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EXPERIMENTAL ARTICLES

Thermotropic Behavior of Lipids and the Morphology of *Yersinia pseudotuberculosis* Cells with a High Content of Lysophosphatidylethanolamine

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Abstract—The content of lysophosphatidylethanolamine (LPE) in *Y. pseudotuberculosis* cells was found to increase during their growth at 8°C under stationary conditions (without stirring the medium) and at 37°C when the medium contained glucose. The maximum level of LPE (up to 45% of the total phospholipids) was observed in cells grown at 8°C under stationary conditions. Such cells showed decreas growth rate, a reduced yield of biomass, an altered cell morphology, and an increased cell area. The cells contained unsaturated fatty acids, phosphatidylethanolamine (PE), and total phospholipids in small amounts, whereas neutral lipids and diphosphatidylglycerol were abundant. In addition, the cells contained an amount of methylated PE and phospholipids of unknown structure. Irrespective of whether the temperature for growth was low or high, the LPE-rich cells showed a high value (32–36°C) of the maximum temperature of thermal transition of lipids (T_{max}). This finding is indicative of a densification of the membrane lipid matrix of the LPE-rich cells. The suggestion is made that LPE is accumulated in bacterial cells in response to stress caused by oxygen deficiency and pH decrease in the course of glucose fermentatin. The possible relationship between LPE accumulation and the virulence of *Y. pseudotuberculosis* cells grown at low temperatures is discussed.

Key words: phospholipids, lysophospholipids, fatty acids, thermotropic behavior of lipids, morphology, *Yersinia pseudotuberculosis.*

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The cellular content of lysophosphatidylethanolamine (LPE), the major lysophospholipid of bacterial cells, is typically small (2–5% of the total phospholipids). However, some bacterial species accumulate LPE in amounts as large as 50% of the total phospholipids in response to stressful factors, such as high acidity of the medium or high growth temperature [1, 2].

The role of lysocompounds in bacterial cells is far from being well-understood. Some authors believe that LPE accumulated in stressed bacterial cells may play the role of chaperons to soluble proteins, preventing their aggregation [2]. Lysophospholipids are biologically active compounds in mammals, where they perform a number of important regulatory functions in inflammation processes and immunity [3]. Lysophospholipids may influence the structure and the permeability of lipid bilayers. This influence depends on the particular composition and the curvature of the lipid bilayer. For example, lysophospholipids act on lecithin bilayers as detergents, promoting the formation of isotropic phases [4]. On the other hand, lysophospholipids may eliminate the negative curvature and the instability of the bilayers induced by nonlamellar phosphatidylethanolamine (PE) [5]. It should be noted that such a stabilizing effect is typical of lamellar lipids [6].

The accumulation of lysocompounds in bacterial cells may enhance protein secretion and membrane defragmentation (the formation of vesicles) [7]. Recent studies of *Helicobacter pylori* variants with an elevated content of LPE showed that they are very pathogenic [8]. The LPE/PE ratio was proposed as an indicator of *H. pylori* virulence [8].

Yersinia pseudotuberculosis, which is the causal agent of pseudotuberculosis in humans, is a facultative psychrophile that inhabits diverse environments. This bacterium can be either parasitic or saprophytic.

The aim of this work was to study conditions favoring the accumulation of LPE in *Y. pseudotuberculosis*

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cells and to investigate the growth dynamics, morphology, and thermotropic behavior of lipids in LPE-rich cells.

MATERIALS AND METHODS

Bacterial strains and cultivation conditions. Experiments were carried out with the *Yersinia pseudotuberculosis* strain KS 3058, serovar O:Ib, which was derived as described by Shovadaeva et al. [9]. The strain was cultivated in 1-l flasks containing 150 ml of domestic nutrient broth (produced in Makhachkala, Russia) with or without substrate (0.5% glucose). These media were designated as NB+Glc and NB, respectively. Cultivation was performed under aerobic conditions (with good aeration and shaking of the medium at 180 rpm), anaerobic conditions (shaking the medium at 180 rpm without air supply), and stationary conditions (cultivation with air supply but without shaking). Anaerobic conditions were created by sparging argon through the medium, which was preliminarily boiled and vacuumized. Argon was sterilized by filtrating it through a 0.2-μm-pore-size membrane filter. In the stationary growth phase (one day of cultivation at 37°C or six days at 8°C), bacterial cells were killed by adding phenol to a total concentration of 1% and mixing for 20 min. Then, dead cells were harvested by centrifugation.

