ISSN 0006-2979, Biochemistry (Moscow), 2006, Vol. 71, No. 4, pp. 437-440. © Pleiades Publishing, Inc., 2006.
Original Russian Text © L. S. Buzoleva, A. M. Krivosheeva, A. S. Isachenko, L. M. Somova, G. P. Somov, 2006, published in Biokhimiya, 2006, Vol. 71, No. 4, pp. 541-545.

Effect of Temperature on Synthesis of Polyphosphates in *Yersinia pseudotuberculosis* and *Listeria monocytogenes* under Starvation Conditions

L. S. Buzoleva*, A. M. Krivosheeva, A. S. Isachenko, L. M. Somova, and G. P. Somov

Institute of Epidemiology and Microbiology, Siberian Branch of the Russian Academy of Sciences, ul. Sel'skaya 1, 690087 Vladivostok, Russia; fax: 7 (4232) 452-327; E-mail: buzoleva@mail.ru

Received March 3, 2005

Abstract—It was found that at low temperature (6-8°C) in the absence of nitrogen supply and at the presence of phosphate ions in the medium, *Yersinia pseudotuberculosis* and *Listeria monocytogenes* are able to actively synthesize reserve substances as polyphosphates. Most of the bacterial polyphosphates are alkali-soluble, especially at the preliminary stage of cell growth (lag-phase). This is proved by electron microscopic studies of ultrastructure of model microorganisms. During a long starvation period under conditions of carbon and energy source deficit, *L. monocytogenes* and *Y. pseudotuberculosis* consume this biopolymer for biosynthetic and bioenergetic processes.

DOI: 10.1134/S0006297906040122

Key words: *Yersinia pseudotuberculosis*, *Listeria monocytogenes*, polyphosphates, starvation conditions, temperature, ultrastructure

The saprozoonosis agents *Yersinia pseudotuberculosis* and *Listeria monocytogenes* can survive for a long time in environmental niches under poor trophic conditions and widely varying temperature [1, 2]. The ability of these bacteria to multiply under these conditions suggests the existence of special adaptation mechanisms that allow them to withstand unfavorable abiotic environmental factors [2].

It is known that survival under poor trophic environmental conditions is explained by the presence of reserve substances in many saprophytic bacteria; under starvation conditions, the bacteria use these reserve substances as carbon and energy source [3, 4]. Biopolymers, which are accumulated in cells under unbalanced growth when synthesis of proteins and nucleic acids is limited, act as reserve substances [5].

Among various reserve polymers synthesized by bacterial cells, polyphosphates are of interest first of all as a source of one of the most important elements—phosphorus; the latter is a constituent of the main subunits of life: amino acids, nucleotides, sugars, phospholipids, and so on [6]. Accumulation of polyphosphates in bacteria is not only a regular natural phenomenon, but also a process with rather fine regulation. Polyphosphates can be used as energy source, phosphate donors for sugars, and chelators for bivalent cations [7]. The data indicate that polyphosphates play an important regulatory role in bacterial response to stress and unfavorable conditions. These polymers directly participate in regulation of nucleic acid synthesis and their hydrolysis in bacteria [8], in bacterial cell response to nitrogen starvation, and also in adaptation to stationary growth phase and stress [9].

The goal of this work was to study possible accumulation of polyphosphates and their role in maintenance of vitality of *Y. pseudotuberculosis* and *L. monocytogenes* populations at various temperatures in media limited in the main nutrition elements.

MATERIALS AND METHODS

For this study, we used *Y. pseudotuberculosis* (512, H-3515, H-2781-1b serovar) and *L. monocytogenes* (10CN, 2L, P-1/2a serovar) strains characterized by typical cultural and morphological, biochemical, and antigenic properties. *Yersinia pseudotuberculosis* strains were obtained from the museum of Yersiniosis and Pseudotuberculosis Center (Institute of Epidemiology and Microbiology, Siberian Branch of the Russian Academy of Sciences, Vladivostok) and *L. monocytogenes*

* To whom correspondence should be addressed.

strains from Control Institute of Veterinary Preparations (Moscow) and the Gamaleya Epidemiology and Microbiology Institute of the Russian Academy of Medical Sciences (Moscow).

