Formation of Hydroxynorspermidine from Exogenously Added 1,3-Diaminopropan-2-ol in *Vibrio* Species

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When *Vibrio parahaemolyticus* AQ 3627 was grown in the presence of 1,3-diaminopropan-2-ol (OH-Dap), a new compound accumulated in the cells. This was identified as hydroxynorspermidine (OH-Nspd), *N*-(3-aminopropyl)-1,3-diaminopropan-2-ol, by gas chromatography-mass spectrometry and thin-layer chromatography. It was also synthesized enzymatically from OH-Dap by a cell-free preparation from this strain. All other *Vibrio* strains examined also showed the ability to synthesize this compound, but none of the non-vibrio organisms did, indicating that OH-Dap is an *in vivo* substrate for the enzyme responsible for biosynthesis of norspermidine characteristically present in vibrios. These results suggest that the ability to synthesize OH-Nspd from OH-Dap present in the growth medium may be useful as an additional identifying factor for the genus *Vibrio*.

Keywords hydroxynorspermidine; 1,3-diaminopropan-2-ol; polyamine; *Vibrio parahaemolyticus*; *Vibrio* species; generic guide

Most bacteria contain spermidine, and its functional roles have been well documented.[1] In *Escherichia coli*, the aminopropyl moiety of the spermidine molecule is donated from decarboxylated *S*-adenosylmethionine in the reaction catalyzed by aminopropyltransferase, which utilizes putrescine, but not 1,3-diaminopropane (Dap) as an amine substrate.[2] Such an enzymatic activity has also been reported to be distributed in a wide variety of prokaryotic organisms.[3] On the other hand, we have previously reported that members of the genus *Vibrio* contain norspermidine [Nspd, *N*-(3-aminopropyl)-1,3-diaminopropane], instead of spermidine, as a major polyamine,[4] and that this amine is biosynthesized from a Schiff base formed between Dap and *L*-aspartic β-semialdehyde through two enzymatic reactions.[5] Such a biosynthetic pathway has not previously been reported in any microorganism. In this context, it was expected that, if exogenously added, Dap analogs might act as growth inhibitors, as reported for putrescine analogs in *E. coli*.[6,7]

While examining the effect of Dap analogs on growth of *V. parahaemolyticus*, however, we found that addition of 1,3-diaminopropan-2-ol (OH-Dap) to the growth medium brought about the accumulation of a new compound in the cells with almost no inhibitory effect on gowth. This paper describes the identification of this compound as hydroxynorspermidine [OH-Nspd, *N*-(3-aminopropyl)-1,3-diaminopropan-2-ol] and shows that the ability to synthesize it from OH-Dap added to the growth medium may be useful as an additional identifying factor for the genus *vibrio*.

Experimental

Chemicals OH-Dap was obtained from Tokyo Kasei Kogyo (Tokyo, Japan) and Nspd from Aldrich Chemical Co. (Milwaukee, Wis., U.S.A.). Synthesis of OH-Nspd was accomplished by reacting OH-Dap with *N*-(3-bromopropyl)-phthalimide (Aldrich) and hydrolyzing the product with 12 M HCl, a method similar to that described by Okada *et al.*[8] OH-Nspd was purified by chromatography on a column of Dowex 50 W × 8 (2.7 × 16 cm) with a linear gradient of HCl (0—4 M). The fractions eluted with 3—3.5 M HCl were combined, and after evaporation of HCl *in vacuo* the residue (trihydrochloride salt) was recrystallized from H_2O–EtOH, mp 244—245 °C. The identity of the preparation was established by gas chromatography-mass spectrometry (GC-MS).[9] *Anal.* Calcd for $C_6H_{20}Cl_3N_3O$: C, 28.08; H, 7.86; N, 16.38. Found: C, 28.13; H, 7.49; N, 15.99. All other chemicals were commercial products of reagent-grade quality.

Growth Conditions Unless otherwise noted, an overnight culture was added as an inoculum (2%) to 200 ml of a synthetic medium[10] supplemented with 0.5% glucose, and then 2 ml of the filter-sterilized stock solution of OH-Dap (1 M), which had been adjusted to pH 7.5 with HCl, was added to give a final concentration of 10 mM. The strains of *V. cholerae* Non O1 were grown at 0.5% NaCl and the others at 2% NaCl. Growth was performed at 37 °C with a reciprocal shaker operating at 120 strokes/min. Usually, cells were harvested at the mid-log phase (6 h) by centrifugation at 5300 × *g* for 10 min at 4 °C, washed by resuspending them in 5 ml of ice-cold NaCl solution containing 10 mM $MgCl_2$ at an NaCl concentration equal to that used for cultivation, and centrifuged again. The cells were suspended in ice-cold 2% NaCl solution containing 10 mM $MgCl_2$ and the total volume was made up to 10 ml with the same solution. An aliquot, usually 0.1 ml, of the cell suspension was saved for protein determination which was done as described previously.[10]