Bacterial growth was monitored by measuring culture turbidity at 600 nm.

Bacterial morphology was studied by examining gram-stained *Y. pseudotuberculosis* cells under an Axiostar light microscope (Germany).

The cell area was calculated with the aid of the Image Tool program, v. 2, for Windows (UTHSA, San Antonio, Texas).

Lipid isolation and analysis. Lipids were extracted twice from cells with a chloroform– methanol (2 : 1, v/v) mixture at 8°C for 2 hours. Polar lipids were separated by two-dimensional TLC as described by Vaskovsky and Terekhova [10]. Phospholipids were identified using authentic samples of individual phospholipids [11]. The total content and the amount of individual phospholipids in cells were determined by measuring phosphorus in particular lipid extracts [12]. The results were expressed in % of dry cell weight and % of total phospholipids, respectively.

Fatty acids were prepared by means of hydrolysis of microbial biomass in 6 M NaOH at 100°C for 4 h and analyzed as methyl esters by gas–liquid chromatography and chromatography–mass spectrometry [12].

Differential scanning calorimetry. Total lipids were dissolved in chloroform and the solution was placed in standard microcuvettes. Chloroform was removed by vaporization under vacuum. Samples dried to a constant weight (approximately 5 mg) were suspended in two parts of 50% ethylene glycol in the microcuvettes, which were then sealed and placed in a DSM-2M differential scanning calorimeter (NPO Biopribor, Pushchino, Russia). The reference microcuvette contained pure 50% ethylene glycol in a volume equal to that of the sample suspension. The samples were heated/cooled within a range of –100 to 60°C at a rate of 16°C/min. Sensitivity was 40 mW. The temperature of phase transition (T_{max}) was determined as the maximum of the heat capacity vs. temperature plot. The temperature scale was calibrated using authentic samples of naphthalene, mercury, and indium.

The data presented in this paper are the means of three or more independent measurements. The experimental error did not exceed ± 5%.

RESULTS AND DISCUSSION

The effect of cultivation conditions on the accumulation of LPE in *Y. pseudotuberculosis* cells. The bacterium was grown under good and poor aeration (with and without shaking of the growth medium, respectively) at two contrasting temperatures (8 and 37°C) in nutrient broth either containing glucose or not. A comparative analysis of the phospholipid content of cells grown under different conditions (Table 1) showed that glucose added to the cultivation medium raised the phospholipid content of cells by a factor of 1.4–7.5 at any growth temperature and aeration conditions. Upon cultivation at 8°C, the phospholipid content of cells grown under stationary conditions (i.e., under poor aeration) was 6 to 16 times higher than under the conditions of intense shaking. At 8°C, the two LPE-promoting factors (glucose and poor aeration) acted additively.

Presumably, oxygen deficiency rather than the absence of medium shaking is the factor responsible for the accumulation of LPE in *Y. pseudotuberculosis* cells. Indeed, the bacterial cells grown under anaerobic conditions and intense shaking contained LPE in amounts as large as 52% of the total phospholipids (data not shown).

In contrast, at 37°C, the effect of aeration on the LPE content of cells was negligible and observable only in the presence of glucose in the cultivation medium. These results can be explained by the low content of dissolved oxygen in the medium incubated at 37°C, even if it was shaken at a high rate.

Bacterial cells with a high level of LPE are characterized by a low content of phosphatidylethanolamine (PE) and total phospholipids, a high content of neutral lipids and diphosphatidylglycerol (DPG), and the presence of methylated PE and phospholipids of unknown structure. Such a lipid composition is typical of bacteria exposed to stress [14, 15]. The LPE-rich cells grown at 8°C (Table 1, columns 3 and 4) showed an enhanced level of anionic phospholipids. Due to this fact, the (PE + LPE)/(PG + DPG) ratio decreased to a value typical of bacterial cells grown at 37°C (in the above ratio, PG stands for phosphatidylglycerol).

