Influence of Amino Acids on Growth and Cell Wall Composition of Methanobacteriales

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Methanogenic bacteria with pseudomurein sacculi incubated with elevated concentrations of amino acids showed growth inhibition, changes in morphology and, under certain conditions, lysis. *Methanobacterium thermoautotrophicum* incorporated the amino acids glycine, threonine, ornithine and, in small amounts, aspartic acid into the sacculi, but not D-alanine, meso-diaminopimelic acid or the amino sugar galactosamine.

INTRODUCTION

Analogues of amino acids that build up the peptide moiety of the murein of eubacteria can interfere with the biosynthesis of peptidoglycan. These analogues either inhibit specific enzymes or replace the corresponding amino acid in the nascent peptidoglycan. A well-investigated example of the first group is D-cycloserine, an analogue of D-alanine, which inhibits alanine racemase, D-alanine:D-alanine ligase and D-amino acid transaminase (Strominger *et al.*, 1960; Neuhaus, 1967). The second group of analogues is represented by glycine (Schleifer *et al.*, 1976; Hammes *et al.*, 1973; for a review see Neuhaus & Hammes, 1981) and D-serine (Whitney & Grula, 1964; Trippen *et al.*, 1976). These amino acids can partially replace D-alanine and are incorporated into the nascent peptidoglycan via UDP-activated precursors. Elevated concentrations of these amino acids in the culture medium inhibit the growth of the bacteria, and produce morphological changes and spheroplasts due to a decrease of the cross-linkage of the peptide subunits (reviewed by Neuhaus & Hammes, 1981).

The ability to take up the amino acids glycine, threonine and isoleucine has been demonstrated for the pseudomurein-containing methanogen *Methanobacterium thermoautotrophicum* strain Marburg (Gilles *et al.*, 1983; Eikmanns *et al.*, 1983*a, b*).

This paper describes the influence of elevated concentrations of amino acids on methanogenic bacteria of the order Methanobacteriales.

METHODS

Organisms and growth conditions. Stock cultures of *Methanothermobacter fervidus* (DSM 2088) and *Methanobrevibacter arboriphilus* (DSM 1125) were gifts from K. O. Stetter and J. Winter, respectively. *Methanobacterium thermoautotrophicum* strain ΔH (DSM 1053) was obtained from the Deutsche Sammlung von Mikroorganismen (DSM), Göttingen, FRG. *Methanobacterium uliginosum* (DSM 2956) is our own isolate. *Methanobrevibacter arboriphilus* (37 °C), *Methanobacterium uliginosum* (37 °C; König 1984), *Methanobacterium thermoautotrophicum* (64 °C) and *Methanothermobacter fervidus* (84 °C; Stetter *et al.*, 1981) were grown in serum bottles with H₂/CO₂ (80:20; 300 kPa) at 100 r.p.m. or in a 10-litre fermenter (New Brunswick) sparged with H₂/CO₂ (80:20) in medium 1 of Balch *et al.* (1979), which contains mineral salts, FeSO₄·7H₂O (0.0002%), sodium acetate (0.25%), sodium formate (0.25%), yeast extract (0.2%), Trypticase (0.2%), L-cysteine·HCl (0.05%) and Na₂S·9H₂O (0.05%), plus trace elements and vitamins.

Analytical methods. Walls were isolated as described by Schleifer & Kandler (1967) or in smaller amounts according to Stetter *et al.* (1981). Amino acids and amino sugars in wall hydrolysates (4 M-HCl, 16 h, 100 °C) were determined with an amino acid analyser (Biotronik LC 5000) programmed for 'hydrolysates of bacteria'.

(Biotronik). N-terminal amino acids were determined by dinitrophenylation (Takebe, 1965). The occurrence of D-alanine in the walls was tested with D-amino acid oxidase (Boehringer-Mannheim; Larson *et al.*, 1971). C-terminal amino acids were determined by hydrazinolysis (Ghuysen *et al.*, 1966).

Chromatography. The dinitrophenylated amino acids were identified on cellulose thin-layer plates using 1.5 M-potassium phosphate buffer pH 6.0 as solvent. The peptides in partial wall hydrolysates (4 M-HCl, 30 min, 100 °C) were separated by thin-layer chromatography (TLC) on cellulose thin-layer plates (Polygram CFL 400, Macherey & Nagel). The following solvents were used: 1, α -picoline/ammonia/water (70:2:28, by vol.); 2, n-propanol/ammonia/water (60:30:10, by vol.); 3, 75% (w/v) phenol in water. The running distance was 8 cm.

