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OCCURRENCE OF NORSPERMIDINE IN SOME SPECIES OF GENERA VIBRIO AND BENECKEA

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SUMMARY: An unusual polyamine in some species of the genera Vibrio and Beneckea was identified by gas chromatography-mass spectrometry as norspermidine[3,3'-diaminodipropylamine] and its cellular concentration determined by gas chromatography. Norspermidine was detected as a major component in these species investigated.

The polyamines such as putrescine, spermidine and spermine are well known to be of wide occurrence in nature and their physiological functions have been reviewed (1).

Recently, the presence of two new polyamines, norspermidine and norspermine[N,N'-bis(3-aminopropyl)-1,3-diaminopropane], in some thermophiles has been comfirmed by Oshima (2) and Rosa et al. (3). Of particular interest are the results that the increased thermal resistance of some bacterial species could be in part associated with the presence of such polyamines(4,5) and also spermine has the ability to prevent osmotic lysis in the obligate halophilic species, Achromobacter fischeri (6). This prompted us to investigate the composition of polyamines in some halophiles. The analyses of polyamines were carried out according to our method using gas chromatography (7). As a result, an unusual polyamine was detected as the major component in the genera Vibrio and Beneckea species and this was identified to be norspermidine by gas chromatography-mass spectrometry.

This paper describes the identification of an unusual polyamine and its cellular concentration in some species of the genera Vibrio and Beneckea.

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MATERIALS AND METHODS

Chemicals 1,3-Diaminopropane, putrescine·2HCl, cadaverine·2HCl, spermidine·3HCl, spermine·4HCl and 1,8-diaminooctane as an internal standard were obtained from Nakarai Chemicals, Kyoto, Japan: 1,3-diaminopropane was purified by distillation, and 1,8-diaminooctane by recrystallization from water. Norspermidine and norspermine were kindly furnished by Dr. S. Matsuzaki (Gunma University, Japan). Isobutyl chloroformate was obtained from Eastman Organic Chemicals, Rochester, NY and used without further purification. Diethyl ether was treated with acidic iron(II) sulphate solution followed by distillation in an all glass apparatus. All other chemicals were the purest available grades from standard commercial sources.

Microorganisms Vibrio parahaemolyticus WP-1 (RIMD 2210086), V. anguillarum NCMB 878, V. piscium NCMB 571, Beneckea campbellii ATCC 25920, B. harvei ATCC 14126, B. vulnifica ATCC 27562, Proteus morganii NCTC 10041, Escherichia coli B and Salmonella typhimurium were used in this study. These organisms were cultivated on Modified MOF agar (MMOF agar) medium at 37°C for 16 hours. MMOF agar medium contains following constituents (in grams per liter): yeast extract (Difco), 4.5; Casitone (Difco), 1; tris(hyroxymethyl)amino methane, 0.5; boric acid, 0.11; ammonium sulphate, 0.5; disodium phosphate, 0.004; ammonium nitrate, 0.0008; sodium chloride, 9.7; magnesium chloride, 4.4; sodium sulphate, 1.6; potassium bromide, 0.04; strontium chloride, 0.017; sodium silicate, 0.002; sodium fluoride, 0.0012 and agar, 15. The medium was adjusted to pH 7.5. Cells grown on the MMOF agar medium were harvested and suspended in 2% sodium chloride solution. The suspension was centrifuged at 9,000 x g for 15 min and the resulting cell pellet was washed twice with 2% sodium chloride solution.

Extraction and isolation of polyamines The extraction and isolation of polyamines from the cells was carried out as earlier described (7). Wet cells (0.4-0.8 g) were reptured with 35 g of glass beads and 15 ml of 2% $HClO_4$ in the stainless-steel chamber of a Vibrogen (Edmund Bühler) at full speed for 10 min. The beads were removed by centrifugation, washed with 2 x 10 ml of 2% $HClO_4$ and centrifuged again. The three supernatants were combined and the whole was made up to 50 ml with 2% $HClO_4$. A 10 ml portion of this solution was loaded on to a column (9 mm I.D.) containing 3-ml bed volume of Amberlite CG-120 (H^+) cation-exchange resin. The column was washed with 30 ml of 0.1M sodium phosphate buffer pH 8 containing 0.1M sodium chloride, and then with 30 ml of 1N HCl. Polyamines were eluted with 40 ml of 6N HCl, and to the eluate was added 1 ml of the internal standard solution (125 nmoles of 1,8-diaminooctane). The eluate containing the internal standard was evaporated to dryness in a rotary evaporator under reduced pressure and the residue was transferred to a 10-ml polyethylene-stoppered vial with 1.5 ml of water for quantitative assay.