Determination of Polyamines The remainder of the cell suspension was extracted with an equal volume of 4% $HClO_4$, and the supernatant of the extract was applied to an ion-exchange column to obtain the polyamine fraction and then to prepare the *N*-ethoxycarbonyl derivatives of polyamines as described previously.[11] However, slight modifications were introduced: 10% Na_2CO_3 was used instead of 10% NaOH to avoid the partial derivatization of a hydroxy group in OH-Nspd, and chloroform was used as a solvent to extract efficiently the derivative of OH-Nspd as well as those of the other polyamines. The resulting derivatives thus obtained were analyzed by GC.[11a] The recoveries of all polyamines added to the initial $HClO_4$ extracts were over 93%. All polyamine values are the means of two independent cultures grown in parallel under the same conditions, and all experiments were repeated at least once more with similar results.

Identification of OH-Nspd The remainder of the GC sample was used for identification of OH-Nspd by GC-MS.[9] On the other hand, according to the procedure of Smith and Best,[12] the polyamine fraction obtained after the ion-exchange column chromatography was dansylated and thin-layer chromatography (TLC) of the resulting products was carried out on a precoated Silica gel 60 plate (Merck, 0.25 mm thickness) with a solvent system of chloroform–triethylamine (5 : 1).

Formation of OH-Nspd by Using a Cell-free Extract Preparation of a cell-free extract from *V. parahaemolyticus* AQ 3627 and assay for enzymatic formation of OH-Nspd were performed as described previously,[5] except that OH-Dap was substituted for Dap as a substrate.

Results and Discussion

When *V. parahaemolyticus* AQ 3627 was cultured for 3 h in the medium supplemented with 10 mM OH-Dap, a new peak appeared, as shown in Fig. 1. The retention time of this peak corresponded to that of the authentic OH-Nspd (Fig. 1A and C). TLC analysis of the dansylated polyamine fraction also demonstrated the presence of an unknown component (*Rf* 0.25) which could be clearly distinguished from OH-Dap (*Rf* 0.10), Nspd (*Rf* 0.67) and spermidine (*Rf* 0.71). The *Rf* value of the authentic OH-Nspd was identical to that of the unknown compound. To further corroborate

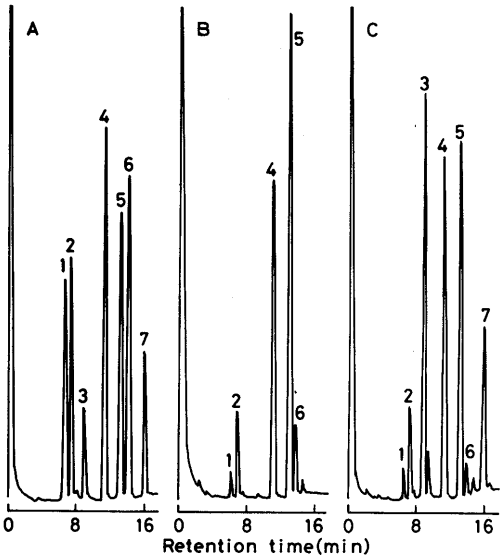

Fig. 1. Gas Chromatograms Showing the Formation of OH-Nspd from OH-Dap in *V. parahaemolyticus* AQ 3627

The organism was grown for 3 h in the absence (B) and presence (C) of 10 mM OH-Dap. A: standards; polyamines (each 125 nmol) were derivatized, the derivatives were dissolved in 0.1 ml of ethyl acetate, and 4 μl of this solution was injected. Peaks: 1, putrescine; 2, cadaverine; 3, OH-Dap; 4, internal standard (1,8-diaminooctane); 5, Nspd; 6, spermidine; 7, OH-Nspd. GC conditions: a glass column (0.5 m × 3 mm i.d.) packed with 0.5% KT-300 on Uniport HP; oven temperature, programmed at 10 °C from 120 to 275 °C; N_2 flow rate, 80 ml/min.

