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OCCURRENCE OF *sym*-HOMOSPERMIDINE AS THE MAJOR POLYAMINE IN NITROGEN-FIXING CYANOBACTERIA

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SUMMARY: We analyzed the amount of polyamines in a variety of cyanobacteria including nitrogen-fixing and nonfixing species. All the cyanobacteria capable of fixing nitrogen, contained *sym*-homospermidine as the major polyamine. The concentration of putrescine, spermidine and spermine was extremely low in these cyanobacteria. The cyanobacteria which normally fail to fix nitrogen contained spermidine as the major polyamine, while the *sym*-homospermidine content was very low or under the limits of detection. Apparently there is a close relationship between the *sym*-homospermidine content and the ability to fix nitrogen in cyanobacteria.

More than ten aliphatic polyamines have been detected in living organisms to date and they are thought to play important roles in various cellular processes (1). Putrescine, spermidine and spermine are widely distributed both in animals and plants and they have been studied extensively over the past decade. In addition to these polyamines, other "unusual" polyamines have been demonstrated in certain organisms. For example, norspermine and norspermidine, which were also referred to as thermine (2) and caldine (3) respectively, were first found in thermophilic bacteria and they were thought to play a specific role in thermal tolerance of the bacteria. Further studies, however, have revealed that these two unusual polyamines are present in

Abbreviations used : ATCC, American Type Culture Collection ; IAM, Institute of Applied Microbiology, Tokyo University ; MDM, Modified Detmer medium ; MA, Microcystis-Aureginosa ; SOT, Spirulina-Ogawa-Terui ; NaCB, $NaNO_3$-Closterium-Bicine.

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halophilic bacteria (4), eukaryotic algae (5-7) and even arthropods (8, 9). Recently another unusual polyamine, *sym*-homospermidine, has been detected in some bacteria (10-12), eukaryotic algae (5-7), animals (13-15) and higher plants (16). When this polyamine is found in any organism, it is usually present only as a minor component among the polyamines. Naturally the biological functions of *sym*-homospermidine have been little understood so far.

Cyanobacteria (blue-green algae) are prokaryotic algae and grossly divided into two groups on the basis of nitrogen metabolism: *i. e.*, the ones which are capable of fixing nitrogen and the others which fail to fix nitrogen. The occurrence of spermidine in the latter group has already been reported (5, 6, 17), but the analysis of minor polyamines was rather incomplete in these studies. Nitrogen-fixing cyanobacteria have never been analyzed for their polyamine content. Recently we have succeeded in separating almost all the naturally occurring polyamines and in determining their picomole levels with a single high-performance liquid chromatography (15). In the course of the study of polyamines in cyanobacteria utilizing this method, we have observed that *sym*-homospermidine is present as the major polyamine exclusively in nitrogen-fixing species.

MATERIALS AND METHODS

Chemicals

sym-Homospermidine [$NH_2(CH_2)_4NH(CH_2)_4NH_2$] was synthesized according to the method of Okada *et al.* (18). Norspermidine [$NH_2(CH_2)_3NH(CH_2)_3NH_2$] and norspermine [$NH_2(CH_2)_3NH(CH_2)_3NH(CH_2)_3NH_2$] were purchased from Eastman Organic Chemicals. Other polyamines were of the purest available grades from standard commercial sources.

Cultures

Pure cultures of cyanobacteria were supplied by the Institute of Applied Microbiology, Tokyo University, the American Type Culture Collection or isolated in DIC Ltd., Ichihara. *Anabaena cylindrica* (IAM, M-1), *Anabaena variabilis* (DIC), *Nostoc muscorum* (IAM, M-131), *Calothrix brevissima* (IAM, M-7), *Fremyella diplosiphon* (IAM, M-100), *Tolypothrix tenuis* (IAM, M-29), *Plectonema boryanum* (IAM, M-101), *Plectonema calothrichoides* (IAM, M-120), *Plectonema tenue* (IAM, M-129), *Oscillatoria neglecta* (IAM, M-83) and *Anacystis nidulans* (ATCC 6301) were cultured in MDM medium (19). *Spirulina siamese* (DIC) and *Spirulina platensis* (salt-water form) (IAM, M-135) were grown in SOT medium (20). A fresh-water form of *Spirulina platensis* (IAM, M-184) was grown in MA medium (21). *Microcystis aeruginosa* (IAM, M-176) was cultured in NaCB medium (21).

