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LACK OF DETECTABLE POLYAMINES IN AN EXTREMELY HALOPHILIC BACTERIUM

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SUMMARY. Polyamines (putrescine, spermidine, spermine and other analogs) were not detectable by the dansylation procedure coupled with HPLC analysis in an extremely halophilic bacterium, Halobacterium halobium. Based on the detection limit of this analytical method, we estimated that the polyamine content in H. halobium, if present, was less than 0.06% of that of E. coli. Putrescine uptake and the metabolic conversion of ornithine or arginine to polyamines were negligible in this bacterium. In a H. halobium cell-free extract, a saturated amount of KCl was needed for poly(U) directed polyphenylalanine synthesis; neither putrescine nor spermidine could replace KCl. These results suggest that polyamines may play an insignificant role in the growth of this halophilic bacterium.

Halobacterium halobium belongs to a group of bacteria requiring near saturated solutions of NaCl for growth (1,2). When they are grown in the presence of ~4.0 M NaCl and 0.032 M KCl, the intracellular concentrations of Na^+ and K^+ are greater than 1 M and 4 M, respectively (2,3). This high internal ionic concentration is 10-20 times higher than that found in nonhalophiles. Thus the internal metabloic processes appear to occur in an environment of extremely high ionic strength.

Polyamines (putrescine, spermidine, and spermine) are naturally occurring organic cations widely distributed in living organisms (reviewed in 4-6). Due to their polycationic charges and their hydrocarbon backbone, polyamines can interact specifically with macromolecules involved in gene expression processes and have also been shown to affect a multitude of enzymatic reactions(4-7). Munro and co-workers (8,9) have reported that the putrescine content of E. coli varies inversely with the osmolarity of the medium. Cohen and associates (10) have demonstrated a five- to eightfold increase of putrescine synthesis in potassium-deficient E. coli maintained in potassium-free medium. In light of these studies it is possible that polyamine

ABBREVIATIONS: ODC, ornithine decarboxylase; TCA, trichloroacetic acid.

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metabolism in H. halobium may be altered to suit the extreme growth condition. Bayley and co-workers (11-13) have studied the cell-free protein synthetic system derived from Halobacterium cutirubrum and found that a concentration of KCl as high as 3.8 M is required for maximal activity. Whether polyamines can affect protein synthesis in a cell-free system derived from H. halobium is not known.

In view of the wide ranging effects of polyamines in nonhalophiles, it is of interest to determine the concentration and to investigate the function of polyamines in halophilic bacteria, as their intracellular ionic environment is known to differ significantly from other prokaryotes and eukaryotes. In the present study we have measured the polyamine content and putrescine uptake in H. halobium. The possible metabolic conversion of ornithine and arginine to polyamines, and the effects of polyamines on poly(U) directed polyphenylalanine synthesis in a cell-free system derived from H. halobium were also investigated. In addition, we have examined possible existence of unusual polyamines in this organism.

MATERIAL AND METHODS

Bacterial Strains. A culture of Halobacterium halobium, strain R_1 was obtained from Dr. W. Stoeckenius from the University of San Francisco. Escherichia coli ATCC 25922 was obtained from Difco Laboratores, Detroit Michigan.

Growth and Harvesting. Strain R1 of H. halobium was grown in a complex salt medium containing (per liter): KCl (2.0 g), $MgSO_4 \cdot 7H_2O$ (20.0 g), sodium citrate (3.0 g), NaCl (250.0 g) $CaCl_2$(0.2 g), $FeCl_2 \cdot 4H_2O$ (3.6 mg), $MnCl_2 \cdot 4H_2O$ (0.2 mg) and peptone (Difco 0885-1, 10.0 g). The cells were grown in a New Brunswick shaker at 38°C under constant illumination (cool white fluorescent lamp) until deep pink color developed. Cells were harvested by centrifugation at a speed of 27,000 x g for 20 min. The cell pellet was washed three times in the complex salt medium minus peptone. The washed cells were suspended in phosphate buffered saline. E. coli (ATCC25922) was grown in BBL Trypticase Soy Broth (30.0 g per liter H_2O). Cells were harvested, washed and suspended in phosphate buffered saline.

Identification and Quantitation of Polyamines. Perchloric acid soluble extracts of H. halobium and E. coli were obtained by adding 100 µl of 1N perchloric acid to a 400 µl of the cell suspensions (containing approximately 5-15 mg proteins). The suspension was sonicated and incubated at 4°C for 30 min. The precipitates were then removed by centrifugation. To a 400 µl aliquot of the perchloric acid extract, 100 µl of saturated Na_2CO_3 solution and 300 µl of saturated $NaHCO_3$ solution were added to bring the pH to 9.5. The dansylation procedure was carried out in this solution as previously described (14,15). The dansylated polyamines were then separated on a reverse-phase column (RP-18, 7 µm ODS column, Urimetrics Corp.) connected to a Beckman model 110 A pump. A Schoeffel spectrofluoromonitor (Model FS970) was used to detect the dansylated polyamines and an Integrating Chart Recorder (Linear Instruments Corp.) was used to record the chromatogram. The solvent system used was acetonitrile-H_2O (80:20, v:v). Standard dansylated polyamines were prepared according to method described by Seiler and Wiechman (14).

