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Variations in Cellular Polyamine Compositions and Contents of *Vibrio* Species during Growth in Media with Various NaCl Concentrations

SHIGEO YAMAMOTO,* MOTOKO YOSHIDA, HIROSHI NAKAO, MIYOKO KOYAMA, YOSHIE HASHIMOTO and SUMIO SHINODA

Faculty of Pharmaceutical Sciences, Okayama University, Tsushima, Okayama 700, Japan

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In order to obtain insight into the possible physiological significance of norspermidine [$NH_2(CH_2)_3NH(CH_2)_3NH_2$], which is characteristically present in the genus *Vibrio*, we have determined the cellular polyamine contents of *Vibrio alginolyticus* and *V. parahaemolyticus* during growth in media with various NaCl concentrations. Regardless of the medium NaCl concentration, the norspermidine content, on a per mg of protein basis, was highest in the early exponential phase and then declined with prolonged cultivation, whereas there was only a slight change in spermidine, the content of which was much lower than that of norspermidine throughout growth. Moreover, at higher NaCl concentrations, norspermidine was the major polyamine throughout growth. When the cells were suddenly exposed to media with various NaCl concentrations, the content of norspermidine increased immediately or after an initial precipitous drop with the onset of growth, but that of spermidine did not change markedly. The putrescine content of these vibrios was substantially elevated only when the cells were grown in or transferred to media with lower NaCl concentration. This pattern of polyamines suggests that, instead of spermidine, norspermidine might play some important role(s) in supporting growth, whereas putrescine may be mainly involved in maintaining cellular ionic or osmotic balance against a lower NaCl environment.

Keywords——norspermidine; putrescine; polyamine content variation; *Vibrio alginolyticus*; *Vibrio parahaemolyticus*; medium sodium chloride concentration; *Vibrio*

The biological polyamines, putrescine (Put), spermidine (Spd) and spermine, are ubiquitous in all living cells, and have been implicated in a wide variety of important biological activities including syntheses of nucleic acid and protein, promotion of cell growth and acclimation to environmental stress.[1-3] Recently, many studies involving microbial mutants deprived of polyamine synthetic activity and enzyme inhibitors have suggested that their presence is indispensable for normal growth.[4-7]

The polyamines in mesophilic eubacteria are generally Put and Spd,[8,9] whereas, in *Vibrio* species, most of which are slightly halophilic as well as mesophilic, norspermidine (Nspd) is generally present together with Put and Spd.[10] We have recently shown that Nspd in *Vibrio* is generated by a novel biosynthetic pathway utilizing 1,3-diaminopropane (DAP) as an amine precursor and aspartic β-semialdehyde, not the conventional decarboxylated S-adenosylmethionine, as an aminopropyl group donor.[11] In this context, it would be of particular interest to explore the possible biological significance of this polyamine in *Vibrio*. Therefore, in the hope that examination of cellular polyamine compositions and levels in vibrios may provide a clue to the function of Nspd, we have determined the cellular polyamines of *Vibrio alginolyticus* and *V. parahaemolyticus* at various stages during growth in media with various NaCl concentrations and during the period following sudden changes, either increase or decrease, in external salinity.

Experimental

Chemicals, Microorganisms and Media——All polyamine standards were purchased from Nakarai Chemicals, Ltd. (Kyoto, Japan) except for Nspd, which was from Aldrich Chemicals Co. (Milwaukee, Wis., U.S.A.). All other chemicals were commercial products of reagent-grade quality. The bacterial strains used were *V. alginolyticus* ATCC 17749 and *V. parahaemolyticus* AQ 3627, and they were routinely maintained on slants of nutrient agar, pH 7.0, supplemented with 2% NaCl. To obtain the inocula, the strains were precultured to the stationary phase (12 h) in a medium composed of 0.45% yeast extract (Difco), 0.1% bacto-casitione (Difco) and the basal inorganic salts[10] at 37 °C with a reciprocal shaker. The synthetic liquid medium was the same as that previously described[10] except for arginine, the concentration of which was 0.3 g/l.