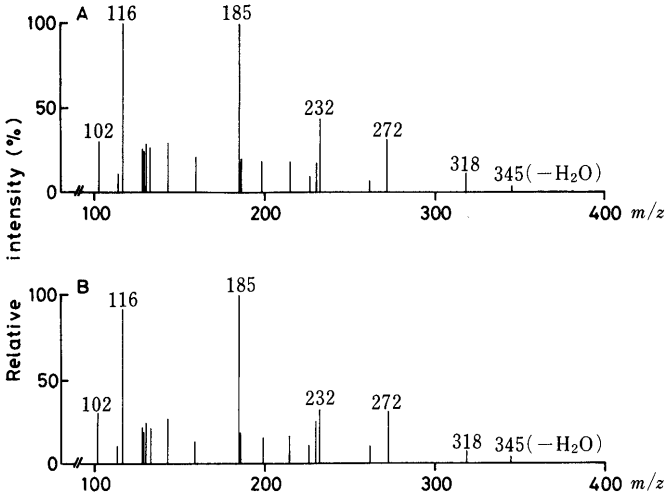

Fig. 2. Mass Spectra of the *N*-Ethoxycarbonyl Derivatives of Authentic OH-Nspd (A) and Peak 7 in Fig. 1 C(B)

the identity of the new compound, peak 7 in Fig. 1C was analyzed by GC-MS. The mass spectrum of this compound was identical to that of the authentic OH-Nspd (Fig. 2). The molecular ion peak (*m/z* 363) for $C_2H_5OCONH(CH_2)_3N(COOC_2H_5)CH_2CH(OH)CH_2NHCOOC_2H_5$ was undetectable, but the highest peak at *m/z* 345 is probably produced by the loss of H_2O from the molecular ion. The loss of $(CH_2)_2NHCOOC_2H_5$ together with H_2O from the molecular ion provides the base peak at *m/z* 185. The chirality of OH-Nspd formed has not yet been determined.

Next, the relationship between the concentration of OH-Dap in the medium and the formation of OH-Nspd was examined in more detail. The contents of OH-Nspd and Nspd were determined with cells which had been grown

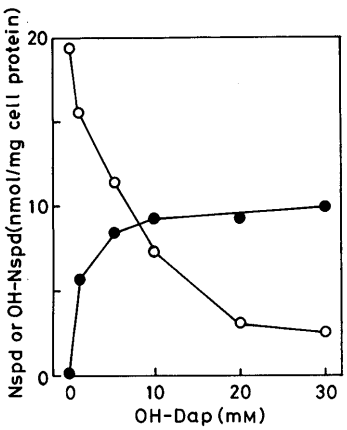

Fig. 3. Effect of Varying the Concentration of OH-Dap on the Contents of Nspd and OH-Nspd in *V. parahaemolyticus* AQ 3627

The organism was grown for 6 h. ○: Nspd; ●: OH-Nspd

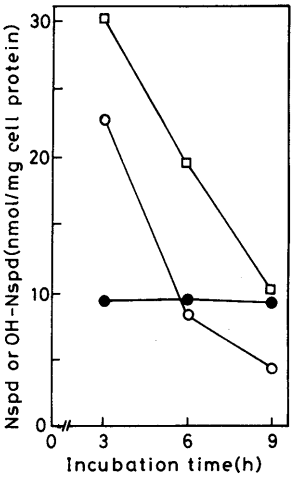

Fig. 4. Time Courses of the Contents of Nspd and OH-Nspd During Growth of *V. parahaemolyticus* AQ 3627 in the Absence or Presence of 10 mM OH-Dap

The organism was grown in batch culture (1 l) and samples (200 ml) were taken at each of the times indicated for determination of polyamines. Nspd (□) in the absence of OH-Dap; Nspd (○) and OH-Nspd (●) in the presence of OH-Dap.

for 6 h at various concentrations of OH-Dap (Fig. 3). The Nspd content decreased with increasing OH-Dap concentration, whereas growth monitored at 600 nm was not significantly affected, even at 30 mM OH-Dap. The content of spermidine, which is present naturally in only a small amount, was also reduced by OH-Dap in a similar manner to Nspd (data not shown). The OH-Nspd content reached near-maximum at 10 mM OH-Dap. Furthermore, addition of OH-Nspd up to 10 mM to the medium did not affect the growth. The role of OH-Nspd is not known, but it seems able to substitute partly for the normal polyamine, Nspd, in the maintenance of cellular growth. The time course of the contents of Nspd and OH-Nspd was examined during growth at 10 mM OH-Dap. The results are shown in Fig. 4 together with those obtained in the absence of OH-Dap. The Nspd content decreased with prolonged cultivation, while OH-Nspd appeared in the first 3 h of growth and thereafter its content was almost unchanged. Figure 4 also shows that the Nspd content in the presence of OH-Dap is lower at any growth phase than that in the absence of OH-Dap.