All the synthetic media contain NO_3^- as a nitrogen source, PO_4^{3-}, SO_4^{2-}, Cl^- and some other rare minerals. Trace amounts of some vitamins are also contained in NaCB medium. Incubation at 25°C in the light was carried out aerobically by shaking. Axenically growing organisms were collected in the stationary phase. *Aphanothece sacrum* cultured in the fields of Amagi City, Fukuoka was purchased from ordinary suppliers.

Polyamine analysis

Cyanobacteria were homogenized in 4 volumes of cold 0.5 N $HClO_4$ after being washed with distilled water. After deproteinization, the supernatants were applied to cation-exchange chromatography on a column (9 x 120 mm) of Hitachi Custom 2612 resin. Free amino acids were first eluted by 0.35 N sodium citrate buffer (pH 5.25) containing 0.5 M KCl and then polyamines were eluted with the second buffer (pH 5.25) containing 1.6 M KCl (7). Amino acids and polyamines were colorimetrically determined after reaction with a ninhydrin solution. Polyamines were also analyzed by high-performance liquid chromatography using a cation-exchange resin (Kyowa Seimitsu, 6221OF) column (4.8 x 80 mm). The three eluting buffers used were 0.35 N potassium citrate buffers containing 0.5 M KCl (pH 5.55), 2.1 M KCl (pH 5.63) and 2.4 M KCl (pH 5.73) (15). The elution pattern of the amines was followed by fluorescence with o-phtalaldehyde. This method is capable of separating and assaying 1,3-diaminopropane, putrescine, cadaverine, 1,6-diaminohexane, norspermidine, spermidine, *sym*-homospermidine, aminopropylcadaverine, norspermine, spermine, and agmatine. Some polyamine metabolites and biogenic amines (15) coelute with the above-mentioned polyamines. The coexistence of acetylpolyamines, hydroxypolyamines, histamine, octopamine, l-methylhistamine and norepinephrine in these polyamine peaks on the column chromatograms was excluded by thin-layer chromatography on silica gel G (Merck) (7, 22). Identification of polyamines was further confirmed by thin-layer chromatography on cellulose (Avicel SF, Funakoshi) (16). Infrared spectra (KBr disc) of polyamines were measured with a Hitachi 215 spectrophotometer using a micro-tablet holder.

RESULTS

Figure 1 shows the polyamine distribution patterns of *Anabaena cylindrica* (A) and *Spirulina platensis* (B). A main peak and a few minor peaks were observed in each chromatogram. The main peaks found in *A. cylindrica* and *S. platensis* were identical to *sym*-homospermidine and spermidine, respectively.

In order to confirm the identification of these two major peaks, fractions corresponding to each peak were collected and desalted on a short column of Dowex 50 W. When analyzed by thin-layer chromatography on cellulose, the peak fractions behaved exactly like *sym*-homospermidine and spermidine (Fig. 2). The fractions contained no other ninhydrin-positive compounds. The identification of these two polyamines as *sym*-homospermidine and spermidine was further confirmed by comparing their infrared spectra with those of authentic samples. Infrared