Table 1. The effect of cultivation conditions on the biomass yield, morphology, and the lipid composition of *Y. pseudotuberculosis* cells

	8°C				37°C			
	180 rpm		Without shaking		180 rpm		Without shaking	
	NB	NB + Glc	NB	NB + Glc	NB	NB + Glc	NB	NB + Glc
	1	2	3	4	5	6	7	8
Biomass, mg/l	978.0	669.1	253.3	287.0	260.8	216.0	215.1	120.7
Cell shape	Cocci	Ovoid	Ovoid	Ovoid	Cocci	Ovoid	Ovoid	Ovoid
Cell area, μm^2	1.107 ± 0.01	1.962 ± 0.03	2.576 ± 0.03	1.984 ± 0.03	1.074 ± 0.01	2.021 ± 0.03	1.870 ± 0.03	2.0 ± 0.03
Lipid yield, % of dry cell wt								
Total lipids	7.2	5.5	7.0	5.8	5.1	4.2	5.0	6.0
Phospholipids	6.8	5.0	2.1	3.1	4.0	2.1	3.6	1.4
Neutral lipids	0.4	0.5	4.9	2.7	1.1	2.1	1.4	4.6
Content, % of total phospholipids								
E	79.6	79.4	22.7	27.2	73.6	50.8	71.6	48.0
LPE	2.1	7.7	33.2	45.6	3.1	23.3	3.5	13.0
PG	7.9	5.1	12.1	8.3	5.8	1.3	6.8	4.7
DPG	8.7	7.9	14.9	16.5	17.5	22.9	18.1	25.7
MePE	1.7		4.8	2.4				
X			8.4			1.7		3.8
$X_1 + X_2$								4.7
(PE + LPE)/(PG + DPG)	5.3	6.7	2.1	2.9	3.3	3.1	3.0	2.4

It is known that the accumulation of LPE in *H. pylori* induces the excretion of HCl to the medium, decreasing its pH to 5.5 [1]. The study of the pH dynamics during the growth of *Y. pseudotuberculosis* under different conditions showed that the pH of the medium in the absence of glucose (irrespective of the other cultivation conditions) tended to increase, whereas it decreased to 4.6–5.0 in the presence of glucose in the medium. The rate of medium acidification depended both on the growth temperature and on the aeration conditions. At 37°C, pH notably decreased as soon as within one day whatever the aeration rate. At 8°C and intense aeration, the pH of the medium decreased more slowly (within two days). Under stationary conditions (poor aeration), the pH of the medium decreased most slowly (within eight days).

Presumably, LPE is accumulated in *Y. pseudotuberculosis* cells in response to stress caused by oxygen deficiency or its complete lack in the medium, as well as by the pH drop in the process of glucose fermentation.

Taking into account the fact that LPE is produced by phospholipase A [16], we may suggest that the LPE content of cells rises due to increase in the enzyme activity or in the level of enzyme expression.

The fatty acid composition of *Y. pseudotuberculosis* cells rich in LPE. The fatty acid patterns of *Y. pseudotuberculosis* cells with a high and low level of LPE are qualitatively the same and typical of this bacterium (Table 2) [17]. However, *Y. pseudotuberculosis* cells grown on glucose at 37°C (Table 2, columns 6 and 8) are distinguished by the presence of $C_{12:0}$ under both conditions of aeration and by the absence of $C_{15:0}$ under stationary cultivation conditions.

At the same time, the quantitative profile of fatty acids in *Y. pseudotuberculosis* cells is subject to cultivation conditions. For example, the content of C_{17cy} (cyclopropanoic acid) and $C_{18:1c7}$ is lower and the content of $C_{18:1c9}$ and saturated fatty acids is higher in *Y. pseudotuberculosis* cells grown at 8°C under stationary conditions than in the cells grown under intense aeration. As a result, the ratio of unsaturated to saturated fatty acids ($\Sigma_{unsat}/\Sigma_{sat}$) is lower in the former cells than in the latter cells. The minimal value of this ratio was observed in the cells grown in nutrient broth with glucose (Table 2, columns 2 and 4). This fact can be explained by oxygen deficiency under stationary cultivation conditions. Oxygen deficiency inhibits the activity of desaturases, which are active only under aerobic conditions [18].

