Polyamine Analysis of Extremely Halophilic Archaebacteria and Methanogenic Archaebacteria

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(Received October 1, 2002 ; Accepted December 19, 2002)

Abstract : The polyamine patterns of 19 species of extremely halophilic archaea (archaebacteria) and 14 species of methanogenic archaebacteria were examined. The acid-extracted cellular polyamines were analyzed by HPLC. Appreciable amount of agmatine was detected as the major polyamine in the neutrophilic halophiles (belonging to the genera *Haloarcula, Halogeometricum, Halobacterium, Halorubrum, Haloterrigena, Natrinema* and *Natrialba*) grown in a polyamine-free synthetic medium. The haloalkaliphilic *Natronorubrum, Natronobacterium* and *Natrialba*, and a neutrophilic halophile, *Halorhabdus*, grown in organic media, contained agmatine in addition to putrescine, cadaverine, spermidine or spermine as ones incorporated from the growth medium. Agmatine was ubiquitously distributed as the major polyamine within the order Halobacteriales; however, its cellular levels in haloalkaliphiles were lower than those in neutrophilic halophiles. Spermidine was the major polyamine in the methanogens, *Methanococcus maripaludis, M. voltae, Methanospirillum hungatei* and *Methanomicrobium mobile.* Homospermidine was major in *Methanosarcina mazei, M. barkeri* and *M. baltica. Methanobrevibacter arboriphilus* contained homospermidine and spermine. *Methanocalculus pumilus* contained spermidine and homospermidine. *Methanobacterium formicicum* and *Methanoculleus chikugoensis* contained spermidine and spermine as the major polyamines. Polyamines were absent in *Methanogenium cariaci.* Thermophilic *Methanothermococcus okinawensis* contained spermidine, spermine and agmatine. Polyamine distribution profile serves as a phenotypic chemotaxonomic marker within the five orders Methanobacteriales, Methanococcales, Methanomicrobiales, Methanosarcinales and Methanopyrales.

Key words : polyamine, halophile, archaebacteria, methanogen, agmatine

INTRODUCTION

It has been shown that the cellular polyamine profiles of bacteria (eubacteria) and archaea (archaebacteria) are variable depend on certain taxonomic levels (*e.g.* at genus-, family- or order-level), suggesting that they could be used as chemotaxonomic markers.[1,2)]

Archaebacterial extreme halophiles represented by members of the order Halobacteriales within the phylum Euryarchaeota grow aerobically and require high concentration of NaCl for growth.[3)] We have reported that agmatine occurred as the cellular major polyamine in the extremely halophilic archaebacterial 11 genera, *Haloarcula, Halobaculum, Halobacterium, Haloferax, Halococcus, Halorubrum, Haloterrigena, Natrialba, Natronobacterium, Natronococcus*, and *Natronomonas.*[4-7)]

Methanogenic archaebacteria spread in five divergent orders (three classes) within the phylum Euryarchaeota.[8)] We have reported the cellular

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polyamine profiles of the six thermophilic members of methanogens, *Methanothermobacter thermautotrophicus* (formerly *Methanobacterium thermoautotrophicum*), *Methanothermus sociabilis, Methanothermus fervidus, Methanothermococcus thermolithotrophicus* (formerly *Methanococcus thermolithotrophicus*), *Methanocaldococcus jannaschii* (formerly *Methanococcus jannaschii*) and *Methanopyrus kandleri*.[9-11]

In the present study, we extend the polyamine analysis by 19 species of the neutrophilic and alkaliphilic extreme halophiles, including the newly described four genera *Halogeometricum, Halorhabdus, Natronorubrum* and *Natrinema*,[3,12] and 14 species of the methanogens belonging to the ten genera *Methanobacterium, Methanobrevibacter, Methanococcus, Methanothermococcus, Methanomicrobium, Methanoculleus, Methanogenium, Methanospirillum, Methanocalculus* and *Methanosarcina*, including thermophiles and psychrophiles[8,13-16] and evaluated their polyamine patterns as a chemotaxonomic marker. Effect of growth conditions, such as medium pH and growth temperature, on the cellular polyamine levels are also discussed.