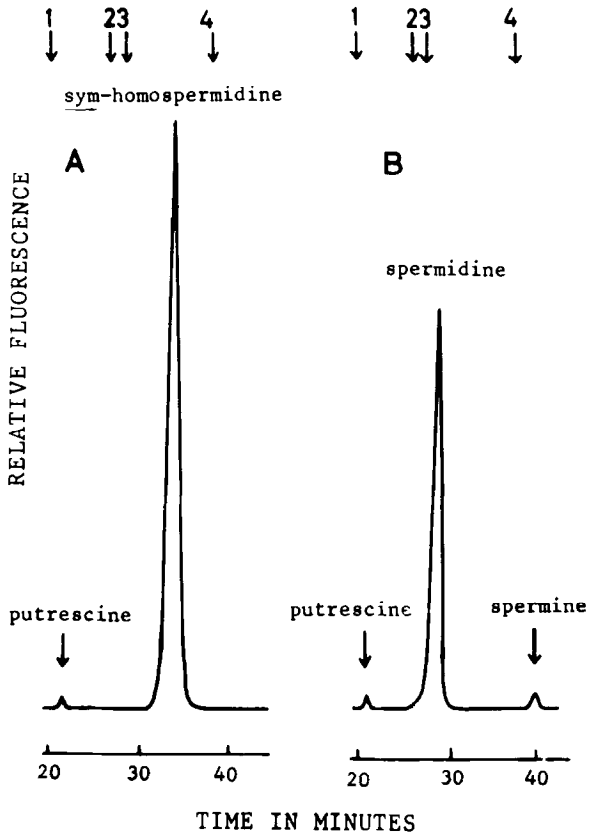

Fig. 1 Separation of polyamines from *Anabaena cylindrica* (A) and *Spirulina platensis* (salt-water form) (B) by high-performance liquid chromatography. The numbers above the arrows indicate the peaks of : (1) 1,3-diaminopropane; (2) norspermidine; (3) cadaverine; (4) norspermine.

spectrum (KBr disc) for the major polyamine of *A. cylindrica* showed absorption bands at 1590, 1470, 1440, 1400, 1350, 1310, 1275, 1255, 1200, 1170, 1150, 1080, 1050, 1025, 940, 855, 760 cm^{-1}. IR (KBr disc) for the major polyamine of *S. platensis*: 1590, 1480, 1450, 1400, 1355, 1310, 1295, 1265, 1240, 1150, 1110, 1075, 1050, 995, 950, 880, 780, 760 cm^{-1}. The IR spectra were quite similar to those of *sym*-homospermidine and spermidine, respectively, as reported by others (12, 16).

Minor peaks detected in cyanobacteria were identified as putrescine and spermine according to their retention time in two systems of column chromatography and their Rf values on thin-layer chromatograms. Other

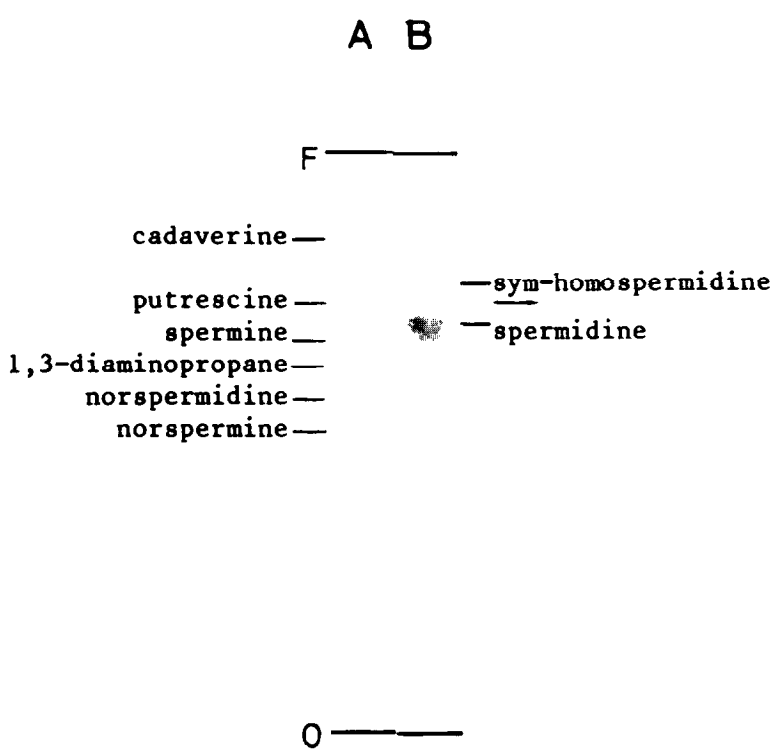

Fig. 2 Thin-layer chromatography on cellulose of the major polyamine fractions from *Anabaena cylindrica* (A) and *Spirulina platensis* (salt-water form) (B) separated by the column chromatography on Hitachi Custom 2612 resin. The solvent system used was 2-propanol-ammonia (7:3, v/v). O, origin; F, solvent front.

polyamines such as norspermidine and norspermine were not detected in pure cultures of cyanobacteria.