Growth and Harvesting of Cells——The bacteria were grown at 37 °C with a reciprocal shaker operating at 120 strokes/min in all cases. For the deterimation of cellular polyamines in various growth phases, 20-ml aliquots of the inoculum cultures (approximately 10^9—10^{10} cells/ml) were added to 1 l of synthetic media with various NaCl concentrations in 2-l flasks and the flask cultures were incubated. At each of the times indicated, a 200-ml aliquot was aseptically withdrawn from the growing culture for polyamine determination. For the determination of cellular polyamine levels in response to a sudden change in the medium NaCl concentration, cells previously prepared by growing them for 12 h in 1 l of the synthetic medium with an NaCl concentration of 2% and by harvesting as described below were treated as follows: the cell pellets were suspended in 50 ml of an ice-cold 2% NaCl solution containing 10 mM $MgCl_2$, and immediately 10-ml aliquots of the suspension were dispensed into 4 flasks, each containing 200 ml of prewarmed fresh synthetic medium adjusted to the indicated NaCl concentration. The remaining suspension (10 ml) was used as a sample at zero time. Each suspension was incubated at 37 °C and the cells were harvested at 1-h intervals for a period of 4 h for polyamine determination.

The cells were harvested by centrifugation at 5300 × *g* for 20 min at 4°C, washed by resuspending them in 5 ml of the ice-cold NaCl solution containing 10 mM $MgCl_2$ at an NaCl concentration equal to that used in cultivation or incubation, and recentrifuged at 8000 × *g* for 10 min. Immediately after removal of the supernatant by decantation, 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.

Polyamine Determination——The remainder of the cell suspension was mixed immediately with an equal volume of 4% $HClO_4$ solution and then left at 4 °C overnight to extract polyamines completely. The precipitate was removed by centrifugation at 3500 × *g* for 10 min, washed with an additional 5 ml of 4% $HClO_4$ solution and recentrifuged. The whole of the combined supernatant was used for polyamine determination. The polyamines separated by ion-exchange column chromatography were converted to their N-ethyloxycarbonyl derivatives, which were analyzed by gas chromatography according to the method previously reported.[12] The recoveries for all polyamines added to the initial $HClO_4$ extracts were over 93% and rinsing of cells with the NaCl solution caused only slight variations in polyamine contents (<7%). All polyamine values expressed as nmol/mg cell protein are the means of two independent cultures grown in parallel under the same conditions. All experiments were repeated at least once with consistent results.

Protein Determination——Protein was determined immediately after cell harvesting by the method of Clark[13] with bovine serum albumin (Sigma) as the standard.

Results and Discussion

It has been found that the polyamine concentration of bacterial cells grown in natural media such as yeast extract or nutrient broth is markedly affected by the polyamines intrinsic to the medium.[8,14] Consequently, a synthetic medium free from polyamines was used in this study. The bacterial strains examined contained mainly Put, Nspd and Spd, and DAP was detected only in a small amount. Cadaverine, which was detected only in a minor quantity, was not included in this study.

The cultures grown in media with various NaCl concentrations within the physiological range were collected at intervals and subjected to polyamine determination. The profiles of changes in polyamine contents are presented in Fig. 1. In both organisms, there was a consistent variation in polyamine composition dependent on the medium NaCl concentration. At any NaCl concentration, the Nspd content was high in the early exponential phase of growth and then decreased with prolonged cultivation. At NaCl concentrations above 2%, the predominant polyamine was Nspd throughout growth. In contrast, Spd was detected only in small amounts at any NaCl concentration and there was only a slight variation in its content throughout growth. Put was maintained at extremely high levels