MATERIALS and METHODS

Neutrophilic extreme halophiles were aerobically grown in the synthetic OMG medium, pH 7.0.[5,7] Besides, JCM media No.168, pH 7.0 and No.294, pH 7.0 (Catalogue of Strains 2002, Japan Collection of Microorganisms) that contain yeast extract were also used for cultivation. Extreme haloalkaliphiles were aerobically grown in JCM medium No.167, pH 9.0 that contains yeast extract. After cultivation at 37℃ or 45℃ in the liquid media, the cells were harvested at stationary phase by centrifugation at 10,000 xg for 30 min, from the culture supernatant containing 20% NaCl. Mesophilic and slightly psychrophilic methanogens were anaerobically grown in the liquid JCM media as shown in Table2 (pH 6.0 - 7.5) at 25 - 38℃ (Catalogue of Strains 2002, Japan Collection of Microorganisms). Thermophilic *Methanothermoccocus okinawensis* was grown at 60℃. The cells were harvested at stationary phase by centrifugation at 2,000 xg for 30 min. Some extreme haloalkaliphiles were also cultivated on agar plates of JCM medium No.167 and cells were collected by scraping from the agar surface.

The cellular polyamines extracted from the packed cells with cold 0.5 M (5%) perchloric acid (PCA) ($HClO4$) were analyzed by high-performance liquid chromatography (HPLC) on a column of cation-exchange resin by Hitachi L6000 High Speed Liquid Chromatograph, as described previously.[5,7,17] For the analysis of medium polyamines and extracellular secreted polyamines in the cultures of extreme haloalkaliphiles grown in JCM liquid medium No.167, cation-exchange resin (Dowex 50W) (5g) was added into the culture supernatant (200 ml). The polyamines were eluted with 6 M HCl from the resin and analyzed by HPLC.

RESULTS AND DISCUSSION

Extreme halophiles

Agmatine was exclusively detected in the polyamine fractions extracted from most of the neutrophilic extreme halophiles grown in OMG and JCM 168 media, at pH 6.8-7.0, as shown in Fig. 1 and Table 1. *Haloarcula aidinensis, H. quadrata, Halogeometricum borinquense, Halorubrum distributum, H. trapanicum, Haloterrigena thermotolerans, Natrinema pellirubrum, N. versiforme* and *Natrialba aegyptia* were included in this group. The same polyamine profile has been observed in many other neutrophilic extreme halophiles belonging to the genera *Haloarcula, Haloferax, Halobaculum, Halorubrum, Haloterrigena, Halococcus*, and *Halobacterium*.[5,7] *Natronomonas pharaonis*, previously analyzed, is slightly alkaliphilic (growing at pH 7.0-8.5) and newly analyzed two *Natrinema* species are nutrophilic. The species of *Natrialba* are composed of neutrophiles (*N. aegyptia* and *N. asiatica*) and alkaliphiles (*N. hulunbeirensis, N. chahannaoensis* and *N. magadii*). *Natronobacterium, Natronorubrum* and *Natronococcus* species analyzed in our previous and present studies are alkaliphilic. The haloalkaliphilic strains of *Natronorubrum, Natrialba* and *Natronobacterium* contained agmatine as major or minor component. Furthermore, most of them contained some of polyamines other than agmatine, that seemed to be derived from the growth medium, as shown in Fig. 1 and Table 1. Spermidine was detected in some of the haloalkaliphiles growing in 167 medium and slightly thermophilic *Natrialba hulunbeirensis* was rich in spermidine.

Medium 167 contained a little amount of agmatine in addition to spermidine and spermine (Fig. 1). The polyamine level decreased when the haloalkaliphiles were cultured and harvested on JCM 167 agar plates (Fig. 1). The polyamine contents obtained from the cultures in the liquid 167 medium were shown in the Table 1. Although cellular agmatine level varied within the alkaliphilic extreme halophiles, intracellular agmatine was not secreted into the culture supernatant and some polyamines were incorporated into the cells from the medium, as show in Fig. 1. We observed that about a half of agmatine intracellularly produced was secreted into the culture

Table1 Cellular polyamines in extremely halophilic archaebacteria.