Table 2. The effect of cultivation conditions on the fatty acid composition of *Y. pseudotuberculosis* cells

	8°C				37°C			
	180 rpm		Without shaking		180 rpm		Without shaking	
	NB	NB + Glc	NB	NB + Glc	NB	NB + Glc	NB	NB + Glc
	1	2	3	4	5	6	7	8
$C_{12:0}$						1.0		1.0
$C_{14:0}$	0.8	2.6	1.6	3.1	7.1	5.1	5.9	3.9
$C_{15:0}$	2.3	0.6	1.5	2.4		1.3		
$C_{16:1c7}$	32.0	35.2	32.4	16.7	5.6	7.1	6.7	5.2
$C_{16:0}$	24.1	27.3	30.7	35.6	41.7	40.2	41.0	41.4
C_{17cy}	15.4	9.3	3.8	4.8	39.9	33.8	39.1	41.4
$C_{18:1c9}$	1.6	2.1	5.4	16.5	1.9	5.5	2.3	2.1
$C_{18:1c7}$	21.7	17.5	18.2	10.6	1.4	2.0	1.9	2.4
$C_{18:0}$	2.2	2.6	6.4	10.5	2.3	4.0	3.1	2.7
$C_{20:1}$		2.8						
Σ_{unsat} FAs	55.3	57.6	55.9	43.7	8.9	14.6	10.9	9.7
Σ_{sat} FAs	29.4	33.1	40.3	51.6	51.2	51.7	50.0	49.0
Σ_{unsat} FAs/Σ_{sat} FAs	1.88	1.74	1.39	0.85	0.17	0.28	0.22	0.20

Note: Data shown in the table are % of total fatty acids. FAs, fatty acids.

Thus, *Y. pseudotuberculosis* cells grown under oxygen deficiency show an altered temperature dependence of the degree of unsaturation of membrane lipids [19]. This circumstance may affect the physical properties of the cellular membranes.

The quantitative fatty acid composition of *Y. pseudotuberculosis* cells grown at 37°C is almost the same irrespective of their LPE content. However, the $\Sigma_{unsat}/\Sigma_{sat}$. ratio is considerably lower (due to a decrease in the level of unsaturated fatty acids) and the level of cyC17:0 is considerably higher in the LPE-rich cells grown at 37° than in the cells grown at 8°C (Table 2, columns 3, 4, 6, 8).

Similar changes in the fatty acid composition of microbial cells in response to elevated temperatures were observed for other microorganisms. Presumably, such changes are necessary to maintain an appropriate degree of membrane fluidity at a given temperature [19].

The growth and morphology of *Y. pseudotuberculosis* rich in LPE. The cultivation conditions that favor LPE accumulation in *Y. pseudotuberculosis* cells inhibit their growth and reduce their yield. For example, at 37°C, the presence of glucose in the cultivation medium diminished the biomass yield by 1.2 to 1.8 times (Table 1). Furthermore, the bacterium grown at 8°C under stationary conditions showed a biomass yield 2.3 to 3.9 times lower than the bacterium grown under intense aeration (Table 1, Fig. 2). The growth curve in the former case does not have a distinct logarithmic phase (Fig. 2).

The low biomass yield at 37°C, in the glucose-containing medium is likely to be due to a drastic decrease in the pH of the medium after one cultivation day (Fig. 1), since low pH values are known to be inhibitory to bacteria [20]. When *Y. pseudotuberculosis* was cultivated at 8°C under stationary conditions, the pH of the medium changed insignificantly even after six days of cultivation in nutrient broth with and without glucose (Fig. 1). This observation can be explained by oxygen deficiency under these cultivation conditions. It is known that oxygen deficiency is the major factor that limits the growth of aerobic bacteria [21].

Optical microscopic studies showed that, whatever cultivation temperature, LPE-rich cells are ovoid and have two times greater size (the difference in the cell size is statistically significant with $P < 0.05$ and $n = 100$) than LPE-low cells, which are coccoid in shape (Table 1). The only exception is bacterial cells grown on glucose, which are ovoid in shape and have a constant area whatever the aeration and temperature conditions. It should be noted that the cell size is maximum under the conditions unfavorable to bacterial growth and division [22].