In Vitro Translation Assay. The H. halobium cell-free extract for in vitro translation assay was prepared exactly according to the procedure of Bayley and

Griffiths (9). Complete assay medium contained NaATP, 38 nmols; Na phosphoenol pyruvate, 0.15 µmol; NaGTP, 23 nmols; mixture of 19 amino acids, omitting phenylalanine, 0.67 nmol each; 1 µCi [^{3}H]phenylalanine; NH_4Cl, 12 µmols; NaCl, 30 µmols; KCl, 22 µmols; $MgCl_2$, 0.3 µmol and 1 mg protein in a total volume of 0.06 ml. The assay was carried at 37°C for 60 min. The amount of Poly(U) (Sigma) used in this experiment was 100µg per assay. The amount of polyphenylalanine formed was measured by standard filter paper assay method.

RESULTS AND DISCUSSION

As a comparison and to validate the quantitation procedure used in this study, parallel experiments on E. coli were carried out. Fig. 1 shows a representive HPLC chromatogram of dansylated extracts of H. halobium and E. coli. Our results indicated that all three polyamines, (putrescine, spermidine and spermine) were not detectable in the acid-soluble extract of H. halobium. In contrast an E. coli sample with a size only 2% of that of H. halobium gave prominent putrescine and spermidine peaks (Fig. 1). The amounts of putrescine and spermidine present in E. coli were estimated to be 20±5 nmol per mg protein and 3.9±1.6 nmol per mg protein, respectively. Since the detection limit of the present analytical method is about 2 pmol for each individual polyamine, the absence of polyamines in extracts of H. halobium may be relative rather than absolute. We have estimated that the

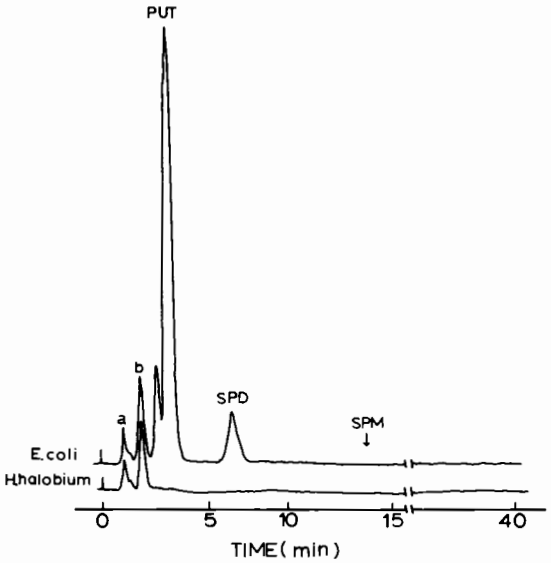

Figure 1 HPLC chromatogram of dansylated amine extracts derived from H. halobium and E. coli. PUT, SPD, and SPM indicate peak positions of dansylated putrescine, spermidine and spermine respectively. Peak a, solvent front; peak b, dansylamide. The amounts of proteins present in the E. coli and H. halobium extracts used for this HPLC analysis were 1.6 µg and 80 µg respectively.

maximal amount of polyamines in H. halobium, if present, would be 10 pmol per mg protein. This value is less than 0.06% of the total polyamines present in E. coli. Unusual polyamines have been demonstrated in some organisms grown under extreme conditions such as thermophilic bacteria (16). In order to examine possible existence of any unusual polyamine in H. halobium, we have extended the time of analysis of the HPLC up to 40 min (Fig. 1). Over the whole range of the retention time examined, no dansylated amine peak was observed suggesting a lack of detectable polyamines, either usual or unusual forms, in H. halobium.

To determine if polyamines may exist in a conjugated form in H. halobium, the cell homogenate from H. halobium was treated with 6N HCl and digested at 108°C for 20 hrs prior to subjecting it for polyamine assay. Our results showed that putrescine and spermidine were not detectable in the acid-hydrolysate (data not shown), suggesting that neither free nor bound polyamines exist in any appreciable amount in H. halobium.