		Medium JCM No.	(pH)	Temp (℃)	Polyamines(nmol/g wet cell) Put	Cad	Spd	Spm	Agm
Genus *Haloarcula*									
H. aidinensis	JCM 10024(P. Zhou A)	OMG	(7.0)	37	–	0.10	–	–	1.60
		168	(7.0)	37	–	–	–	–	0.90
	JCM 10025(P. Zhou B2)	168	(7.0)	37	–	–	–	–	0.25
H. quadrata	JCM 11048^T(DSM 11927)	OMG	(7.0)	37	–	0.05	–	–	0.70
		168	(7.0)	37	–	–	–	–	0.46
Genus *Halogeometricum*									
H. borinquense	JCM 10706^T(ATCC 700274)	OMG	(7.0)	37	–	–	–	–	0.35
		168	(7.0)	37	–	–	–	–	0.57
Genus *Halorhabdus*									
H. utahensis	JCM 11049^T (DSM 12940)	294	(7.0)	37	–	0.11	–	–	0.44
Genus *Halobacterium*									
Halobacterium sp.	JCM 11081(ATCC 700922)	OMG	(7.0)	37	–	–	–	–	0.20
		168	(7.0)	37	–	–	–	–	0.07
Genus *Halorubrum*									
H. distributum	JCM 10118^T	OMG	(7.0)	37	–	–	–	–	0.40
		168	(7.0)	37	–	–	–	–	1.05
	JCM 10247 (VKM B-1739z)	OMG	(7.0)	37	–	–	–	–	0.40
		168	(7.0)	37	–	–	–	–	0.35
H. trapanicum	JCM 10477 (NCIMB 13488)	168	(7.0)	37	–	–	–	–	0.70
Genus *Natronorubrum*									
N. bangense	JCM 10635^T(Y. Xu A33)	167	(9.0)	37	–	–	0.04	0.02	1.35
N. tibetense	JCM 10636^T(Y. Xu GA33)	167	(9.0)	37	–	–	0.75	0.02	0.06
Genus *Haloterrigena*									
H. thermotolerans	JCM 11050^T (DSM 11552)	OMG	(7.0)	37	–	0.07	–	–	1.90
		168	(7.0)	37	–	–	–	–	1.15
Genus *Natrinema*									
N. pellirubrum	JCM 10476^T (NCIMB 786)	OMG	(7.0)	37	–	–	–	–	1.25
		168	(7.0)	37	–	–	–	–	2.60
N. versiforme	JCM 10478^T (P. Zhou XF10)	OMG	(7.0)	37	–	–	–	–	1.80
		168	(7.0)	37	–	–	–	–	0.32
Genus *Natrialba*									
N. hulunbeirensis	JCM 10989^T (Y. Ma X21)	167	(9.0)	45	–	–	1.17	0.02	0.16
N. chahannaoensis	JCM 10990^T (Y. Ma C112)	167	(9.0)	37	–	–	0.16	0.01	0.02
N. magadii	JCM 8861^T(a)	167	(9.0)	37	0.50	–	0.60	0.10	0.30
N. aegyptia	JCM 11194^T (DSM 13077)	OMG	(7.0)	37	–	–	–	–	1.75
		168	(7.0)	37	–	–	–	–	0.75
N. asiatica	JCM 9576^T(a)	OMG	(7.0)	37	0.90	–	–	–	2.50
Genus *Natronobacterium*									
N. nitratireducens	JCM 10879^T (P. Zhou C231)	167	(9.0)	37	–	–	–	–	0.08
	JCM 10880 (P. Zhou C42)	167	(9.0)	37	–	–	–	–	0.10
N. gregoryi	JCM 8860^T(a)	167	(9.0)	37	0.40	–	0.70	0.10	0.40

Note: Put, putrescine; Cad, cadaverine; Spd, spermidine; HSpd, homospermidine; Spm, spermine; Agm, agmatine; 3(3)4, N^4-aminopropylspermidine; 3(3)(3)4, N^4-bis(aminopropyl)spermidine; JCM, Japan Collection of Microorganisms, RIKEN, Wako, Japan; ATCC, American Type Culture Collection, Manassas, Virginia, USA; ^{T}, type strain; –, not detectable (<0.005); Temp, growth temperature; a, data were cited form Hamana et al.[7] Cellular polyamine levels of the extreme halophiles were shown as nmol/g wet weight, whereas the polyamine levels of other non-halophilic archaebacteria were shown as μmol/g wet weight, because the packed cell pellets of the extreme halophiles contain about 2 M NaCl.