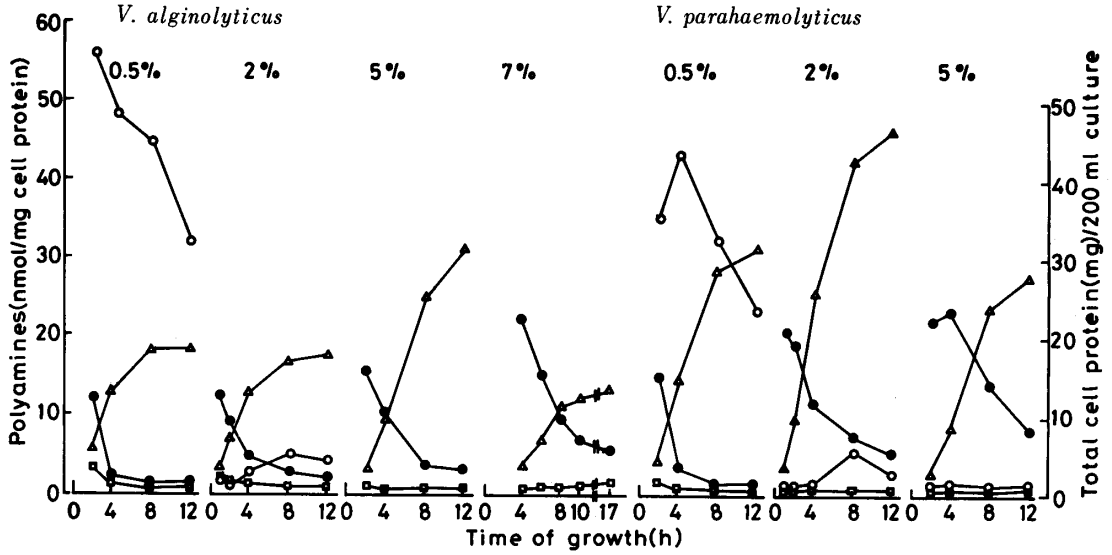

Fig. 1. Time Courses of Polyamine Compositions and Contents of *Vibrio* Species during Growth in Synthetic Media with Various NaCl Concentrations

Cells precultured in a medium of 2% NaCl were inoculated into synthetic media containing the indicated NaCl concentrations. Each point represents the mean value obtained in duplicate experiments. The putrescine contents of *V. alginolyticus* grown at 5% and 7% NaCl were less than 0.05 nmol/mg cell protein. ○, putrescine; ●, norspermidine; □, spermidine; △, total cell protein.

during growth at lower NaCl concentrations, while growth at increased NaCl concentrations resulted in a dramatic reduction in its level. It should be noted that, in *V. alginolyticus* grown at 5% and 7% NaCl, only negligible amounts of Put (<0.05 nmol/mg cell protein) could be detected. To examine the possibility that Put may be excreted into the medium, the supernatant of a culture of *V. alginolyticus* grown for 6 h at 5% NaCl was analyzed. However, Put was detected in only a trace amount. In accordance with these observations, the cells grown at 5% NaCl showed much lower specific activities of both ornithine decarboxylase and arginine decarboxylase-agmatine ureohydrolase (responsible for the formation of Put) compared with those grown at 0.5% NaCl.[15] DAP was present in small amounts (0.99—1.88 nmol/mg cell protein or less) only in the early phase of growth. This is presumably because of its immediate utilization for Nspd production.

In order to determine whether Spd exists in these bacteria as a conjugated form such as a monoacetylated derivative,[16] the supernatant fractions of the cell sonicates were treated with 6 N HCl at 108 °C for 12 h and the hydrolyzates were analyzed for polyamines. The results showed no increase in Spd content, suggesting that neither free nor conjugated Spd exists in any appreciable amount in these species.

Cells previously grown at 2% NaCl to the stationary phase, and harvested, were resuspended at the same cell concentrations in fresh media at various NaCl concentrations. This resulted in slow growth and made it easier to evaluate variations in polyamine contents at the initial stages of growth and during adaptation. The results are presented in Fig. 2. Soon after incubation at 0.5% NaCl, a rapid and remarkable elevation of Put took place in both strains with a maximum at 2 h; a more than 15-fold increase relative to the control at zero time occurred during this period. When the cells were incubated in a medium composed of only basal inorganic salts (free from amino acids) where the vibrios did not grow, the Put content also rapidly increased, but to a lesser extent (about 5-fold after 3 h). The prompt and remarkable increase of Put suggested possible involvement of this amine in growth adaptation to a lower NaCl medium. A similar phenomenon has been demonstrated not only in prokaryotes,[17,18] but also in eukaryotes,[19,20] whereby cells can rapidly restore intracellular

