Vol. 150, No. 1, 1988 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
January 15, 1988 Pages 185-191

POLYAMINE METABOLISM IN AN OBLIGATELY ALKALOPHILIC BACILLUS ALCALOPHILUS THAT GROWS AT PH 11.0

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Received November 25, 1987

Summary: Bacillus alcalophilus, an obligately alkalophilic bacterium that grows at pH 11.0, has an intracellular pH of 9.5 or less. Unlike all other living organisms, polyamines (putrescine, spermidine and spermine) in B. alcalophilus, if present, will be largely unprotonated. HPLC analysis indicated that spermidine is the major polyamine in B. alcalophilus, accounting for more than 90% of total polyamines, and the level of spermidine varies during growth. Ornithine decarboxylase activity was not detectable in B. alcalophilus under all conditions examined. When [^{3}H]arginine was added to the culture medium, the radioactivity can be recovered from polyamine pool; the distribution is 3% for putrescine, 94% for spermidine, and 3% for spermine, suggesting the presence of arginine pathway for polyamine biosynthesis. The polyamine transport system in B. alcalphilus appears to be Na^+-dependent and is highly sensitive to the inhibition of gramicidin S and valinomycin.

Polyamines (putrescine, spermidine, and spermine) are naturally occurring organic cations widely distributed in living organisms [1,2]. Abundant evidence in literature indicates that polyamines are essential for growth in both prokaryotes and eukaryotes [3-5]. Many metabolic pathways are involved in modulating polyamine contents in cells [3-5]. Polyamines interact with proteins and nucleic acids and have been shown to affect a wide spectrum of biological reactions in vitro [3-6]. Because of the abundance and versatility of polyamines in interacting with other biological molecules, it has been difficult to define precisely the function of individual polyamines in vivo. Recently, attention has been directed to the study of polyamine metabolism in unusual organisms such as extreme halophilic bacteria [7,8] and thermophilic bacteria [9,10] in the hope that comparative studies may shed more light on

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the function and physiological significance of polyamines.

Bacillus alcalophilus belongs to a group of bacteria that have an optimal pH of growth at 10.5 or above, and a cytoplasmic pH of 9.5 or below [11]. Values of the dissociation constants (pKa) for various polyamines are in the ranges of 8.5 to 10 [12,13]. Thus, for spermidine, each nitrogen is fully protonated at pH 7.0 but only ~30% will be protonated at pH 10.5 [13]. In view of this, polyamines in B. alcalophilus, if present, will be much less polybasic than that in other organisms. If the polybasic property of polyamines is a key to their physiological function, differences in polyamine metabolism between extreme alkalophiles and other organisms may be expected.

In this paper, we have determined the polyamine contents and examined the biosynthetic pathways of polyamines in B. alcalophilus. In view of the "reversed" proton gradient [11,15] in this organism, we also studied its polyamine transport system.

MATERIAL AND METHODS

Bacterial Strains. A culture of Bacillus alcalophilus was a gift of Dr. T.A. Krulwich, Mt. Sinai Medical School, NY. Escherichia coli (ATCC 25922) was previously obtained from Difco Lab, Detroit, MI.

Growth and Harvesting. The basal growth medium for B. alcalophilus consisted of 25mM Na_2CO_3 buffer, 1mM KH_2PO_4/K_2HPO_4, 0.1% (w/v) $(NH_4)_2SO_4$, and 0.1 mM $MgSO_4 \cdot 7H_2O$ in H_2O; KOH was used to obtain the final pH of 11.0 prior to the addition of $MgSO_4$. This basal medium was supplemented with 0.1% yeast extract, 1% (v/v) trace metal solution [14], and 50 mM sodium L-malate added from separate sterile solutions [15]. Samples of 5 to 10 ml were harvested by centrifugation at 27,000xg for 10 min; samples of less than 1.5 ml were centrifuged at 12,000 rpm for 3 min using Beckman Microfuge. Cell pellets were washed twice with basal growth medium. E. coli was grown in BBL Trypticase Soy Broth. Cells were harvested, washed and suspended in phosphate buffered saline unless indicated otherwise.

Identification and Quantitation of Polyamines. The procedure of Seiler and Weichman [16] was used to dansylate polyamines extracted from B. alcalophilus and E. coli. The dansylated polyamines in cell extracts were separated by HPLC on a reverse-phase columnm and quantitated by a Schoeffel spectrofluorimeter as we previously described [7,17]. For metabolic conversion experiments, dansylated polyamines were separated on Whatman K5 silica gel TLC plates in $CHCl_3$: $(C_2H_5)_3N$ (5:1 v/v) [18]. Spots corresponding to standard polyamines were scraped from the plate for radioactive counting.

