Microbiology, Vol. 69, No. 6, 2000, pp. 652–654. Translated from Mikrobiologiya, Vol. 69, No. 6, 2000, pp. 770–773.

EXPERIMENTAL ARTICLES

Metabolism of Poly-β-Hydroxybutyric Acid in *Yersinia pseudotuberculosis* and *Listeria monocytogenes* Cultivated at Different Temperatures

L. S. Buzoleva and A. D. Chumak

Research Institute of Microbiology and Epidemiology, Siberian Division, Russian Academy of Medical Sciences

Pacific Fisheries Research Center (TINRO Center), Vladivostok, 690600 Russia

Received December 14, 1999

Abstract—A comparative investigation of the intracellular content of poly-β-hydroxybutyric acid showed that *Yersinia pseudotuberculosis* strains accumulated, on the average, lower amounts of this reserve substance than *Listeria monocytogenes* strains. The intracellular pool of poly-β-hydroxybutyric acid was responsible for the growth of the bacteria at low temperatures (4–6°C) in the absence of any exogenous carbon and energy source.

Key words: poly-β-hydroxybutyric acid, *Yersinia pseudotuberculosis*, *Listeria monocytogenes*, temperature, growth

The isolation of bacteria of the genera *Yersinia* and *Listeria* from bodies of water during the cold seasons has been reported by some authors [1, 2]. Experimental contamination of tap and distilled water showed that these bacteria can survive and even grow slightly in contaminated water for at least one year (longer observations were not made) [3, 4]. The cultivation of *Yersinia pseudotuberculosis* and *Listeria monocytogenes* in phosphate buffer (pH 7.4–7.6) at 4–6°C (cold enrichment) is the most promising method of isolation for these bacteria [5]. The survival (and sometimes reproduction) of these bacteria under nutritional limitation is indicative of their high tolerance to extreme environmental conditions. It is known that the survival of saprophytic bacteria in oligotrophic environments is due to intracellular storage substances, such as poly-β-hydroxybutyrate (PHB), which are used by the cells as carbon and energy sources under conditions of nutritional deficiency [6–10]. PHB accumulates in cells under conditions of unbalanced growth, when biosynthetic processes are limited and require little energy [11, 12]. PHB serves as a sink for excess energy, which is stored in the form of this osmotically and chemically neutral substance [13].

PHB was revealed in both gram-positive and gram-negative bacteria belonging to different systematic groups. The most studied PHB producers are hydrogen-oxidizing [9, 14] and methanol-oxidizing [6, 15, 16] bacteria. Like these bacteria, *Y. pseudotuberculosis* and *L. monocytogenes* can utilize molecular hydrogen, carbon dioxide, methanol, and other C_1 compounds [17]. With this knowledge, the goal of this study was to measure the ability of different strains of the two bacteria to synthesize PHB and to utilize this reserved polymer during batch cultivation in phosphate buffer (pH 7.4–7.6) at 37, 20, and 6°C.

MATERIALS AND METHODS

The strains used in this study were *Yersinia pseudotuberculosis* H-2781, 3515, 512, 907, 282, 158, and 557, obtained from the Collection of the All-Russia Center of Yersiniases, the Research Institute of Epidemiology and Microbiology, Siberian Division, Russian Academy of Medical Sciences, Vladivostok, and *Listeria monocytogenes* P, K, A, 10CN, 546, 2L, and 4L, from the All-Russia Control Institute of Veterinary Preparations, Moscow. All of these strains are morphologically, culturally, and antigenically typical representatives of the two species.

