cells) and centrifuged. The folate content was reproducible among different extracts and stable on storage (−20°C). Extreme care was taken to avoid contamination of glassware and media with exogenous folate. Most media components were tested for folate content with *Streptococcus faecalis*, and in particular, vitamin-free Casamino Acids (Difco) contained less than 2 pmol of folic acid per g. *Streptococcus faecalis* and *Lactobacillus casei* were grown in folic acid assay medium and folic acid Casei medium (Difco), respectively (7, 10). All assays were done in triplicate. The A_{540} of cultures was read after 24 to 48 h. Unknown values were estimated from the linear portion of the folate standard curves, between 0.1 and 1 ng/ml for

TABLE 1. Bacterial strains used in this study

Organism	Strain no.[a]	Source[a]
Archaebacteria		
Methanobacterium formicicum	ATCC 33274	W. J. Jones
Methanobacterium thermoautotrophicum Marburg	DSM 2133	DSM, H. Hippe
Methanococcus voltae	DSM 1537	W. J. Jones
Methanosarcina barkeri	DSM 800	M. J. McInerney
Sulfolobus solfataricus	ATCC 35091	ATCC
Halobacterium volcanii	ATCC 29605	R. Gupta
Halobacterium salinarum	ATCC 19700	ATCC
Eubacteria		
Bacillus subtilis	OU 10093	K. Kealy
Lactobacillus casei	ATCC 7469	ATCC
Pseudomonas fluorescens	OU 10025	K. Kealy
Streptococcus faecalis (*faecium*)	ATCC 8043	ATCC

[a] Abbreviations: ATCC, American Type Culture Collection, Rockville, Md.; DSM, Deutsche Sammlung von Mikroorganismen, Göttingen, Federal Republic of Germany; OU, Department of Botany and Microbiology Culture Collection, University of Oklahoma, Norman, Okla.

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TABLE 2. Growth of indicator strains on pteridine compounds in the folate bioassays

Compound (concn [ng/ml])	Growth (A_{540}) of indicator organism	
	Streptococcus faecalis	*Lactobacillus casei*
Folic acid (0.4)	0.420	0.873
Folic acid (0.5)	0.457	
Folic acid (0.5)–trimethoprim (10)	0	
Thymidine (51)	1.5	
Thymidine (51)–trimethoprim (10)	1.3	
Pteroic acid (0.4)	0.232	0
Methanopterin (1,300)	0	0.048
Acid-hydrolyzed methanopterin (650)	0.032	0.176

Streptococcus faecalis (A_{540}, 0.09 to 0.97) and 0.01 and 0.4 ng/ml for *Lactobacillus casei* (A_{540}, 0.04 to 0.567). Unknowns with the lowest levels of folate yielded A_{540}s of less than 0.05, with considerable scatter among replicates. For every condition assayed, uninoculated sterile controls did not exhibit growth after 96 h. All unknowns were tested for the inhibition of folate-dependent growth of the assay strains. Tubes with folate (0.4 to 0.5 ng/ml) were challenged with 0.05 ml of extracts and read at 24 h. Conjugase was prepared from desiccated hog kidney (Sigma Chemical Co., St. Louis, Mo.), and treatment at pH 4.5 was done essentially as described previously (10). Correction for folate in the enzyme and controls which lacked enzyme (sham conjugase) were done. Protein determinations were done (25) after cells were heated in a base. Folate levels were expressed on a dry weight basis (assuming 0.55 g of protein per g [dry weight] [13]).

Growth of indicator strains. *Streptococcus faecalis* grew on folate, pteroic acid, and thymidine (since all other products of folate metabolism were present in the medium). Folate-dependent growth was inhibited by the antifolate compound trimethoprim, whereas thymidine-dependent growth was not (Table 2) (28). The organism is known to grow on $PteGlu_1$, $PteGlu_2$, and reduced derivatives (26, 31). *Lactobacillus casei* was supported by folate and methanopterin and utilizes $PteGlu_2$ and $PteGlu_3$ (26, 31). Both *Lactobacillus casei* and *Streptococcus faecalis* subsisted on acid-hydrolyzed methanopterin (Table 2), but the methanopterin compounds were at least 3 orders of magnitude less potent than folate. These compounds did not inhibit growth of *Streptococcus faecalis*.

Folate and pteroylpolyglutamate content. Levels of folate activity in representative species grown in folate-free media are presented in Table 3. Values for free folate activity in more than 35 strains of eubacteria have been reported (11, 14, 21, 29, 32); these values range from 0.68 (*Bacillus sphaericus*) to 261 (*Pseudomonas convexa*) nmol/g (dry weight), with most being between 1 and 10 nmol/g (dry weight). Very high levels of $H_4PteGlu_3$ have been found in the purinolytic *Clostridium acid-urici* (3,900 nmol/g [dry weight]) (6). The results for *Bacillus subtilis* and *Pseudomonas fluorescens* are in good agreement with those obtained with different strains and assay systems (11, 14, 21, 32). The halophilic archaebacteria contained levels of folate activity similar to those of the eubacteria, whereas the methanogens and the thermoacidophile *Sulfolobus solfataricus* contained considerably less. The value for folate in *Methanococcus voltae* was similar to that reported previously (21), while that in *Methanobacterium thermoautotrophicum* Marburg was higher than in strain ΔH, possibly because of strain, growth, or assay differences (21).