These data suggest that cell growth and morphology depend on cultivation conditions. Stationary conditions (which cause oxygen deficiency) and the presence of glucose in the medium (which decreases its pH in the

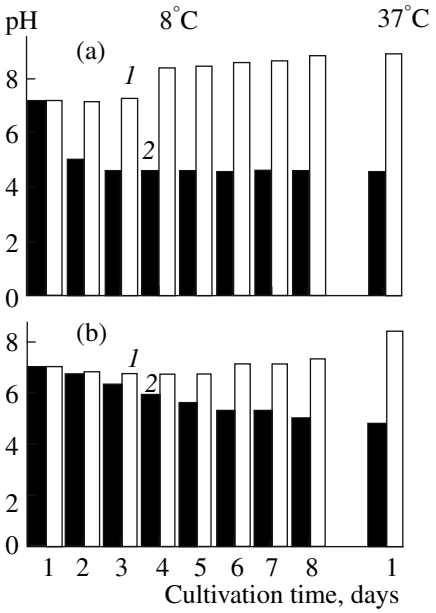

Fig. 1. Changes in the pH of the cultivation medium (*1*, nutrient broth; *2*, nutrient broth with glucose) of *Y. pseudotuberculosis* grown at 8 and 37°C with (a) and without (b) shaking the medium.

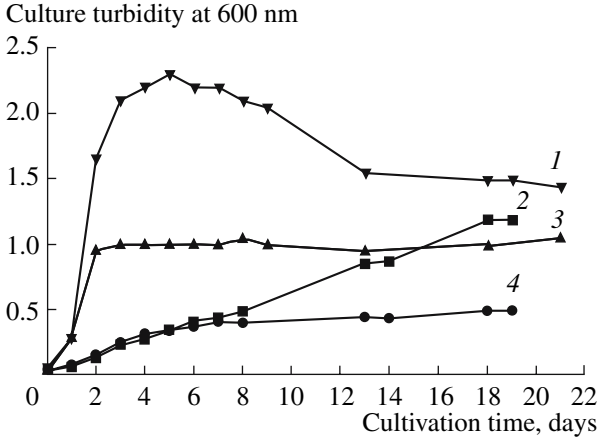

Fig. 2. Growth curves of *Y. pseudotuberculosis* grown at 8°C in nutrient broth (*1*, *2*) and in nutrient broth with glucose (*3, 4*) under aeration (*1, 3*) and under stationary conditions (*2, 4*).

process of cultivation) are stressful to *Y. pseudotuberculosis* cells.

Thermotropic behavior of lipids. A comparative analysis of lipid thermograms showed that the total lipids isolated from bacterial cells grown under stationary conditions at different temperatures with and without glucose in the cultivation medium are characterized by about the same maximum temperature of thermal transition of lipids. Namely, T_{max} is equal to 32 and 34°C for the lipids isolated from glucose-grown cells and 36°C for the lipids isolated from cells grown in the absence of glucose in the medium (Fig. 3).

The high temperature of phase transition of lipids and, hence, greater stability of membranes in bacterial cells grown at 8°C under poor aeration can be explained by the accumulation of LPE in such cells. This suggestion seems to be reasonable in view of the fact that nonlamellar PE comprises the major portion of bacterial lipids and that this PE increases the curvature and instability of the lipid bilayer. The elevated content of LPE, whose molecule greatly differs from the molecule of PE esterified with unsaturated fatty acids, may not only straighten and stabilize the bilayer [5], but also make lipid molecules more closely packed and, consequently, raise the temperature of their phase transition. The decreased curvature of the bilayer may enlarge the volume and the area of cells and thereby change their shape (Table 1).

The stabilization of lipid bilayers in the LPE-rich cells grown at low temperatures under stationary conditions may also be due to an enhanced content of unsaturated fatty acids in these cells and a high level of acidic phospholipids, PG and DPG (Table 1), which have a tendency to form lamellar phases under normal physiological conditions [23]. The close packing of lipids and the stable lamellar phase are probably responsible for the high degree of cooperative transitions in the lipids of bacterial cells grown under stationary conditions.

The high phase transition temperature of lipids in bacterial cells grown at 37°C under stationary conditions can be explained by the relatively high degree of lipid saturation rather than by the accumulation of LPE. At the cultivation temperature of 37°C, when the activity of desaturases and, hence, the degree of fatty acid unsaturation is low, the stabilizing effect of LPE on the membranes is probably not important. On the other hand, the high values of T_{max} typical of lipids isolated from bacterial cells grown at 37°C may be due to a high content of cyclopropanoic acid, since the formation of this acid through the methylation of unsaturated fatty acids is known to augment T_{max} and stabilize the lipid matrix of bacterial membranes under unfavorable cultivation conditions [24].