Distilled water, physiological solution, and phosphate buffer were used as starvation media; they were inoculated with *Listeria* and pseudotuberculosis bacteria. To obtain the needed amount of biomass, we used rather high initial density of living bacteria (0.5 mg/ml); this is due to the sensitivity of the method [10].

Periodic cultivation was performed at 18-20 and 6-8°C, samples for plotting the growth curve for the studied bacteria and assay of total amount of polyphosphates at various growth phases were taken every day. Polyphosphate fractions were isolated by the Langen–Liss method as modified by Kulaev et al. [10]. Polyphosphates were sequentially extracted from biomass by acidic (0.5 M $HClO_4$ at 0°C), salt (5 g $NaClO_4$ and 5 mg of 1 M $HClO_4$ for every 10 g of initial material), and alkaline (cold 0.05 M NaOH) solutions and the following fractions were thus obtained: acid-soluble (PP1), salt-soluble (PP2), and alkali-soluble (PP3) polyphosphates. Amount of labile phosphorus in all polyphosphate fractions was determined by the orthophosphate content formed as a result of acidic hydrolysis in the presence of 1 M HCl for 10 min at 100°C.

For electron microscopic studies, bacterial samples were fixed according to Ito and Karnovski [11] with additional fixation in 1% OsO_4 solution in 0.2 M cacodylate buffer, pH 7.3, sequentially dewatered in alcohol at increasing concentrations (50, 60, and 70% at 4°C and 90 and 100% at 20°C for 15 min in each portion) and placed in epon araldite. Ultrafine slices were prepared using an LKB-V ultramicrotome, contrasted with saturated uranyl acetate solution in 8% buffered formalin and additionally with 0.02% lead citrate. Preparations were studied using a JEM-100S transmission electron microscope from Jeol (Japan) at accelerating voltage 80 kV.

The data were statistically processed using a standard Microsoft Excel version 7.0 package. On electron microscopic studies, number of bacterial cells which contained structures not revealed in control culture was estimated at magnification ×10,000. To reveal the difference in compared data sets (culture version varying with temperature, with various polyphosphate content), the Student *t*-criterion was applied.

RESULTS AND DISCUSSION

It should be noted that when growing *L. monocytogenes* and *Y. pseudotuberculosis* on distilled water, the polyphosphate content was almost the same as when physiological solution was used. On cultivation in phosphate buffer, significant difference in the total amount of polyphosphates in *L. monocytogenes* and *Y. pseudotuberculosis* was not detected: 500-550 and 450-500 μg phosphorus per g dry cell mass ($p > 0.001$), respectively. The maximal amount of this polymer in *E. coli* was about 1000 μg/g [7].

It is known that the presence of phosphate ions in culture medium stimulates synthesis of polyphosphates in bacterial cells [7]. In support of this statement, the total amount of polyphosphates in the studied bacteria cultivated in phosphate buffer was 2-2.5 times higher than in bacteria cultivated in physiological solution. This may possibly explain the use of phosphate buffer as an efficient storage medium, as suggested by Paterson and Cook in 1963 for isolation of pseudotuberculosis bacteria.

The data allow correlation of the processes of polyphosphate storage with bacterial growth and multiplication; this is consistent with earlier data [7, 12]. The dynamics of polyphosphate accumulation by the studied bacteria in phosphate buffer is presented in Fig. 1. The maximal amount of polyphosphates in *L. monocytogenes* and *Y. pseudotuberculosis* cells is formed in the early lag phase. Then they are stored during preliminary and logarithmic phases (during active multiplication of bacteria). In the stationary growth phase, some accumulation of polyphosphates is again observed. Consequently,