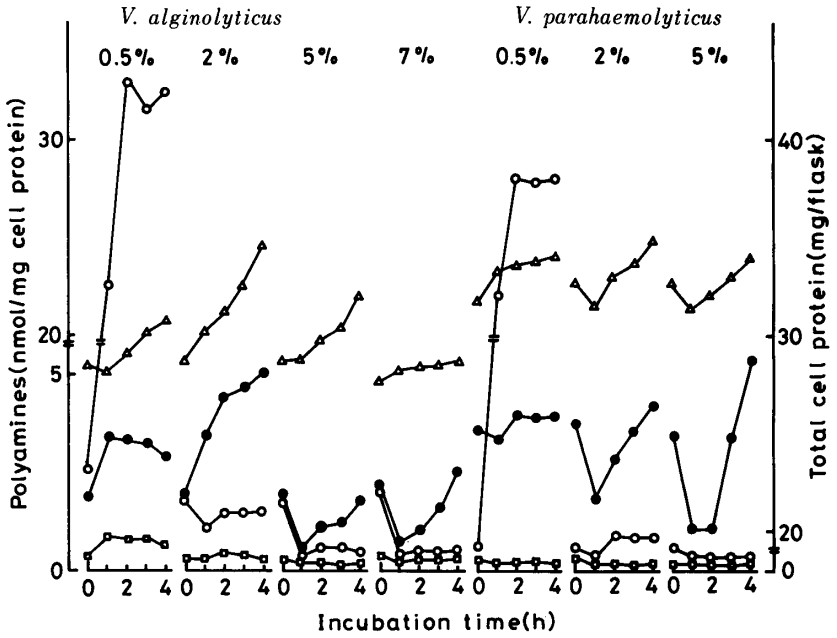

Fig. 2. Variations in Polyamine Contents of *Vibrio* Species during Growth Following Sudden Changes in Medium NaCl Concentration

Cells previously grown to the stationary phase (12 h) in synthetic medium containing 2% NaCl and harvested, were transferred to synthetic media with the indicated NaCl concentrations. Each point represents the mean value obtained in duplicate experiments. ○, putrescine; ●, norspermidine; □, spermidine; △, total cell protein.

ionic imbalance induced by a hypotonic environment.

Exposure of the cells to media of higher NaCl concentration first produced a marked decline in the Put and Nspd contents, and then Nspd, but not Put, increased characteristically with the onset of cell growth. The Spd content, on the other hand, did not change. This observation that the level of Nspd decreased during the adaptational period is not in agreement with that of Munro *et al.*[17] who found that, in *Escherichia coli*, a sudden increase in the osmolarity of the medium led to a rapid excretion of cellular Put without decreasing Spd. It is interesting to speculate that, in *Vibrio*, Nspd may be consumed in protecting the cells from a sudden high-osmotic stress and transformed to an unextractable form. When the basal amino acids were absent from the medium, the increase in Nspd was not observed at any NaCl concentration, but the initial precipitous drop in Nspd occurred at higher NaCl concentrations. These results suggested that the increase in Nspd is associated with the initiation of cell growth.

In conclusion, it appeared that, in the vibrios examined, Nspd exists as a predominant triamine species and its content is high in the early exponential phase, that is, the period of rapid growth. Furthermore, Nspd is the major polyamine in all stages of growth at higher NaCl concentrations. In view of the presence of a novel biosynthetic pathway for Nspd in *Vibrio*[11] as well as the results presented here, it seems possible that Nspd in *Vibrio* assumes the roles that are played by Spd in other organisms. Nspd has been reported to be equivalent to Spd in supporting the growth of polyamine auxotrophic strains of *E. coli*.[6] In addition, Nspd may play some specific role(s) in supporting the growth of *Vibrio* under halophilic conditions. Thus, the precise roles of this polyamine in the biological reaction(s) of *Vibrio* seem to be worthy of further study.

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