Assay for Ornithine Decarboxylase (ODC) Activity. Aliquots of 5 ml E. coli or 10 ml B. alcalophilus cells were removed from cultures at various times after a 1:10 dilution with fresh growth medium, centrifuged, washed, and resuspended in a Tris buffer (50 mM Tris, pH 7.4 or 9.5) containg 50 μM pyridoxal-5'-phosphate, 0.1 mM EDTA, and 5 mM dithiothreitol (TEPD buffer). The cells were

sonicated and centrifuged at 12,000 rpm for 5 min. The supernatant was used for assay of ODC activity by a procedure previously described [17].

Putrescine Uptake. B. alcalophilus cells were washed with basal growth medium and resuspended in the same medium containing [^{3}H]putrescine and various drugs. At designated time points, 1.5 ml of cell suspension was removed, filtered (Millipore 1225 Sampling Manifold) through Millipore nitrocellulose filters, and washed. The filters were air dried and counted in Aqualyte by a liquid scintillation spectrometer.

RESULTS AND DISCUSSION

As an initial step to investigate polyamine metabolism in B. alcalophilus, we examined the levels of individual polyamine (putrescine, spermidine and spermine) in this organism during its growth at pH 11.0. Results obtained from two separate experiments were shown in Fig. 1. Spermidine was the major polyamine in B. alcalophilus, accounting for more than 90% of total polamines. In contrast, 80% of total polyamines in E. coli is putrescine [19]. Spermidine level in B. alcalophilus increased and reached a peak level 1-2 hrs after the initiation of growth. Levels of putrescine and/or spermine, however, remained either low or barely detectable throughout the entire time course of growth (Fig. 1). Since most prokaryotic cells contain only putrescine and spermidine [1-3], the presence of minute quantity of spermine in B. alcalophilus could be due to contamination from yeast extracts in the supplemented growth medium. To test this possibility, bacterial cultures were

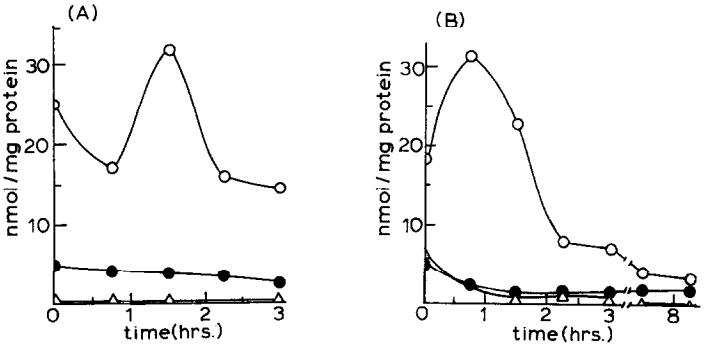

Fig.1. Changes of polyamine contents in B. alcalophilus during growth. Overnight cultures were diluted 10-fold with fresh supplemented growth medium for (A) 1.5 hrs or (B) 3 hrs and further diluted 5-fold with fresh supplemented growth medium. Polyamine contents in two cultures were then determined at various times after the second dilution as described under MATERIAL and METHODS. Putrescine (△), spermidine (○). spermine (●).

incubated with [^{3}H]arginine for various times and the distribution of radioactivity in polyamine pool was analyzed by TLC and liquid scintillation spectrometry. For a comparison, we also carried out a similar metabolic conversion experiment with E. coli. Radioactivity in the polyamine pool of B. alcalophilus appeared in putrescine, spermidine, and spermine with more than 90% of this radioactivity recovered as spermidine (Table 1). In contrast, more than 95% of the radioactivity in the polyamine pool of E. coli appeared as putrescine, and no radioactivity was detectable in the spot corresponding to spermine (Table 1). Thus, our data suggest the presence of biosynthetic pathway for spermine in B. alcalophilus but not in E. coli.

In prokaryotes, two synthetic pathways are known to be involved in putresine production, one uses arginine and the other uses ornithine as precursor [20]. The key enzyme for the ornithine pathway is ornithine decarboxylase (ODC, EC 4.1.1.17). Specific ODC activity was induced in E.coli during growth but was not detected in B. alcalophilus throughout the entire period of growth, whether the assay was carried out at pH 7.2 or 9.5 (Fig. 2).

TABLE 1

Metabolic Conversion of Arginine to Polyamines in B. alcalophilus

Sample	Putrescine	Spermidine	Spermine
	(Counts per minute per spot)		
B. alcalophilus			
3-hour	7±2[a]	232±94	8±2
6-hour	10±6	202±108	8±7
E. coli			
3-hour	416±252	12±12	ND[b]
6-hour	477±12	10±2	ND

[a]The number is the difference of counts between dansylated polyamine spots and backgrounds. Each number represents an average of two separate experiments.

[b]ND, nondetectable.