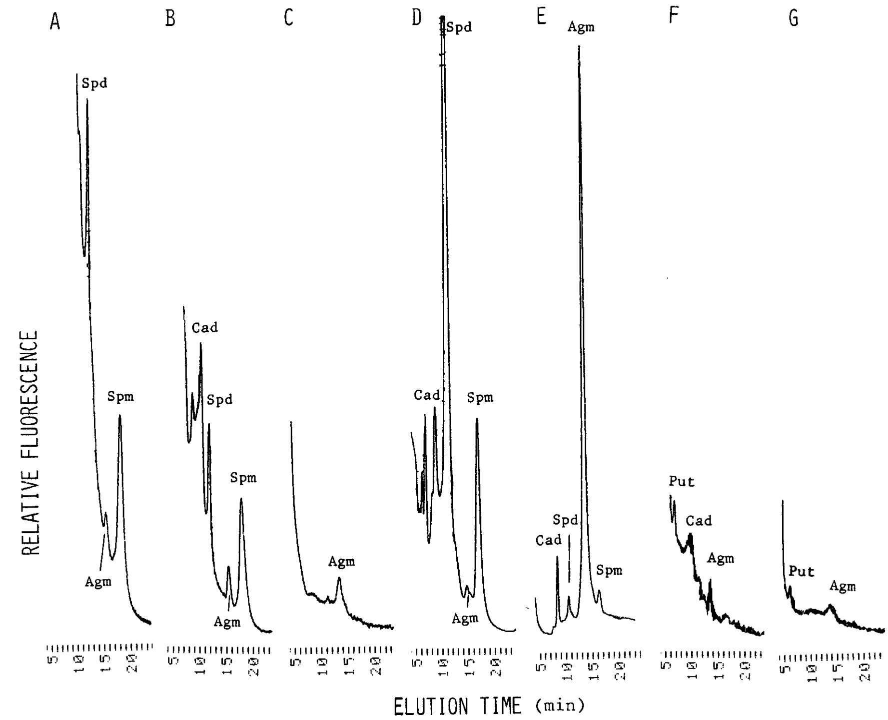

Fig. 1 HPLC chromatograms of the concentrated polyamine fraction of liquid JCM medium No.167 (A), the culture supernatant of *Natronobacterium nitratireducens* JCM 10880 grown in the liquid 167 medium (B), the cellular polyamines extracted form *N. nitratireducens* JCM 10880 (C), the culture supernatant of *Natronorubrum bangense* JCM 10635 grown in the 167 medium (D), the cellular polyamines extracted from *N. bangense* JCM 10635 (E), the cellular polyamines extracted from *Natronobacterium nitratireducens* JCM 10879 grown in the liquid 167 medium (250ml) (F), and the cellular polyamines extracted from *N. nitratireducens* JCM 10879 grown on the 167 agar medium (5 plates) (G). Abbreviations for polyamines are shown in Table 1. Figs F and G were monitored by a high sensitivity.

medium, when neutrophilic extreme halophiles were cultivated in synthetic liquid OMG medium, pH 7.0.[5,6)]

The present data on the cellular polyamines of archaebacterial extreme halophiles, support the ubiquitous occurrence of agmatine, in the order Halobacteriales. However, cellular levels of agmatine and other polyamines were varied by their growth pH and growth temperatures.

Methanogens

Methanogenic archaebacteria are phylogenetically distributed into the five orders Methanobacteriales, Methanococcales, Methanomicrobiales, Methanosarcinales and Methanopyrales. The typical HPLC chromatograms obtained in the present study are show in Fig. 2. Cellular concentrations of polyamines in the neutrophilic methanogens, grown at pH 6.0-7.5, analyzed are shown in Table 2.