RESULTS

Growth in the presence of amino acids

Growth of the autotrophic methanogen *Methanobacterium thermoautotrophicum* was tested in the presence of different concentrations of amino acids (Table 1). It grew in 1% (w/v) solutions of all amino acids tested. While 3% (w/v) solutions of the apolar and polar amino acids glycine, alanine, serine and threonine inhibited growth, 5% (w/v) of the acidic amino acids glutamic acid and aspartic acid and more than 5% (w/v) of the basic amino acids lysine and ornithine were required for growth inhibition. In contrast, *Methanobacterium uliginosum* did not grow in the presence of a 0.5% (w/v) concentration of the amino acids tested except ornithine, which was inhibitory at concentrations of 1% (w/v). When glycine was added to growing cultures of this organism, cells started to lyse. The OD₅₇₈ decreased after the addition of 1% glycine (w/v; final concentration) from 0.53 to 0.21 during an incubation period of 33 h. *Methanobrevibacter arboriphilus* and *Methanothermus fervidus* grew like *Methanobacterium thermoautotrophicum* in the presence of 1% (w/v) glycine, the only amino acid tested.

Morphology

Methanobacterium thermoautotrophicum normally occurs in irregular long rods, often forming filaments (Zeikus & Wolfe, 1972). However, in the presence of 1% (w/v) glycine most of the cells were highly crooked or coccoid, and the thickness of the walls could be increased up to about fourfold (see also Kandler & König, 1978). Lysed cells with defective wall structures also occurred. When mannitol (0.8 M, final concentration) together with glycine (1%, w/v, final concentration) was added to growing cultures of *Methanobacterium uliginosum*, rod-shaped cells with a very thin (4–5 nm) pseudomurein layer, or with no such layer, were obtained.

Incorporation of amino acids into the pseudomurein

Methanobacterium thermoautotrophicum grown in the presence of an amino acid (1%, w/v) normally not present in the wall (König *et al.*, 1982) incorporated glycine, threonine, ornithine and, in smaller amounts, aspartic acid into the pseudomurein (Table 2), while D-alanine, meso-diaminopimelic acid and the amino sugar galactosamine were not used for cell wall biosynthesis (data not shown). Analysis of walls isolated from *Methanothermus fervidus* and *Methanobrevibacter arboriphilus* revealed that glycine, the only amino acid tested, could be used as a cell wall constituent (Table 2). This was also true for *Methanobacterium uliginosum* (data not shown).

In order to determine the position of glycine in the peptide moiety, the walls of *Methanobacterium thermoautotrophicum* were subjected to dinitrophenylation, hydrazinolysis and partial acid hydrolysis. Glutamic acid (41%), alanine (12%) and ϵ -NH₂-lysine (12%) were found to be the N-terminal amino acids and glutamic acid (0.16%), glycine (17%), alanine (3.7%) and lysine (1.4%) the C-terminal amino acids. The N-terminal glutamic acid residues are increased in glycine-containing walls compared to normal walls (König *et al.*, 1982). In addition, a peptide was isolated from partial acid hydrolysates with *R_F* values of 0.39, 0.53 and 0.51 in solvent systems 1, 2 and 3, respectively, and an *R_{NH₃}* value of 0.94 on the amino acid analyser. It was composed of glycine, alanine and lysine in a molar ratio of 1.20:1.23:1.00 determined after acid hydrolysis. The hydrolysis products of the dinitrophenylated peptide were glycine, DNP-alanine and α -DNP-lysine, indicating that the peptide has the primary structure ϵ -Ala-Lys-Gly. Thus the C-terminal position of glutamic acid (König *et al.*, 1982) is partially substituted by glycine, but this amino acid is also incorporated in other positions of the peptide moiety.

Table 1. *Growth of Methanobacterium thermoautotrophicum in the presence of elevated concentrations of amino acids*

The experiments were done in triplicate; representative results are shown.

Amino acid	Concn (% _{w/v})	Growth (OD ₅₇₈)			
		Incubation time (h):			
		0	24	47	72
Control*		0.03	0.69	1.04	1.35
Ala	1	0.02	0.26	0.72	1.17
	3	0.02	0.05	0.05	0.04
Asp	1	0.03	0.47	1.04	1.40
	3	0.02	0.16	0.81	1.34
	5	0.01	0.02	0.03	0.03
Glu	1	0.04	0.44	0.98	1.29
	3	0.02	0.11	0.58	1.16
	5	0.04	0.04	0.06	0.07
	10	0.05	0.04	0.04	0.04
Gly	1	0.03	0.75	1.12	1.69
	3	0.02	0.04	0.03	0.03
Lys	1	0.03	0.24	0.95	1.19
	3	0.02	0.26	0.69	0.75
	5	0.01	0.03	0.06	0.11
	10	0.03	0.04	0.04	0.04
Orn	1	0.03	0.53	1.19	1.57
	3	0.03	0.27	0.92	1.24
	5	0.02	0.02	0.06	0.13
	10	0.04	0.03	0.03	0.04
Ser	0.5	0.03	0.12	0.44	0.86
	1	0.03	0.11	0.14	0.03
	3	0.02	0.03	0.08	0.02
Thr	0.5	0.02	0.44	1.01	1.21
	1	0.02	0.20	0.27	0.30
	3	0.03	0.04	0.08	0.02

* Medium 1 of Balch *et al.* (1979). The amino acids were added to the medium before inoculation.