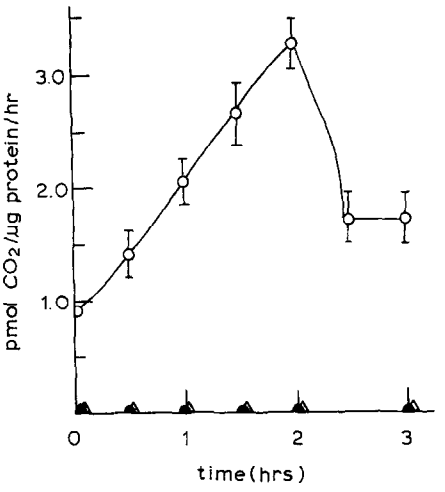

Fig.2. Ornithine decarboxylase activity in E. coli (○) and B. alcalophilus (● , △). Overnight cultures of E. coli and B. alcalophilus were diluted 10-fold with fresh supplemented growth medium. Aliquotes of cells were removed at various times after dilution for ODC assay. For B. alcalophilus, ODC activity was measured at both pH 7.2 (●) and pH 9.5 (△).

In accordance with this observation, no appreciable metabolic conversion was observed in B. alcalophilus when [^{3}H]ornithine was added to the growth medium (data not shown). Together, these results suggest that, unlike most other prokaryotes, the ornithine pathway in B. alcalophilus is either missing or not operational.

An additional source of polyamines in living organisms comes from the transport of polyamines from growth medium [3,20]. Active transport system for polyamines have been described for E. coli [19] and cultured mammalian cells [21]. Because of the low proton motive force in B. alcalophilus [11], it is generally thought that this organism may possess unusual transport systems for amino acids and other nutrients. Studies carried out by Krulwich and associates have demonstrated that obligately alkalophilic bacteria possess Na^+/solute symports rather than H^+/solute symports that are typically found in other bacteria [11,15]. In light of their work, and considering the chemical nature of polyamines at pH 11, it is of interest to examine the polyamine transport system in B. alcalophilus. We found that putrescine uptake in B. alcalophilus followed a Michaelis-Menten kinetics with apparent Km and Vmax values of 3.3 µM and 31 nmol/mg protein/hr, respectively, (data not shown),

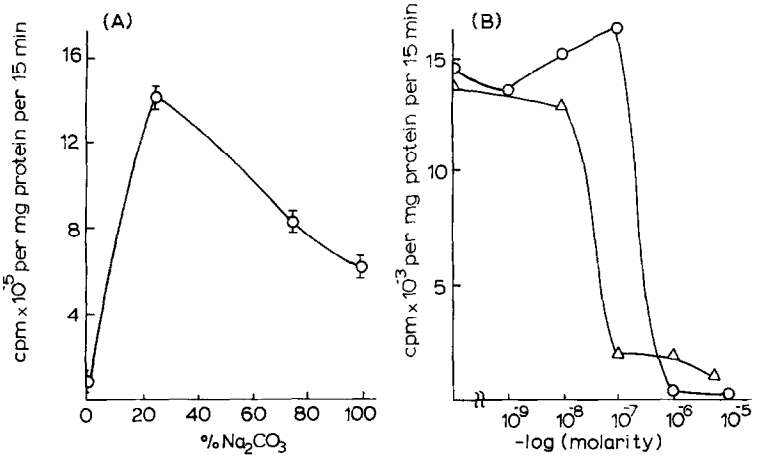

Fig.3. The sodium dependency and the effect of ionophores on putrescine transport in B. alcalophilus. (A) The uptake of [^{3}H]putrescine (0.5 μCi/ml, 5 μM) in B. alcalophilus was measured for 15 min in the basal growth medium where Na_2CO_3 was displaced by K_2CO_3. At the end of incubation at 36°C, cells were washed and cell-associated radioactivity was determined as described under MATERIAL and METHODS. Each point represents an average of duplicate meaurements. (B) The uptake of [^{3}H]putrescine (10 nCi/ml, 5 μM) in B. alcalophilus was measured for 15 min in the basal growth medium containing gramicidin S (-0-) or valinomycin (-Δ-) at various concentrations.

suggesting the presence of a carrier mediated transport system. This transport system exhibited a biphasic Na^+-dependency with maximal uptake occurring at 30% Na_2CO_3 (Fig. 3A). Ionophores such as gramicidin S and valinomycin, known to disrupt the normally existing transmembrane Na^+/K^+ balance, were found to be potent inhibitors of putrescine uptake (Fig. 3B). Thus, the polyamine transport in B. alcalophilus appears to depend on an electrochemical gradient, possibly via a Na^+/putrescine symport mechanism.

Polyamine metabolism in extremely alkalophilic bacteria has not been previously investigated. Our results indicated that spermidine is the major polyamine in B. alcalophilus and that its biosynthesis depends solely on arginine pathway. We also showed that putrescine uptake in this organism involves a carrier system and an electrochemical gradient. These data should serve as a basis for further characterization of the regulation of polyamine metabolism and functions of polyamines in this unusual organism.

ACKNOWLEDGMENT

We are grateful to Drs. T.A. Krulwich and A.A. Guffanti for providing us B. alcalophilus strain and helping us to establish the culture in our laboratory. This work was supported by grants from Charles and Johanna Busch Memorial Fund

and Rutgers Research Council. S.C. was a Henry Rutgers Scholar during the tenure of this work.

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