Polyamine transport systems, which have been demonstrated in both prokaryotes (17) and eukaryotes (18), may be involved in the homeostasis of cellular polyamine content (19). The lack of detectable polyamines in H. halobium prompted us to examine whether a polyamine transport system is present in H. halobium. For comparative purposes, we also measured the uptake of putrescine in E. coli. As shown in Fig. 2, while there was significant uptake of putrescine in E. coli, there was little or no

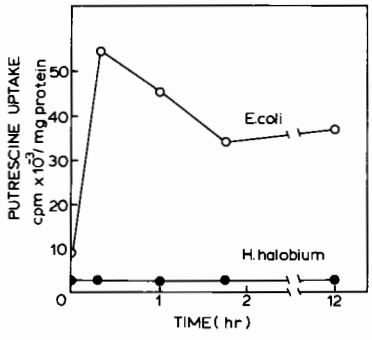

Figure 2 Putrescine uptake in H. halobium and E. coli. H. halobium and E. coli at log phase of growth were used. [^{3}H]Putrescine was added to the culture at time zero to a final concentration of 0.01 μM (0.4 μCi per ml) and the incubation was continued with shaking at 37°C. At various time points, 10 ml aliquots were transferred to pre-cooled centrifuge tubes and rapidly centrifuged at 27,000 x g for 5 min. Cells were then washed two times and resuspended in distilled water. Aliquots of the cell homogenate were used for radioactivity counting and protein determination.

uptake of putrescine in H. halobium even after a 12 hr incubation period at 37°C. In contrast to the negligible putrescine uptake, leucine uptake was demonstratable in H. halobium (data not shown). Our result on leucine uptake is in agreement with that of previous studies which showed that amino acids are taken up by H. halobium even in the presence of a high ionic strength incubation medium (20,21). It is not clear whether the lack of appreciable putrescine uptake in H. halobium is due to a lack of transport mechanism or an inactivation of transport mechanism under the present experimental condition.

Both arginine and ornithine are amino acid precursor of polyamines in various prokaryotic cells (4-6). Although polyamines were not detectable in H. halobium, the possibility that H. halobium may possess enzyme systems which can convert ornithine or arginine into polyamines was examined. H. halobium culture was incubated with [^{3}H]ornithine or [^{3}H]arginine (0.5μCi per ml) for various periods of time up to 24 hrs , cells were harvested at various time periods and processed for polyamine quantitation by dansylation of cell extract followed by analysis on thin layer chromatography. After 24 hrs of incubation more than 70% of added radioactivity ([^{3}H]ornithine or [^{3}H]arginine) was taken up by cells. However, none of the radioactivity was recovered in spots corresponding to authentic dansylated polyamines, suggesting none or minimal metabolic conversion of either ornithine or arginine or polyamines. In accordance to these observations we could not detect ornithine decarboxylase (ODC) activity in H. halobium whether assayed in the absence or in the presence of 3.8M KCl (data not shown).

A cell-free protein synthetic system was set up from H. halobium according to the procedure of Bayley and Griffiths (9). The addition of poly(U) to this cell-free system gave a three-fold stimulation of the incorporation of [^{3}H]phenylalanine into material insoluble in hot trichloroacetic acid (10%) (Table 1). In agreement with the observation of Bayley and Griffiths (9) the cell-free protein synthetic system derived from H. halobium had a specific requirement for high KCl concentration; approximately 4.2 M of KCl was necessary to achieve a maximal rate of incorporation of radioactivity. Exogenously added polyamines did not further stimulate the polypeptide synthesis (Table 1). Neither

TABLE 1: Effects of Polyamines on the Poly(U) Directed Polyphenylalanine Synthesis in H. halobium Cell-Free Extract

	Radioactivity (cpm) Incorporated in Hot TCA residue	
	-Poly(U)	+Poly(U)
Complete[a]	259	245
Complete + 1mM Putrescine[b]	223	231
Complete + 1mM Spermidine	200	203
Complete + 1.26M Putrescine	104	118
Complete + 0.66M Spermidine	110	124
Complete + 4.2M KCl[c]	780	2297
Complete + 4.2M KCl + 1mM Spermidine	760	2108

a. The composition of the complete assay medium was described in METHODS.
b. Putrescine and spermidine were used in their hydrochloride salt form.
c. To achieve saturation of KCl in the assay mixture 20 mg solid KCl was added to the assay mixture.

putrescine nor spermidine could replace KCl in this cell-free system (Table 1).

The data presented above indicate that polyamines were not detectable in H. halobium by the present analytical method (detection limit 2 pmol) suggesting that polyamines were either nonexistent or present in an amount less than 10 pmol per mg protein (or 2 nmol per g wet weight). A polyamine deficient mutant of E. coli has been isolated by Tabors and their co-workers (22). The residual putrescine content in that mutant has been estimated to be less than 39 nmol per g wet weight. Thus even if polyamines existed in H. halobium, the contents would be considerably lower than that in the polyamine deficient mutant of E. coli. Our data also indicate that several aspects of polyamine metabolism (i.e. putrescine uptake, metabolic conversions from ornithine and arginine to polyamines, ODC activity and effects of polyamines on in vitro translation assay) were either nonexistent or lower than the limits of resolution in H. halobium.

Although polyamines appear to be ubiquitous and play important roles in the growth regulation of both prokaryotes and eukaryotes; the data presented above suggest that at least in H. halobium, with an internal K^+ concentration as high as 4M, the presence of an appreciable amount of polyamines may not be necessary to sustain its growth and cellular physiology.

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