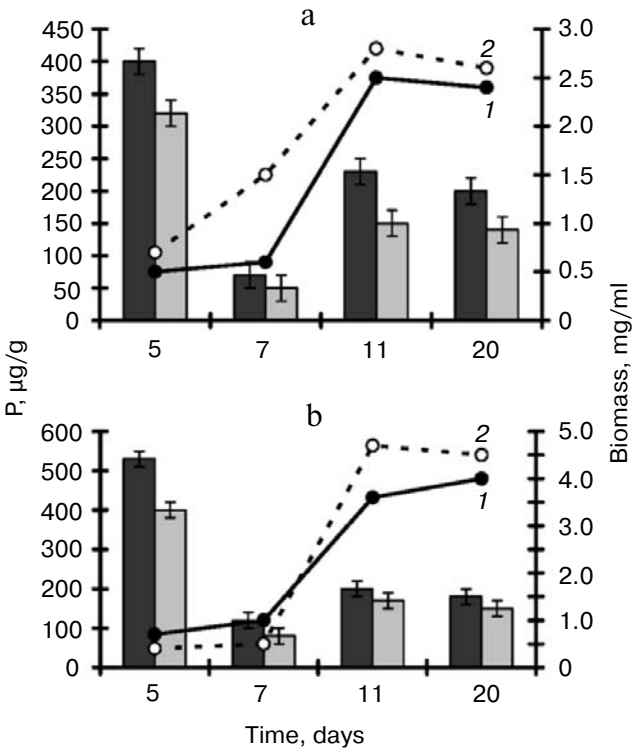

Fig. 1. Total polyphosphate content (calculated for dry cell mass) in *Y. pseudotuberculosis* (strain 512) (a) and *L. monocytogenes* (strain 10CN) (b) cells under periodic cultivation in phosphate buffer, pH 7.2-7.4, at 6-8°C (black columns) and 18-20°C (gray columns) ($n = 3$, $p < 0.01$). Curves *1* and *2* correspond to biomass changes at 6-8 and 18-20°C, respectively.

increased amount of polyphosphates preceded intensive culture growth, and then they were again consumed by growing and dividing cells. Analogous results were obtained for *E. coli* and *Klebsiella aerogenes* under amino acid starvation conditions [12].

It should be noted that at lower cultivation temperature (6-8°C) polyphosphate content increased in both *L. monocytogenes* and *Y. pseudotuberculosis* cells compared with that at room temperature (18-20°C) (Fig. 1).

In physiological solution, total polyphosphate content in *Y. pseudotuberculosis* did not increase significantly in the absence of biomass increase (Fig. 2a). *Listeria monocytogenes* culture accumulated this polymer during the interval of the 7-10th growth day, and this also was not related to the multiplication process (Fig. 2b). Decrease in polyphosphate content by the 20th day may be rationalized by possible consumption of this polymer for maintaining culture vitality under starvation conditions.

Consequently, the main function of polyphosphates in vital activity of the studied bacteria is intracellular reservation of significant amounts of activated phosphate