Polyamine distribution patterns were examined in 16 species of cyanobacteria (Table I). *sym*-Homospermidine was the most abundant polyamine in all the nitrogen-fixing cyanobacteria examined, while spermidine was the major polyamine in cyanobacteria which fail to fix nitrogen. The polyamine distribution patterns were nearly the same in both the fresh-water form (grown in MA medium with low salt concentration) and the salt-water form (grown in SOT medium with high salt concentration) of *S. platensis*, suggesting that osmolarity little affects polyamine metabolism. Culture media (MDM with a low salt concentration and SOT) and culture time little influenced the relative distribution patterns of polyamines in *A. cylindrica*, *Anabaena variabilis*, *Nostoc muscorum*

TABLE 1

Polyamine contents of nitrogen-fixing cyanobacteria (A) and nitrogen-nonfixing cyanobacteria (B)

	Cyanobacteria	Polyamines (μmoles/g wet weight)			
		Putrescine	Spermidine	*sym*-Homo-spermidine	Spermine
A	*Anabaena cylindrica*	0.060	0.006	2.182	0.000
	Anabaena variabilis	0.015	0.036	3.854	0.018
	Nostoc muscorum	0.098	0.112	4.780	0.089
	Tolypothrix tenuis	0.024	0.050	0.482	0.001
	Calothrix brevissima	0.027	0.019	1.000	0.000
	Fremyella diplosiphon	0.100	0.028	0.880	0.000
	Aphanothece sacrum	0.108	0.226	0.453	0.000
B	*Spirulina platensis**	0.004	0.588	0.000	0.052
	*Spirulina platensis***	0.004	0.604	0.000	0.020
	Spirulina siamese	0.004	0.796	0.000	0.016
	Oscillatoria neglecta	0.080	1.200	0.097	0.007
	Plectonema boryanum	0.040	2.712	0.000	0.012
	Plectonema calothrichoides	0.008	1.827	0.000	0.000
	Plectonema tenue	0.033	1.940	0.000	0.000
	Anacystis nidulans	0.003	0.229	0.000	0.000
	Microcystis aeruginosa	0.216	7.830	0.012	0.001

The ability of nitrogen fixation is based on the data under the usual aerobic growth conditions (23) (24). *, salt-water form; **, fresh-water form.

and *Anacystis nidulans*. The polyamine content of several substrains of *A. variabilis*, *N. muscorum* and *S. platensis* was also measured, and shown to be nearly identical to the figures listed in Table I.

DISCUSSION

In the present study we have demonstrated that the major polyamine found in nitrogen-fixing cyanobacteria is *sym*-homospermidine. The occurrence of spermidine in some cyanobacteria which do not fix nitrogen has already been reported by others (5, 6, 17) and we confirmed this in our present study. *sym*-Homospermidine has been reported to be present not only in eukaryotic algae (5-7) but also in thermophilic bacteria (12), higher plants (16) and animals (13-15). In these organisms it is only a

minor component of the polyamines detected. On the other hand, *sym*-homospermidine is the most abundant polyamine in nitrogen-fixing bacteria *Rhizobium* (10) and *Rhodopseudomonas* (11). It is of special interest to note that the occurrence of *sym*-homospermidine is closely associated with nitrogen fixation in both cyanobacteria and bacteria. The results seem to suggest that *sym*-homospermidine plays some specific role in the regulation of nitrogen fixation.