Table 2. *Amino acid composition of the pseudomurein after growth of the cells in the presence of different amino acids*

The experiments were repeated two or three times; representative results are shown.

Species	Amino acid added	Cell wall composition (molar ratio)*							
		Asp	Glu	Ala	Lys	GlcN	Gly	Orn	Thr
<i>Methanobacterium thermoautotrophicum</i>	Gly	ND	2.41	1.31	1.00	1.55	0.47	ND	ND
	Thr	ND	1.59	0.43	1.00	0.70	ND	ND	0.29
	Asp	0.10	1.78	0.54	1.00	0.83	ND	ND	ND
	Orn	ND	2.69	1.51	1.00	1.54	ND	0.49	ND
<i>Methanothermus fervidus</i>	Gly	ND	2.76	0.96	1.00	0.78†	0.89	ND	ND
<i>Methanobrevibacter arboriphilus</i>	Gly	ND	2.10	0.88	1.00	0.53‡	0.36	ND	ND

ND, Not detected.

* For the normal cell wall composition of Methanobacteriales see König *et al.* (1982) and König (1984).

† Containing 31 mol % GalN.

‡ GalN.

DISCUSSION

The incorporation of different amino acids into the pseudomurein by the autotrophic methanogenic bacteria *Methanobacterium thermoautotrophicum* strain ΔH , *Methanothermobacter* *fervidus* and *Methanobrevibacter arboriphilus* shows that members of three genera of Methanobacteriales can take up amino acids on a large scale from the culture medium. In methanogenic habitats like the rumen or sewage sludge, where high concentrations of amino acids may temporarily occur, the amino acids can probably be used as a cell carbon source. Eikmanns *et al.* (1983a) pointed out that the mechanism of amino acid uptake by *Methanobacterium thermoautotrophicum* is probably not facilitated diffusion or active transport because relatively high concentrations (10 mM) are necessary for maximal rates of incorporation. On the other hand, it is well known that eubacteria can re-use D-alanine from the outside of the cytoplasmic membrane which is released from precursors during murein synthesis (Schwartz *et al.*, 1959). Such re-utilization of D-alanine plays an important role in the cellular economy (Neuhaus & Hammes, 1981). *Methanobacterium thermoautotrophicum* also releases L-alanine during growth and cell wall synthesis (Schönheit & Thauer, 1980), but this methanogen seems not to possess a transport system for L-alanine, which prevents the accumulation of L-alanine in the culture medium (Schönheit & Thauer, 1980).

The lack of, or very low, incorporation into the pseudomurein of some of the tested compounds, e.g. aspartic acid, may not be due to a higher specificity of the corresponding ligases; the uptake may be too low for measurable incorporation to be obtained, as is the case for glutamic acid (Gilles *et al.*, 1983). Some enzymes, e.g. the ligases of eubacteria and, as shown here, the equivalent enzymes of methanogenic archaeobacteria, which are involved in wall biosynthesis do not appear to have a high specificity for a particular amino acid. This raises the question as to how during cell wall biosynthesis the correct amino acid is incorporated, because among the methanogenic bacteria only one example is known, where two amino acids can replace each other. This occurs in *Methanobrevibacter ruminantium*, where threonine can partly or completely replace alanine (König *et al.*, 1982).

The methanogens tested here varied in their behaviour in the presence of elevated concentrations of amino acids, although all exhibited changes in morphology, probably due to a disturbance of cell wall biosynthesis. The most sensitive species was *Methanobacterium uliginosum*, isolated from a marshy soil (König, 1984). It did not grow in the presence of a 0.5% (w/v) concentration of most of the amino acids tested, and addition of 1% (w/v) glycine to growing cultures caused lysis. However, the lysis could be prevented by the addition of mannitol. The resulting stabilized cells without or with a very thin pseudomurein layer may be useful for studies of membrane functions. The fact that different amino acids can be incorporated into the pseudomurein may also be helpful in the study of the *de novo* synthesis of pseudomurein *in vitro*.

This paper is dedicated to Prof. Dr Otto Kandler on the occasion of his 65th birthday.

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