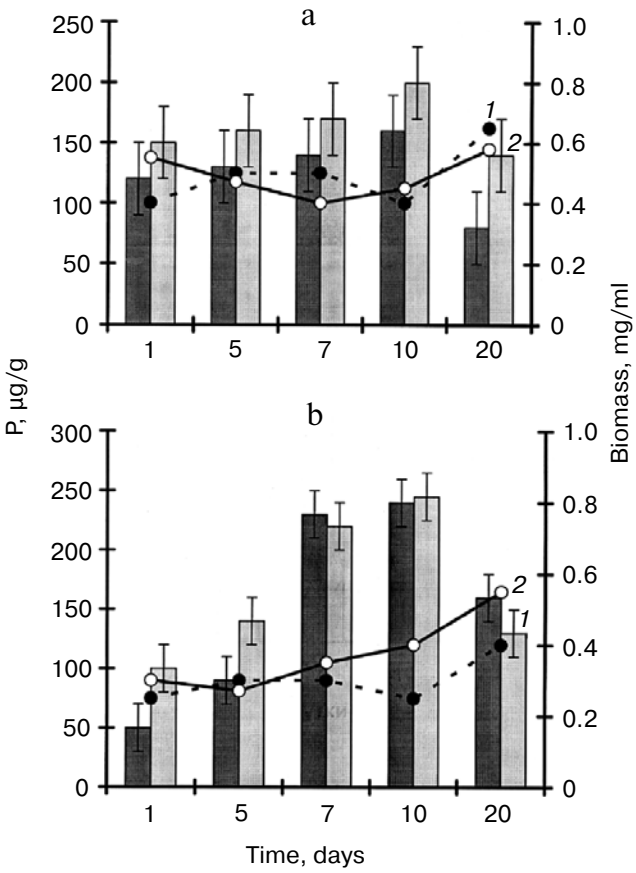

Fig. 2. Total polyphosphate content (calculated for dry cell mass) in *Y. pseudotuberculosis* (strain 3515) (a) and *L. monocytogenes* (strain 10CN) (b) cells under periodic cultivation in physiological solution at 6-8°C (black columns) and 18-20°C (gray columns) ($n = 3$, $p < 0.01$). Curves *1* and *2* correspond to biomass changes at 6-8 and 18-20°C, respectively.

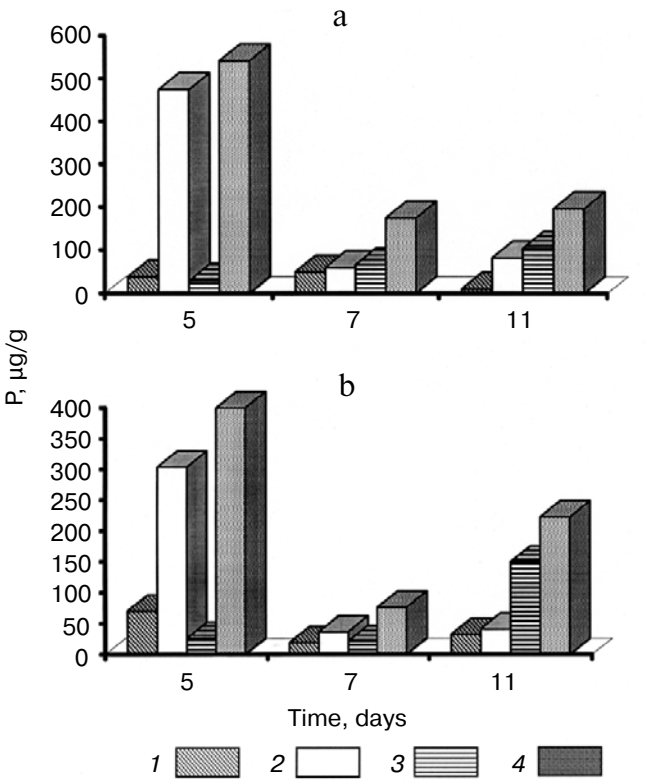

Fig. 3. Polyphosphate content (calculated for dry cell mass) of various fractions extracted from *Y. pseudotuberculosis* (strain 512) (a) and *L. monocytogenes* (strain 10CN) (b) cultivated in phosphate buffer, pH 7.2-7.4 ($n = 3$, $p < 0.01$): *1*) salt fraction; *2*) alkaline fraction; *3*) acidic fraction; *4*) total polyphosphates.

necessary for energetic processes. This function of polyphosphates is especially important for *L. monocytogenes* and *Y. pseudotuberculosis*, because their life support depends on external conditions, especially when energy sources are absent from the medium. Earlier we found that the studied bacteria have many common features when they inhabit environmental objects together with oligotrophic microorganisms [2]. Analogously it is suggested that highly polymeric phosphate fractions in *L. monocytogenes* and *Y. pseudotuberculosis* functionally substitute for ATP in certain catabolic reactions [13].

