POLYAMINE METABOLISM IN AN OBLIGATORILY ALKALOPHILIC

BACILLUS ALKALOPHILUS THAT GROWS AT pH 11.0

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Summary: Bacillus alkalophilus, an obligately alkaliphilic 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.
alkalophilus, if present, will be largely unperturbed. HPLC analysis
indicated that spermine is the major polyamine in B. alkalophilus,
accounting for more than 90% of total polyamines, and the level of spermidine
varies during growth. Ornithine decarboxylase activity was not detectable in
B. alkalophilus under all conditions examined. When L-arginine was added to
the culture medium, the radioactivity can be recovered from polyamine pool;
the distribution is 7% for putrescine, 90% for spermidine, and 3% for
spermine, suggesting the presence of arginine pathway for polyamine
biosynthesis. The polyamine transport system in B. alkalophilus appears to be
Na+-dependent and is highly sensitive to the inhibition of gramicidin S and

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 vivo [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
the function and physiological significance of polyanines.

*R. alcalophilus* belongs to a group of bacteria that have an optimal pH of growth at 10.5 or above, and a cytochrome pH of 9.5 or below [11]. Values of the dissociation constants (pKa) for various polyanines 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, polyanines in *R. alcalophilus* if present, will be much less polybasic than that in other organisms. If the polybasic property of polyanine is a key to their physiological function, differences in polyanine metabolism between *alcalophilus* and other organisms may be expected.

In this paper, we have determined the polyanine contents and examined the biosynthetic pathways of polyanines in *R. alcalophilus*. In view of the "reversed" proton gradient (11,12) in this organism, we also studied its polyanine transport system.

**MATERIAL AND METHODS**

**Bacterial Strains.** A culture of *R. alcalophilus* was a gift of Dr. T.A. Kruclwich, Mt. Sinai Medical School, NY. *Bacillus subtilis* (ATCC 29922) was previously obtained from Biceps Lab, Detroit, MI.  

**Growth and Harvesting.** The basal growth medium for *R. alcalophilus* consisted of 25 mM NaNO₃ buffer, 100 mM potassium glutamate, 0.14 M (w/v) (NH₄)₂SO₄, and 0.1 M MgSO₄·7 H₂O in H₂O; KNO₃ was used to obtain the final pH of 11.0 prior to the addition of MgSO₄. This basal medium was supplemented with 0.1% yeast extract, 1% (v/v) trace metal solution [14], and 50 mM sodium L-lactate added from separate sterile solutions [15]. Samples of 5 to 10 ml were harvested by centrifugation at 27,000 rpm 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 ERL Trypticase Soy Broth. Cells were harvested, washed and suspended in phosphate buffered saline unless indicated otherwise.

**Identification and Quantitation of Polyanines.** The procedure of Beller and Gleichman [16] was used to demulsify polyanines extracted from *R. alcalophilus* and *E. coli*. The demulsified polyanines in cell extracts were separated by HPLC on a reverse-phase column and quantitated by a Scheffer spectrophotometer as we previously described (7,17). For metabolic conversion experiments, demulsified polyanines were separated on Whatman K5 filter paper TLC plates in CH₃Cl·(CH₂)₄NO (5:1 v/v) [18]. Spots corresponding to standard polyanines were scraped from the plate for radioactive counting.

**Assay for Ornthidine Dehydrogenase (ODDH) Activity.** Aliquots of 5 ml of *E. coli* or 10 ml of *R. 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 9.3) containing 50 mM benzidine-5'-phosphate, 0.1 M EPPS, and 5 mM dithiothreitol (TDE buffer). The cells were

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Fig. 1. Changes of polyanine. Overlaid cultures - growth medium or t with fresh supplemented cultures were then a dilution as described spermidine (O).
suspended 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 Deprote.* B. alcalophilus cells were washed with basal growth medium and resuspended in the same medium containing 5% putrescine and various drugs. At designated time points, 1.5 ml of cell suspension was removed, filtered (Millipore 1225 Sampling Manifold) through Millipore microcellulose 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 polyamines (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 94% of total polyamines. 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

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**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 (●).

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incubated with [14C]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 40% of this radioactivity recovered as spermine (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 putrescine 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 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

<table>
<thead>
<tr>
<th>Sample</th>
<th>Putrescine</th>
<th>Spermidine</th>
<th>Spermine</th>
</tr>
</thead>
<tbody>
<tr>
<td>B. alcalophilus</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3-hour</td>
<td>73.6</td>
<td>232.96</td>
<td>8.2</td>
</tr>
<tr>
<td>4-hour</td>
<td>104.6</td>
<td>202.408</td>
<td>8.3</td>
</tr>
<tr>
<td>E. coli</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3-hour</td>
<td>416.732</td>
<td>121.32</td>
<td>ND</td>
</tr>
<tr>
<td>5-hour</td>
<td>472.12</td>
<td>104.2</td>
<td>ND</td>
</tr>
</tbody>
</table>

*The number in the difference of counts between denatured polyamine spots and background. Each number represents an average of two separate experiments.

ND, not detectable.

In accordance with this observation in B. alcalophilus (data not shown), together with the lack of ornithine decarboxylase activity, the ornithine pathway is not operational.

An additional source of polyamines for E. coli cells [21]. Because of this, it may be that other systems for amino acids and solute transport may be responsible for polyamines such as spermine in prokaryotes. The nature of polyamines at pH 9.5 and 31 mM values of 3.3 μM and 31 mM...
Fig. 7. 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. Aliquots 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 9.3 (△).

In accordance with this observation, no appreciable metabolic conversion was observed in B. alcalophilus when [NH₃]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 [1,20]. Active transport systems 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 Kruwich and associates have demonstrated that obligately alcalophilic 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).
suggesting the presence of a carrier mediated transport system. This transport system exhibited a biphasic Na⁺ dependency with maximal uptake occurring at 30mM Na₂CO₃ (Fig. 3A). Isocitrates such as granicidin 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 $\mathcal{E}$. alcalophilus appears to depend on an electrochemical gradient, possibly via a Na⁺/putrescine support mechanism.

Polyamine metabolism in extremely alkaliphilic bacteria has not been previously investigated. Our results indicated that spermidine is the major polyamine in $\mathcal{E}$. alcalophilus and that its biosynthesis depends solely on an 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.

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REFERENCES