When extracts were challenged with trimethoprim in the *Streptococcus faecalis* assay, absorbances decreased by more than 70% (to <0.02) for methanogens and by more than 95% (to <0.01) for halophiles (data not shown), indicating that little of the folate activity was due to the presence of thymidine. Folate was stable in *Sulfolobus solfataricus* extracts, as more than 80% of the authentic folic acid that was mixed with cells was recovered. All extracts were tested for possible inhibition of the folate-dependent growth of the assay organisms. One *Methanococcus voltae* preparation inhibited both strains (decreasing growth by >80%). The organism did not grow well in folate-free medium, and this extract was high in FeS, which may explain the inhibition.

Extracts were tested for pteroylpolyglutamates by treatment with conjugase (Table 3). The results of trimethoprim challenge of conjugase-treated extracts indicated that additional growth was due to folate activity (data not shown). The eubacteria *Bacillus subtilis* and *Pseudomonas fluorescens* contained additional folate activity (about 4- and 10-

TABLE 3. Folate content of extracts of representative bacteria

Organism	Folic acid activity (nmol/g [dry wt])[a] determined with:					
	Streptococcus faecalis			*Lactobacillus casei*		
	Untreated	Sham conjugase	Conjugase	Untreated	Sham conjugase	Conjugase
Methanobacterium formicicum	0.09 ± 0.07	0.48 ± 0.02	0.39 ± 0.09	0.16 ± 0.07	1.1 ± 0.5	1.8 ± 0.9
Methanobacterium thermoautotrophicum	0.36 ± 0.18	4.3 ± 0.7	4.8 ± 1.6	0.34 ± 0.14	NM[b]	NM
Methanococcus voltae	0.03 ± 0.02	NM	NM	NM	NM	NM
Methanosarcina barkeri	0.09 ± 0.04	0.75 ± 0.02	0.82 ± 0.23	0.09 ± 0.02	0.23 ± 0.07	0.11 ± 0.07
Sulfolobus solfataricus	0.05 ± 0.02	0.11 ± 0.05	0.45 ± 0.25	0.09 ± 0.07	0.15 ± 0.05	—[c]
Halobacterium halobium	5.9 ± 3.2	5.7 ± 0.5	76 ± 5	24 ± 5	38 ± 17	38 ± 15
Halobacterium salinarum	58 ± 32	60 ± 5	99 ± 16	26 ± 3	46 ± 16	60 ± 33
Halobacterium volcanii	5.4 ± 4.1	4.8 ± 3.2	81 ± 16	11 ± 6	2.2 ± 0.8	21 ± 8
Bacillus subtilis	2.5 ± 1.4	3.6 ± 0.5	9.5 ± 1.1	3.2 ± 0.8	2.5 ± 0.3	24 ± 2
Pseudomonas fluorescens	1.5 ± 0.6	7.0 ± 0.9	43 ± 21	5.4 ± 2.1	17 ± 2	50 ± 17

[a] Results of triplicate determinations repeated 2 to 6 times, expressed as ±standard deviation of the mean.
[b] NM, Not measured.
[c] —, Conjugase-treated extract inhibited growth of *Lactobacillus casei* (five repetitions).

fold, respectively) in both assays, indicating the presence of $PteGlu_n$, with $n > 3$. It has been reported that conjugase treatment causes increases in folate activity of 45-fold for *Bacillus subtilis* (11) and 15-fold for *Streptococcus pneumoniae* (29).

Conjugase-treated extracts of *Halobacterium halobium* and *Halobacterium volcanii* contained additional growth factor for *Streptococcus faecalis*, but not that for *Lactobacillus casei*, which indicates that $PteGlu_3$ was present. However, since the folate activity of *Halobacterium salinarum* extracts did not increase significantly after digestion, the predominant forms present were $PteGlu_1$ and $PteGlu_2$. It is not likely that the dimeric pterin sulfohalopterin II (from *Halobacterium marismortui*) would be active if it was present in these halophiles (22). *Sulfolobus solfataricus* extracts contained more folate activity in both assays after incubation at pH 4.5 (sham treatment). After conjugase treatment, the extracts contained even slightly more activity in the *Streptococcus faecalis* system but became inhibitory for the folate-dependent growth of *Lactobacillus casei*, causing a decrease in the A_{540} of more than 65%, which may reflect the presence of novel natural products in this archaebacterium. One thermoacidophile contains tetrahydromethanopterin (30), and *Sulfolobus solfataricus* contains a novel pterin (23), suggesting that such pterins may be the source of folate activity.

Methanogen extracts contained significantly more folate activity after conditions of conjugase treatment (pH 4.5, heating), which is consistent with the results for acid-hydrolyzed methanopterin (Table 1). With one exception, methanogens contain very high levels of tetrahydromethanopterin (1,300 to 9,600 nmol/g [dry weight]) (15). Since methanopterin is far less potent than folate, the results suggest that it is the source of folate activity in methanogens.

Halobacterium volcanii contains folates and was the only archaebacterium listed in Table 3 that was susceptible to the antifolates methotrexate and trimethoprim (unpublished data). This is consistent with the report that it is the only trimethoprim-sensitive halobacterium and that it appears to contain a trimethoprim-sensitive dihydrofolate reductase (27).

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