As the material for inoculation, we used bacteria grown on nutrient agar at 20°C for two days. Once grown, these cells were collected, washed three times with physiological saline solution, and placed in a phosphate-buffered saline (pH 7.4–7.6) in an amount of 1000 cells/ml (according to the optical turbidity standard) [5]. The choice of PBS for bacterial cultivation was dictated by the fact that it provided a biomass yield sufficient for investigating the PHB accumulation. Bacteria were cultivated in batch mode without any exogenous carbon source and in the presence of 1% glucose. They were cultivated in 0.5-l glass flasks filled to 20% with the growth medium at 37, 18–20, and 4–6°C. To determine the cellular content of PHB, the biomass was

0026-2617/00/6906-0652$25.00

treated using the method of Braunegg *et al.* [18]. The methyl ester from 3-hydroxybutyric acid was obtained by hydrolysis and methylation of the biomass and was analyzed on a Shimadzu-16A gas–liquid chromatograph equipped with a CPB-5 column (50 m × 0.33 mm ID; mobile phase thickness 0.5 μm). The column was kept at 85°C. The temperature of the detector and injector was 240°C. The carrier gas was helium, and the flow rate was 50 ml/min. The internal standard was sodium salt of DL-β-hydroxybutyric acid (ICN, United States). Chromatograms were processed using a specialized Shimadzu-CR-4A computer.

The dynamics of bacterial growth and changes in the intracellular material of PHB were observed for 30 days. At regular time intervals, 1-ml aliquots of cultures were taken to determine the cellular PBS content. For these measurements, the washed biomass was dried at 105°C to a constant weight. To plot the growth curve, 0.1 ml aliquots of cultures were plated on to the respective differentiating media (Serov's medium was used for yersinia, and casein and yeast extract agar was used for listeria). The number of bacterial colonies grown on the differentiating solid media at 37°C was expressed as the logarithm of colony-forming units (CFU) per 0.1 ml of the culture. Measurements were repeated at least three times. The results were processed on an IBM-compatible PC using Microsoft Excel, v. 7. Statistical evaluations were made using the student's *t*-test.

RESULTS AND DISCUSSION

To ascertain the ability of *Y. pseudotuberculosis* and *L. monocytogenes* to accumulate PHB, these bacteria were cultivated in nitrogen-free PBS supplemented with 1% glucose for 5 days at 18–20°C. These growth conditions were chosen because it is known that PHB primarily accumulates in the bacterial cells cultivated under an excess of carbon and energy sources and a deficiency of nitrogen sources. In this case, excess energy in the form of the reduced equivalents (NADH and NADPH) was spent by the cell for PHB synthesis to occur [10, 19]. These experiments showed that *Y. pseudotuberculosis* and *L. monocytogenes* are able to synthesize PHB.

Comparative study of the intracellular content of PHB showed that the *L. monocytogenes* cells accumulated two times more of the polymer than the *Y. pseudotuberculosis* cells (see table). Some quantitative differences in the intracellular PHB pools were also observed for different strains of the same species. The highest concentrations of PHB were revealed in the *Y. pseudotuberculosis* strain 3515 and in the *L. monocytogenes* strains 2L and 546. The lowest concentrations of PHB were observed in the *Y. pseudotuberculosis* strain H-2781 and in the *L. monocytogenes* strain A (see table). The intracellular content of the polymer in the bacteria studied turned out to be considerably lower than in some chemotrophic and phototrophic saprophytes [8, 10, 19]. The intensity of PHB synthesis in the *Y. pseudotuberculosis* and *L. monocytogenes* cells, which depends on the parameters of cultivation [7, 10, 13], can probably be increased if required.

The poly-β-hydroxybutyrate content of different strains of *Y. pseudotuberculosis* and *L. monocytogenes*

Y. pseudotuberculosis, strain	PHB, μg/g dry cells	*L. monocytogenes*, strain	PHB, μg/g dry cells
H-2781	2.12 ± 0.05	4L	5.37 ± 0.02
3515	5.49 ± 0.03	2L	6.39 ± 0.03
512	3.08 ± 0.03	546	6.35 ± 0.03
907	3.00 ± 0.12	10CN	4.76 ± 0.01
282	2.53 ± 0.002	A	4.04 ± 0.05
158	2.99 ± 0.01	K	6.30 ± 0.01
557	2.73 ± 0.05	P	5.11 ± 0.03

To understand the role of PHB reserves in the growth of bacteria in the absence of exogenous sources of carbon and energy, the bacterial cultures were grown in PBS without glucose for 30 days at 37, 18–20, and 4–6°C. Since the results obtained for the two bacterial species differed insignificantly, the figure presents only data for *Y. pseudotuberculosis.*

It should be noted that, in the absence of carbon and energy sources, the bacteria grew at 18–20°C and 4–6°C but failed to grow at 37°C. During cultivation at 18–20°C, the intracellular PHB content increased with the concentration of bacterial cells in the culture (Fig. 1a). In our opinion, the growth of bacterial cells at this temperature was not due to PHB but to some unknown reserve substance.