In the order Methanobacteriales, mesophilic *Methanobacterium formicicum*, as well as thermophilic *Methanothermobacter thermoautotrophicus* contained putrescine, spermidine and spermine.[9)] *Methanobrevibacter*

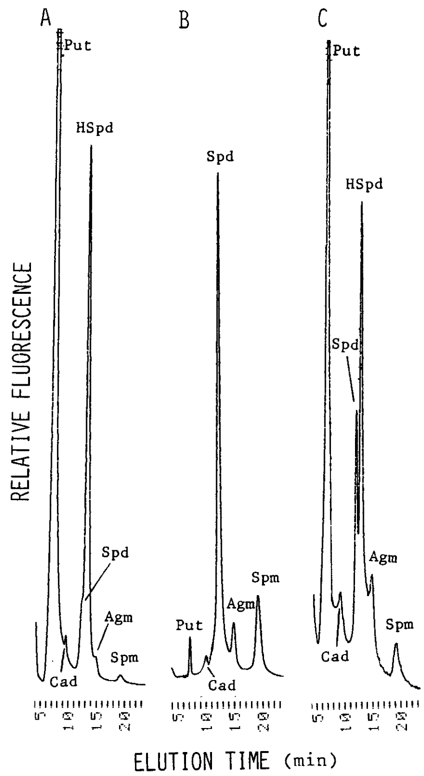

Fig. 2 HPLC chromatgrams of the cellular polyamines extracted from *Methanosarcina mazei* JCM 9314 (A), *Methanococcus voltae* JCM 10010 (B), and the mixture of the two polyamine samples (C). Abbreviations for polyamines are shown in Table 1.

Table 2 Cellular polyamines in methanogenic archaebacteria.

		Medium JCM No.	(pH)	Temp (℃)	Put	Cad	Spd	HSpd	Spm	Agm	3(3)4	3(3)(3)4
Genus ***Methanobacterium***												
M. formicicum	JCM10132^T(DSM1535)	242	(7.0)	37	0.01	–	0.10	–	0.45	–	–	–
Methanobacterium sp.	JCM10400(K.Adachi 3544-4)	198	(7.4)	37	0.09	–	2.30	–	–	–	–	–
Genus Methanothermobacter												
M. thermautotrophicus	ATCC29096^T(a)	231	(7.2)	60	0.30	–	0.17	–	0.69	–	–	–
	JCM10044^T(a)	231	(7.2)	65	0.02	0.03	0.25	–	0.50	–	–	–
Genus ***Methanobrevibacter***												
M. arboriphilus	JCM9316(Y.Koga A2)	198	(7.4)	37	–	–	–	0.20	0.60	–	–	–
Genus ***Methanothermus***												
M. fervidus	JCM10308^T(b)	251	(6.5)	80	–	–	0.33	–	0.35	0.23	–	–
M. sociabilis	JCM10723^T(c)	251	(6.5)	85	–	–	0.45	–	1.55	0.03	–	–
Genus ***Methanococcus***												
M. maripaludis	JCM10722^T(DSM1539)	265	(6.5)	37	–	–	0.85	–	–	0.20	–	–
M. voltae	JCM10010(W.B.Whitman A3)	228	(7.0)	37	0.36	–	4.40	–	–	0.40	–	–
Genus ***Methanothermococcus***												
M. okinawensis	JCM11175^T	232	(6.0)	60	0.02	–	0.60	–	0.40	0.14	–	–
M. thermolithotrophicus	ATCC35097^T(a)	265	(6.5)	60	0.04	–	1.45	–	0.14	–	–	–
Genus ***Methanocaldococcus***												
M. jannaschii	JCM10045^T(a)	232	(6.0)	80	0.04	–	0.60	–	1.50	–	0.04	0.35
Genus ***Methanomicrobium***												
M. mobile	JCM10551^T(DSM1539)	266	(6.5)	37	–	–	0.13	–	–	–	–	–
Genus ***Methanoculleus***												
M. chikugoensis	JCM10825^T(S.Asakawa MG62)	262	(6.5)	30	–	–	0.10	–	0.85	–	–	–
Genus ***Methanogenium***												
M. cariaci	JCM10550^T(DSM1497)	265	(6.5)	25	0.07	–	–	–	–	–	–	–
Genus ***Methanospirillum***												
M. hungatei	JCM10133^T(DSM864)	242	(7.0)	37	0.43	–	0.95	–	–	–	–	–
Genus ***Methanocalculus***												
M. pumilus	JCM10627^T(M.Hatsu MHT-1)	272	(7.5)	35	0.25	–	0.30	0.43	–	–	–	–
Genus ***Methanosarcina***												
M. mazei	JCM9314(S.Asakawa TMA)	197	(6.8)	37	8.00	–	–	4.80	–	–	–	–
M. barkeri	JCM10043^T(DSM800)	230	(7.0)	38	8.00	–	–	4.30	–	–	–	–
M. baltica	JCM11281(M.Thomm GS1-A)	305	(6.5)	25	–	–	–	2.00	–	–	–	–
Genus ***Methanopyrus***												
M. kandleri	JCM9639^T(b)	216	(6.0)	95	–	–	0.04	–	0.02	0.22	–	–