TABLE I. Formation of OH-Nspd from Exogenously Added NH-Dap in *Vibrio* Strains

No.	Strain	Content (nmol/mg cell protein)	
		Nspd	OH-Nspd
1	*V. alginolyticus* ATCC 17749	20.3 (24.7)[a]	5.4
2	*V. anguillarum* NCMB 6	11.6 (19.2)	6.3
3	*V. anguillarum* NCMB 828	5.1 (5.4)	2.5
4	*V. cholerae* Non O1 NCTC 4716	13.0 (26.5)	8.0
5	*V. cholerae* Non O1 RIMD 2214046	23.3 (26.5)	6.4
6	*V. cholerae* Non O1 RIMD 2214092	25.4 (30.5)	4.6
7	*V. cholerae* Non O1 RIMD 3214082	25.0 (27.9)	5.9
8	*V. parahaemolyticus* AQ 3627	7.5 (20.0)	10.2
9	*V. parahaemolyticus* OV-24	14.7 (24.9)	8.9
10	*V. parahaemolyticus* WP-1	5.2 (8.7)	17.0
11	*V. parahaemolyticus* MY 73-2	23.5 (35.7)	16.2
12	*V. parahaemolyticus* OKA A-11	6.4 (12.1)	4.1
13	*V. parahaemolyticus* OKA A-20	4.8 (8.6)	5.5
14	*V. vulnificus* CDC A3490	2.1 (9.7)	23.3
15	*V. vulnificus* CDC A6546	14.6 (23.7)	5.1
16	*V. vulnificus* CDC B2828	15.4 (17.4)	24.2
17	*V. vulnificus* CDC B9626	13.7 (22.1)	26.1
18	*V. vulnificus* CDC B3547	12.0 (24.6)	14.0
19	*V. vulnificus* E-29	5.1 (6.0)	5.3
20	*V. vulnificus* O19	7.4 (15.4)	8.3
21	*V. vulnificus* O20	9.8 (12.4)	1.2
22	*V. vulnificus* O24	20.3 (24.7)	3.3

Strains No. 5—11 were supplied by Research Institute for Microbial Diseases, Osaka University (Osaka, Japan) and strains No. 12, 13, 19—22 were isolated from sea-water in our laboratory. All strains were grown at 37 °C for 6 h in the presence of 10 mM OH-Dap, except for strains of *V. cholerae* Non O1 which were grown at 30 °C. *a*) Values in parentheses represent the contents of Nspd when the strains were grown in the absence of OH-Dap.

When OH-Dap was substituted for Dap as a substrate with the cell-free system from *V. parahaemolyticus* AQ 3627, a peak having the same retention time on the gas chromatogram as that of the authentic OH-Nspd was observed. The yield of OH-Nspd in this enzymatic reaction was only about 5% of that of Nspd obtained with Dap as a substrate, this difference in reactivity between Dap and OH-Dap explaining the requirement for the relatively high concentration of the latter. The results obtained with *V. parahaemolyticus* AQ 3627 indicate that OH-Dap is transformed into its aminopropyl derivative through the action of the enzymes responsible for biosynthesis of Nspd intrinsically present in this strain.

Other Dap analogs such as 2-methyl-Dap, 2,2-dimethyl-Dap, *N*-methyl-Dap, 2-chloro-Dap were tested for their abilities to serve as *in vivo* substrates under the same conditions as those for OH-Dap, but none were substrates, and they did not inhibit the growth at 10 mM.

If the ability to form OH-Nspd from OH-Dap is specific to *Vibrio*, the determination of its formation would serve as an additional generic identifying factor for identification of new species or isolates of *Vibrio*. In order to verify this possibility, non-vibrio organisms taxonomically related to or morphologically similar to *Vibrio* as well as other *Vibrio* strains were examined for this ability. All of the *Vibrio* strains examined were found to be capable of forming OH-Nspd (Table I). However, no such ability was detected in *Plesiomonas shigelloides*, *Aeromonas caviae*, *A. hydrophila*, *Enterobacter aerogenes*, *Enterobacter cloacae*, *Morganella morganii*, *Proteus vulgaris*, *Pseudomonas aeruginosa*, *Salmonella typhimurium* or *E. coli*. In these non-vibrio species, as in *E. coli*, putrescine and spermidine were the only polyamines detected.

In conclusion, we demonstrated the ability of vibrios to form OH-Nspd from OH-Dap supplemented in the growth medium and we propose that this ability in addition to the presence of Nspd might be useful as a reliable generic guide to the genus *Vibrio*.

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