At the low cultivation temperature (4–6°C), the intracellular PHB pool gradually decreased as the cultures grew (Fig. 1b). This may be related to the partial degradation and utilization of this polymer by cells, which causes growth (exponential phase) or maintainenance in the viable state (stationary phase). This is confirmed by the relevant data concerning other bacterial species [9].

At the middle cultivation temperature (18–20°C), the content of PHB in the cells tended to decrease from the beginning of the stationary growth phase and was completely depleted by the 30th day of cultivation (Fig. 1a). This was probably related to the death of the bacterial culture, since the PHB content of the cells and the number of viable cells in the culture dropped in parallel [15].

In conclusion, the storage polymer poly-β-hydroxybutyrate accumulates in the bacteria *Y. pseudotubercu-*

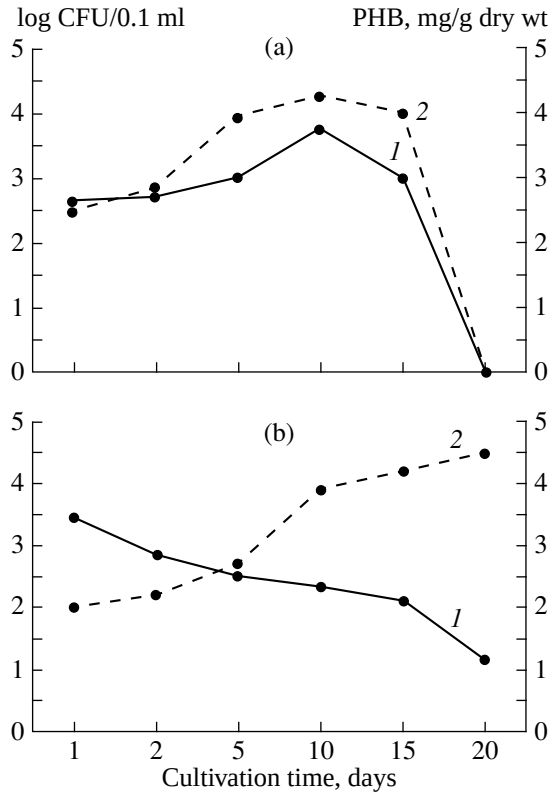

Fig. 1. Utilization of poly-β-hydroxybutyric acid by *Y. pseudotuberculosis* 512 during growth in PBS (pH 7.4–7.6) supplemented with 0.1% glucose at (a) 18–20°C and (b) 4–6°C: (*1*) dynamics of the cellular PHB content and (*2*) changes in the number of viable cells in 0.1 ml of culture.

losis and *L. monocytogenes* accumulate, which provides for their survival and reproduction at low ambient temperatures in the absence of or with a deficiency of exogenous carbon and energy sources.