Note: See Table1. a, data were cited from Hamana et al.[9]
b. data were cited from Hamana et al.[10]
c, data were cited from Hamana et al.[11]

arboriphilus contained homospermidine and spermine. The occurrence of appreciable amounts of spermidine and spermine (and agmatine) in two thermophilic *Methanothermus species* was previously reported.[10,11] Scherer and Kneifel,[18] and Kneifel *et al.*[19] reported the absence of cellular triamines and tetraamines in the genera of the order Methanobacteriales (two species of *Methanobacterium*, two species of *Methanobrevibacter* and a *Methanothermus* species).

In the order Methanococcales, mesophilic *Methanococcus maripaludis* and *M. voltae*, ubiquitously contained spermidine and agmatine as the major polyamines. The two thermophilic *Methanothermococcus* species, *M. okinawensis* and *M. thermolithotrophilcus*, contained spermine as a major polyamine in addition to spermidine. In some other *Methanococcus* and *Methanothermococcus* species, reported by Scherer and Kneifel[18] and Kneifel et al.[19], contained spermidine, or spermidine plus spermine. A tertiary branched tetraamine and a quaternary branched pentaamine were detected in thermophilic *Methanocaldococcus jannaschii*, as described previously.[9]

Several genera are located in the order Methanomicrobiales. *Methanomicrobium mobile* contained spermidine alone at low cellular level. *Methanoculleus chikugoensis* contained spermidine and spermine as the major polyamines. Slightly psychrophilic *Methanogenium cariaci* grown at 25℃

was characterized by low content of putrescine alone, suggesting the absence of significant amount of cellular polyamines. *Methanospirillum hungatei* contained high concentration of putrescine and spermidine. Equal levels of putrescine, spermidine, and homospermidine were found in *Methanocalculus pumilus*. The polyamine patterns of *Methanocorpusculum* species located in this order are characterized by high concentration of diaminopropane by Zellner *et al.*[20], thus, distinct from those of other methanogens. A variety of polyamine distribution profiles was observed in the order Methanomicrobiales and the profiles may be served as differential chemotaxonomic marker at genus- or species- level.

Homospermidine (and putrescine) was the major polyamine component in the three *Methanosarcina* species, *M. mazei, M. barkeri* and *M. baltica* belonging to the order Methanosarcinales. Polyamine level was low in the slightly psychrophilic species, *M. baltica,* grown at 25℃. The occurrence of homospermidine has been reported also in several non-validated *Methanosarcina species.*[18,19]

In our previous report, appreciable amount of agmatine and low level of spermidine and spermine were found in extremely thermophilic *Methanopyrus kandleri* belonging to the order Methanopyrales.[11]

Polyamine distribution profiles of methanogens determined in the present study showed that they varied the composition of the cellular polyamines from taxon to taxon, irrespective of their growth temperatures, and may be served for the chemotaxonomy as a phenotypic marker within methanogenic archaebacteria. More spices of archaeal methanogens should be analyzed to confirm the usefulness for chemotaxonomy.

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