Quantitative analysis of polyamines by gas chromatography Polyamines were converted to their volatile N-isobutyloxycarbonyl (iso BOC) derivatives, which were analyzed by gas chromatography as earlier described (7). To the sample solution in a vial were added 0.5 ml of 10% Na_2CO_3 and 0.1 ml of isobutyl chloroformate, and then the mixture was shaken for 10 min at room temperature. The resulting derivatives of polyamines were extracted and analyzed. Each peak height of polyamines and the internal standard was measured and the amounts of each polyamine in samples were determined by comparing the peak height ratios obtained from samples with those from a standard mixture.

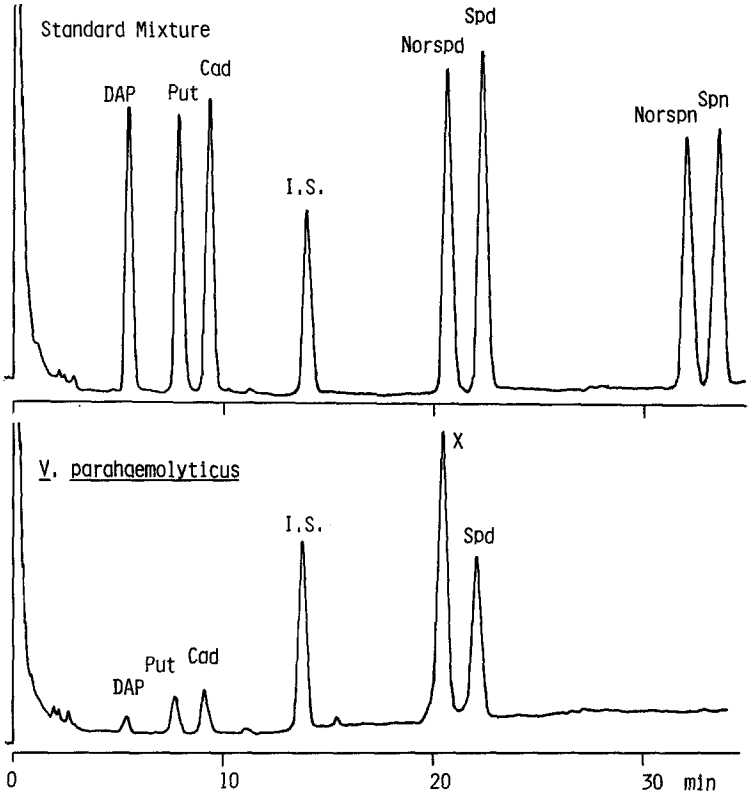

Fig. 1. Gas chromatograms of the N-isoBOC derivatives of polyamines obtained from a standard mixture and V. parahaemolyticus

* For experimental details, see Materials and Methods
** Abbreviations: DAP, 1,3-diaminopropane; Put, putrescine; Cad, cadaverine; Norspd, norspermidine; Spd, spermidine; Norspn, norspermine; Spn, spermine; I.S., internal standard (1,8-diaminooctane)

Gas chromatography-mass spectrometry The mass spectra of the polyamine derivatives were measured using a Shimadzu LKB 9000 gas chromatograph-mass spectrometer, operating at an accelerating voltage of 3.5kV, with an ionizing voltage of 70eV. Gas chromatographic analyses were performed with a glass column (2 m x 3 mm) packed with 2% OV-17 on silanized Gas Chrom P (80-100 mesh) and with a programmed temperature from 170 to 250°C at 4°C/min and a helium carrier gas flow-rate of 20 ml/min.

RESULTS

Preliminary analysis of the N-isoBOC derivatives of polyamines obtained from V. parahaemolyticus by gas chromatography indicated the presence of 1,3-diaminopropane, putrescine, cadaverine,

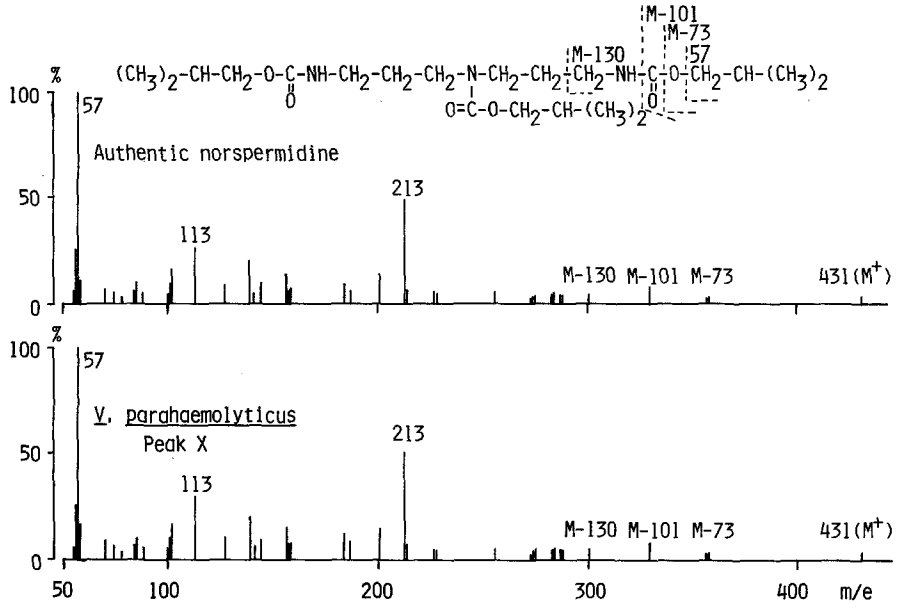

Fig. 2. Mass spectra of the N-isoBOC derivatives of authentic norspermidine and peak X