REFERENCES

1. Kuznetsov, V.G., Isolation of Bacteria of the Genus *Yersinia* from Water Bodies in Primorskii krai, *Gig. Sanit.*, 1983, no. 2, pp. 72–74.
2. Buzolyova, L.S. and Somov, G.P., Listeria's Survival in River and Sea Water, *Proc. Int. Symp. "Earth–Water–Humans,"* Kanazawa (Japan), 1999, pp. 286–292.
3. Somov, G.P., *Dal'nevostochnaya skarlatinopodobnaya likhoradka* (Far Eastern Scarlatinoid Fever), Moscow: Meditsyna, 1979.
4. Gershun, V.I., Viability of Listeria in Water, *Sib. Vestn. S–kh. Nauki*, 1979, no. 6, pp. 48–50.
5. Paterson, J.S. and Cook, R., A Method for the Recovery of *Pasteurella pseudotuberculosis* from Faeces, *J. Path. Bacteriol.*, 1963, vol. 85, pp. 241–242.
6. Slabova, O.I., Nikitin, D.I., and Zagreba, E.D., Poly-β-Hydroxybutyric Acid Degradation in *Methylobacterium organophilum* Cells Oxidizing Gaseous Substrates, *Mikrobiologiya*, 1990, vol. 59, no. 6, pp. 938–941.
7. Bonartseva, G.A., Myshkina, V.A., and Zagreba, E.D., Effect of Various Sources of Carbon and Nitrogen on the Content of Poly-β- Hydroxybutyrate in *Rhizobium* Cells, *Mikrobiologiya*, 1994, vol. 63, no. 1, pp. 78–84.
8. Kondrat'eva, E.N. and Krasil'nikova, E.N., Phototrophic Bacteria as Poly-β-Hydroxybutyrate Producers, *Prikl. Biokhim. Mikrobiol.*, 1989, vol. 25, no. 6, pp. 786–789.
9. Volova, T.G., Kalacheva, G.S., and Puzyr', A.P., Effect of Poly-β-Hydroxybutyrate on the Growth of Hydrogen-Oxidizing Bacteria under Nonoptimum Conditions, *Mikrobiologiya*, 1996, vol. 65, no. 5, pp. 594–598.
10. Semenov, A.M., Ganzlikova, A., and Tenov, N., Accumulation of Poly-β-Hydroxybutyrate by Some Oligotrophic Polyprosthecate Bacteria, *Mikrobiologiya*, 1989, vol. 58, no. 6, pp. 923–925.
11. Anderson, A.J. and Dawes, E.A., Occurrence, Metabolism, Metabolic Role and Industrial Uses of Bacterial Polyhydroxyalkanoates, *Microbiol. Rev.*, 1990, vol. 54, no. 4, pp. 450–472.
12. Dawes, E.A., Growth and Survival, in *Bacteria in Nature*, New York: Plenum, 1989, no. 3, pp. 67–187.
13. Bonartseva, G.A., Myshkina, V.A., and Zagreba, E.D., Effect of Various Concentrations of Oxygen, Sucrose, and Nitrate on the Synthesis of Poly-β-Hydroxybutyrate in *Rhizobium phaseoli* Cells, *Mikrobiologiya*, 1995, vol. 64, no. 1, pp. 40–43.
14. Senior, P.J., Un Thermoplastique Produit par des Bacteries, *Rev. Polytechn.*, 1986, no. 1473, p. 195.
15. Govorukhina, N.I. and Trotsenko, Yu.A., Poly-β-Hydroxybutyrate Content in Methylotrophic Bacteria with Different Pathways of Methanol Assimilation, *Prikl. Biokhim. Mikrobiol.*, 1991, vol. 27, no. 1, pp. 98–101.
16. Tokahiro, S., Tsuneo, Y., and Shoichi, S., Kinetics and Effect on Nitrogen Source Feeding on Production of Poly-β-Hydroxybutyric Acid by Fed Batch Culture, *Appl. Microbiol. Biotechnol.*, 1986, vol. 24, p. 366.
17. Buzoleva, L.S. and Somov, G.P., Autotrophic Assimilation of CO_2 and C_1-compounds by Pathogenic Bacteria, *Biokhimiya*, 1999, vol. 64, no. 10, pp. 61–65.
18. Braunegg, G., Sonnleitner, B., and Lafferty, R.M., A Rapid Gas Chromatographic Method for the Determination of Poly-β-Hydroxybutyric Acid in Microbial Biomass, *Eur. J. Appl. Microbiol. Biotechnol.*, 1996, no. 6, pp. 29–37.
19. Zagreba, E.D., Seliverstova, T.I., Ginovska, M.K., Klintsare, A.Ya., and Yakobson, Yu.O., Differentiation of *Rhizobium leguminosarum* Strains by IR Spectroscopy, *Izv. Akad. Nauk SSSR. Ser. Biol.*, 1982, no. 6, pp. 906–910.