Nitrogenase is an oxygen-labile enzyme which converts one molecule of nitrogen (N_2) into two molecules of NH_3. This enzyme activity is strongly inhibited by the presence of NH_4^+ (23). Our preliminary experiments have shown that the addition of 3 m NH_4^+ to the culture medium results in little change in the *sym*-homospermidine content of nitrogen-fixing cyanobacteria. Nitrogenase activity is induced under anaerobic conditions in some cyanobacteria which normally do not fix nitrogen aerobically (23). Anaerobic cultures of these species under N_2 and CO_2 gas in the absence of NO_3^- in MDM medium, however, gave nearly the same polyamine distribution patterns as those obtained under aerobic conditions in the presence of NO_3^- in MDM medium. These results suggest that *sym*-homospermidine has no obligatory role in the control of nitrogen fixation in cyanobacteria. Possibly the ability of *sym*-homospermidine formation in nitrogen-fixing cyanobacteria is determined genetically. If this is the case, the polyamine distribution patterns may be useful in determining the taxonomical position of certain cyanobacteria.

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REFERENCES

1. Gaugas, J. M. (ed.) (1980) Polyamines in Biomedical Research, John Wiley & Sons, Chichester·New York, Brisbane·Toronto.
2. Oshima, T. (1975) Biochem. Biophys. Res. Commun. 63, 1093-1098.
3. De Rosa, M., De Rosa, S. and Gambacorta, A. (1976) Biochem. Biophys. Res. Commun. 69, 253-261.

4. Yamamoto, S., Shimoda, S. and Makita, M. (1979) Biochem. Biophys. Res. Commun. 87, 1102-1108.
5. Kneifel, H. (1977) Chemiker-Zeitung 101, 165-168.
6. Kneifel, H. (1979) in "Marine Algae in Pharmaceutical Science" (Hoppe, H. A., Levring, T. and Tanaka, Y. eds.) pp. 365-401, Watter de Gruyter, Berlin·New York
7. Hamana, K. and Matsuzaki, S. (1982) J. Biochem. (Tokyo) 91, 1321-1238.
8. Stillway, L. W. and Walle, T. (1977) Biochem. Biophys. Res. Commun. 77, 1103-1107.
9. Zappia, V., Porta, R., Carteni-Farina, M., De Rosa M. and Gambacorta, A. (1978) FEBS Lett. 94, 161-165.
10. Smith, T. A. (1977) Phytochemistry 16, 278-279.
11. Tait, G. H. (1979) Biochem. Soc. Trans. 7, 199-200.
12. Oshima, T. and Baba, M. (1981) Biochem. Biophys. Res. Commun. 103, 156-160.
13. Hamana, K. and Matsuzaki, S. (1979) FEBS Lett. 99, 325-328.
14. Matsuzaki, S., Tanaka, S., Suzuki, M. and Hamana, K. (1981) Endocrinol. Jpn. 28, 305-312.
15. Matsuzaki, S., Hamana, K., Imai, K. and Matsuura, K. (1982) Biochem. Biophys. Res. Commun. 107, 307-313.
16. Kuttan, R., Radhakrishnan, A. N., Spande, T. and Witkop, B. (1971) Biochemistry 10, 361-365.
17. Ramakrishna, S., Guarino, L. and Cohen, S. S. (1978) J. Bacteriol. 134, 744-750.
18. Okada, M., Kawashima, S. and Imahori, K. (1979) J. Biochem. (Tokyo) 85, 1235-1243.
19. Watanabe, A. (1960) J. Gen. Appl. Microbiol. 6, 283-292.
20. Ogawa, T. and Terui, G. (1970) J. Ferment. Technol. 48, 361-367.
21. Ichimura, T. (1979) in "Methods in Phycological Studies" (Nishizawa, K. and Chihara, M. eds.) pp. 294-305, Kyoritsu, Tokyo.
22. Blankenship, J. and Walle, T. (1978) Adv. Polyamine Res. 2, 97-110.
23. Stewart, W. D. P. (1978) Endeavour, New Series 2, 170-179.
24. Bothe, H. (1982) in "The Biology of Cyanobacteria" (Carr, N.G. and Whitton, B. A. eds.) pp. 87-104, Blackwell Sci. Publ., Oxford.