spermidine and an unidentified component (peak X) according to the relative retention times, as shown in Fig. 1. The peak X exhibited the same retention time as that of the authentic norspermidine. In order to prove its identity with norspermidine gas chromatography-mass spectrometry was employed. The peak X displayed the mass spectrum shown in Fig. 2, in which the molecular ion at m/e 431 and the fragmentation pattern consistent with the structure postulated was observed. The mass spectrum of the derivative of authentic norspermidine was indistinguishable from that obtained with the peak X as shown. The identity of the other polyamines could be also definitively accompanished by comparing the mass spectra obtained from a sample with those of the corresponding standard compounds.

In order to elucidate the quantification of this method, recovery experiments were carried out. The polyamine standard solution was added directly to the supernatant after homogenization,

TABLE I

Polyamine concentration in some species of genera Vibrio and Beneckea

Species	DAP*	Put	Cad	Norspd	Spd
	μmoles/wet cell g				
Vibrio parahaemolyticus	0.13	0.40	0.47	2.38	1.07
V. piscium	0.63	trace**	trace	2.86	0.39
V. anguillarum	0.34	2.14	tace	2.98	1.51
Beneckea campbellii	trace	0.40	0.13	1.08	1.39
B. harvei	0.06	0.28	0.35	0.35	0.32
B. vulnifica	0.11	0.09	trace	2.94	0.19

* Abbreviations: the same as in Fig. 1

** trace:<0.05 μmoles/wet cell g

and it was comfirmed that the recovery rate of each polyamine during the isolation and gas chromatographic stages was constant and almost quantitative (>93%).

The amounts of polyamines in some species harvested during late log-phase of growth (16 hours) are reported in Table I. In all the species investigated norspermidine and spermidine were detected as the major components, whereas spermine was present only in trace or practically undetectable in our analytical conditions. Proteus morganii, Escherichia coli and Salmonella typhimurium were employed as the reference species in this study. In these species we were unable to detect any norspermidine.

DISCUSSION

In general, the normally occurring polyamines are putrescine and spermidine in mesophilic bacteria, in which spermine is rather minor component or undetectable (1). The occurrence of two new polyamines, norspermidine and norspermine, and the physiological significance related to thermophility were demonstrated for the first time in some thermophiles (2,3). Recently these polyamines have been also detected in the brine shrimp (Penaeus setiferus)

(8), unicellular green algea (Scenedesmus acutus) (9), Euglena gracilis (10) and Arthropods and so on (11). However, it seems to be difficult to reveal a possible relationship between the occurrence of these polyamines and their physiological functions in these organisms. The identification of norspermidine in the genera Vibrio and Beneckea gives not only a further example of the occurrence of unusual polyamines, but also a first example in mesophilic bacteria. More recently Rosa et al. have been elucidated the biosynthetic pathway of polyamines in Caldariella acidophila, in which norspermidine is proposed to be a precusor of norspermine (5). However, in the present study norspermine was not detected in any species under culture conditions. Furthermore, analyses of polyamine pool of V. parahaemolyticus harvested at regular time intervals during from early log-phase (1 hour) to stationary-phase (30 hours) showed the absence of norspermine. The relatively large amounts of 1,3-diaminopropane were observed in most of all the species (Table I). This is interesting in respect to the biosynthesis of norspermidine in Vibrio and Beneckea species. V. parahaemolyticus and all species of Beneckea are marine or estuary bacteria, and V. anguillarum and V. piscium are isolated from both marine and fresh water. Results in this study seem to suggest that norspermidine probably play a role in salt tolerance or prevention of osmotic lysis of these bacteria, similar to that of spermine in Achromobacter fischeri (6). Further studies on the distribution and physiological role(s) in allied bacteria are in progress.

On the other hand Baumann et al. proposed the transfer of V. parahaemolyticus from the genus Vibrio to Beneckea, because it possesses the peritrichous (lateral) flagella in addition to the polar monotrichous flagella (12,13). V. natriegens was also transferred to the genus Beneckea by them (12). Some properties of the

genus Beneckea are similar to that of the genus Vibrio (14), and taxonomic position of Beneckea is not clear in Bergey's Manual of Determinative Bacteriology (8th edition). Results in this paper suggest the similarity of these two genera and that norspermidine may be a useful marker of chemotaxonomy.

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