The intense aeration of the cultivation medium shifted the thermograms of isolated bacterial lipids to lower temperatures (Fig. 3), especially when bacterial cells were grown at 8°C. For example, the T_{max} of the total lipids isolated from bacterial cells grown in nutrient broth without glucose (Fig. 3a) decreased from 36 to 6°C. The presence of glucose in the cultivation medium decreased T_{max} even more drastically (from 34 to –10°C) (Fig. 3b). In contrast, glucose insignificantly influenced T_{max} when the bacterium was grown under stationary conditions (Figs. 3a, 3b). These data show that the decrease in the T_{max} of lipids isolated from bacterial cells grown at 8°C in glucose-containing nutrient broth [13] can be observed only if these cells are grown under intense aeration.

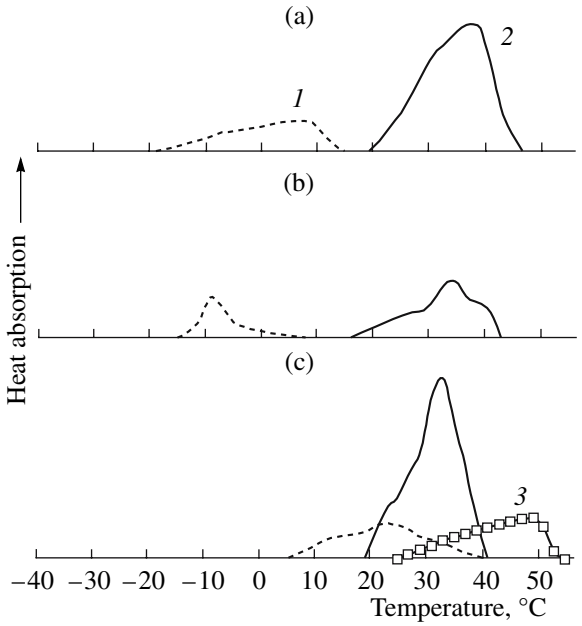

Fig. 3. Thermograms of the total lipids isolated from *Y. pseudotuberculosis* cells grown at 8°C (a, b) and 37°C (c) in nutrient broth without (a) and with (b, c) glucose under intense aeration (*1*) and under stationary conditions (*2*). Curve *3* refers to cultivation at 37°C with shaking the medium lacking glucose. The lipids were mixed with 50% ethylene glycol in a proportion of 1 : 2 (v/v). Lipid samples (5 mg) were scanned at least in triplicate in a differential scanning calorimeter at a temperature rate of 16°C/min. The vertical line represents 25 mW.

At the growth temperature of 37°C, the effect of aeration on the thermotropic behavior of lipids was less pronounced. For example, the aeration of nutrient broth containing glucose diminished T_{max} only by 10°C (Fig. 3c), as compared to 44°C in the case of cultivation at 8°C (Fig. 3b). This difference can be explained by the poorer solubility of oxygen in the cultivation medium at 37°C.

The data presented in this paper suggest that oxygen deficiency during the cultivation of *Y. pseudotuberculosis* at 8°C under stationary conditions induces stress in this bacterium. In response, the bacterium forms an abnormal (too closely packed for this temperature) lipid matrix, which provides for the necessary strength and permeability of the cell membranes and the maintenance of membrane-bound functions in the bacterial cells. We can assume that such bacteria can survive a drastic rise in the growth temperature. It is known that bacteria which usually grow at low temperatures die at high temperatures because their membranes are excessively permeable and insufficiently stable [25]. In nature, such a transition from low to high environmental temperatures takes place when a saprophytic bacterium goes to parasitic life. In this case, the ability of this bacterium to survive a drastic increase of growth temperature must increase its pathogenic potential. The high virulence of *Y. pseudotuberculosis* grown at low temperatures [26] is presumably based on the increased stability of the cell membranes under the influence of stressful factors. The accumulation of LPE in bacterial cells cultivated at low temperatures under oxygen deficiency provides for specific physicochemical properties of the cell membranes and may be considered to be a factor of cell pathogenicity. The high content of LPE in the *Y. pseudotuberculosis* cells grown in the cold may serve as an indicator of their increased pathogenicity.

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