As known, there are several phosphate fractions in cell varying in physicochemical properties, cell localization, and physiological activity [14]. By the method of separation, polyphosphate fractions can be divided into salt-, acid-, and alkali-soluble [10]. It has been shown that accumulation of salt-soluble polyphosphates directly depends on intensity of RNA synthesis, and increased amount of acid-soluble polyphosphates suppresses this nucleic acid synthesis. On the whole, these fractions are related to growth processes [6, 14]. When studying localization of polyphosphates, it was found that compounds of the salt-soluble fraction are in the cell nucleus and those of acid-soluble fraction in cytoplasm [15].

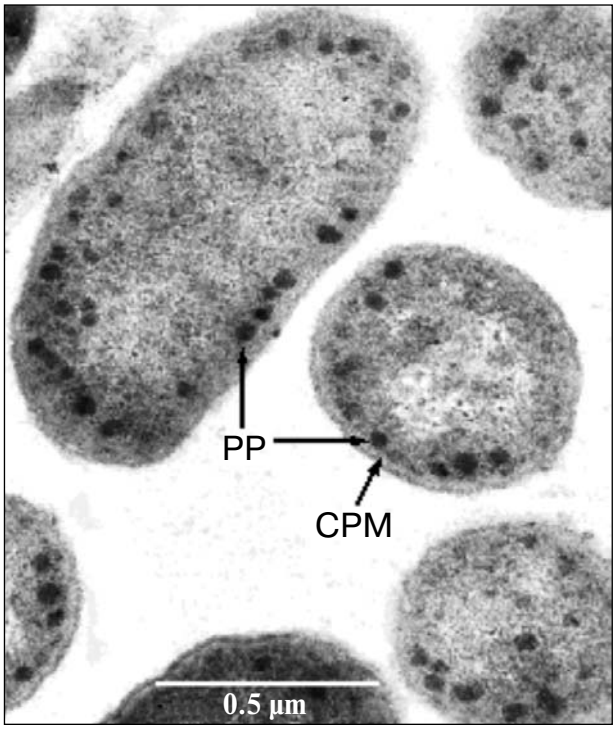

Fig. 4. Granules of polyphosphates (PP) in *Y. pseudotuberculosis* cells localized in cytoplasmic membrane area (CPM).

Compared with salt- and acid-soluble fractions, alkali-soluble fraction is represented by highly polymeric polyphosphates, which are localized in the peripheral cell area in the neighborhood of the cytoplasmic membrane; similar to ATP, these polyphosphates are energy-rich phosphorus-containing compounds participating in energetic processes of the bacterial cell [8].

The results indicate that most of the polyphosphates are alkali-soluble, especially during the preliminary cell growth stage: 90% under bacteria cultivation in phosphate buffer and 70% in physiological solution (Fig. 3). It is probable that under starvation conditions, the main requirements of *L. monocytogenes* and *Y. pseudotuberculosis* for this reserve substance are for their energy consumption.

Studying the ultrastructure of *Y. pseudotuberculosis* cells cultivated in phosphate buffer, we noted the presence of osmium-stained oval inclusions localized as a necklace in the peripheral cytoplasmic area and having ultrastructural features typical of alkali-soluble polyphosphates [15, 16] (Fig. 4). The number of bacteria containing analogous inclusions decreased to the end of lag-phase. The content of electron-dense inclusions in dividing cells (exponential growth phase) was less than that in non-dividing ones. Cells with polymer inclusions were again detected in the stationary growth phase. A correlation of morphological data with amount of polyphosphates at various growth phases of periodic culture indicates that polymer granules became visible as ultrastructures when the amount of phosphorus was more than or equal to 400 μg per g dry cell mass. It is evident that there exists some quantitative threshold of polyphosphate content in the bacterial cell; when it is achieved, polyphosphates can be revealed as structures using electron microscopy.

So, the data indicate that at low temperature in the absence of a nitrogen source and in the presence of phosphate anions in the medium, *L. monocytogenes* and *Y. pseudotuberculosis* are able to actively accumulate reserve substances as polyphosphates. During a long starvation period under conditions of carbon and energy source deficit, *L. monocytogenes* and *Y. pseudotuberculosis* consume this biopolymer for biosynthetic and energetic processes.

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