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EXPERIMENTAL ARTICLES

A Comparative Analysis of the Ice Nucleation Activity of Pseudomonad Cells and Lipopolysaccharides

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Abstract—The paper deals with the study of the ice nucleation activity of the cells, extracellular lipopolysaccharides (ELPSs), lipopolysaccharides (LPSs), and LPS structural components (lipid A, core oligosaccharide, and O-specific polysaccharide) of *Pseudomonas fluorescens, P. syringae, P. fragi*, and *P. pseudoalcaligenes.* Aqueous suspensions of intact cells of *P. syringae* IMV 1951 and IMV 185 began to freeze at –1 and –4°C, respectively. This suggests that these cells possess ice nucleation activity. Aqueous cell suspensions of two other strains, *P. fluorescens* IMV 1433 and IMV 2125, began to freeze at lower temperatures than did distilled water (–9°C), which suggests that the cells of these strains possess antifreeze activity. The ice nucleation activity of the bacterial strains studied did not show any correlation with their taxonomic status. The ice nucleation activity of ELPSs depended little on their concentration (within a concentration range of 0.2–0.4%). In most cases, the ice nucleation activity of ELPSs, LPSs, and LPS structural components differed from that of the intact cells from which these biopolymers were obtained. This may indicate that the biopolymers under study play a role in ice nucleation but this role is not crucial. The relationship between the structure of LPSs and their effect on ice nucleation is discussed.

Key words: P. fluorescens, P. syringae, P. fragi, P. pseudoalcaligenes, lipopolysaccharides, ice nucleation, antifreeze activity.

The presence of particles (including bacterial cells) on the surface of plants increases frost injury [1], as such particles serve as ice nuclei and elevate the temperature of water freezing. Significant ice nucleation activity was observed in the phytopathogenic bacteria *Pseudomonas syringae* and *Erwinia herbicola*, in bacteria of the genus *Xanthomonas*, and in some saprophytic species of the genera *Pseudomonas* and *Comamonas* [1, 2]. The problem of ice nucleation is of great practical interest, being related to the problem of plant defense against frost and the development of affordable cloud condensation reagents [3]. When ingested by Colorado potato beetles, *Pseudomonas fluorescens* cells with ice nucleation activity can significantly reduce the winter survival rate of these frost-hardy beetles [4] and thus increase crop yield. There is great research interest in studying the ice nucleation activity of various microorganisms [1, 3] and the cellular components that are responsible for this activity [5–8].

Turner *et al.* [6] divided such components into three classes: (1) proteins associated with phosphatidylinositol, (2) proteins associated with mannan and glucosamine, and (3) proteins associated with sole mannosyl residues. These proteins are the products of specific genes, *inaZ* in *P. syringae, inaW* in *P. fluorescens,* and *iceE* in *E. herbicola.* The most active ice nucleation structure contains a protein associated with phosphatidylinositol and mannose (it is likely to be a polymer of mannan or glucosamine) [6–8]. Consequently, ice nucleation activity is a specific property of complex cellular components containing carbohydrates.

The major component of the cell wall of gram-negative bacteria is lipopolysaccharide (LPS), which contains a hydrophilic domain composed of O-specific polysaccharide (OPS) and a core oligosaccharide. The latter is covalently bound to lipid A, a hydrophobic domain of the LPS molecule, which links the hydrophilic carbohydrate domain of the LPS to the outer membrane. The LPS molecules are associated with the surface membrane proteins and occur in the outer monolayer of the cell membrane, being in contact with the environment. LPSs are excreted by live microbial cells and appear in the medium after their death [9]. The structure and biological activity of the LPSs of various gram-negative bacteria are well studied; however, little is known about their role in ice nucleation.

Recent studies of ours have been devoted to the structure and biological activity of the LPSs of various *P. fluorescens* homology groups [10]. The aim of this work was to study the ice nucleation activity of intact cells and LPSs of *P. fluorescens, P. syringae, P. fragi*, and *P. pseudoalcaligenes* in order to reveal the relationship between the ice nucleation activity and the structure of the LPSs.

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Table 1. The ice nucleation activity of intact cells of various bacterial strains of the genus *Pseudomonas*

Strain	Percentage of frozen drops of cell suspensions at temperatures from –1 to –19°C																		
	–1	–2	–3	–4	–5	–6	–7	–8	–9	–10	–11	–12	–13	–14	–15	–16	–17	–18	–19
P. fluorescens IMV 4125 (biovar I)									2	4	6	12	40	64	80	100			
P. fluorescens IMV 1433 (biovar I)												2	12	22	64	72	90	100	
P. fluorescens IMV 1152 (biovar I)							2	8	14	18	20	30	64	80	92	100			
P. fluorescens IMV 247 (biovar II)								2	6	6	8	8	22	42	86	94	100		
P. fluorescens IMV 472 (biovar I)					2	8	78	100											
P. fluorescens IMV 1602 (biovar II)						4	4	4	6	8	16	28	38	64	86	100			
P. fluorescens IMV 2125 (biovar III)												2	14	30	80	100			
P. fluorescens IMV 2303 (biovar I)							2	8	8	8	12	20	30	48	78	92	100		
P. fluorescens IMV 2763 (biovar V)						2	6	10	12	12	14	26	78	90	94	100			
P. fluorescens IMV 2111 (biovar IV)							2	10	12	16	18	26	46	64	84	100			
P. fragi IMV 4002							2	2	2	2	6	6	10	18	52	70	94	100	
P. pseudoalcaligenes IMV 4134						2	4	16	24	32	40	44	50	58	84	100			
P. syringae pv. *syringae* CPPB 281							2	4	4	4	6	18	22	40	70	94	100		
pv. *maculicola* IMV 381									4	22	26	32	36	38	44	64	94	100	
pv. *aptata* IMV 185				2	32	40	44	50	52	58	62	68	68	70	80	88	96	100	
pv. *syringae* (*holci*) IMV 1951	2	56	100																
Distilled water									2	6	10	16	22	30	38	70	86	94	100

MATERIALS AND METHODS

Experiments were carried out with 16 strains of four *Pseudomonas* species (*P. fluorescens, P. syringae, P. fragi*, and *P. pseudoalcaligenes*) obtained from the Ukrainian Collection of Microorganisms (UCM) at the Zabolotnyi Institute of Microbiology and Virology. The *P. fluorescens, P. fragi*, and *P. pseudoalcaligenes* strains were grown at 28°C on nutrient agar for 28 h. The *P. syringae* strains were grown at 26–28°C on potato agar.

To prepare LPSs and extracellular lipopolysaccharides (ELPSs), cells were washed off from the agar plates with physiological saline solution, washed in the same solution by centrifugation at 10000 *g* for 30 min, and dehydrated with acetone and diethyl ether. LPSs were isolated from the dry biomass by extraction with a phenol–water mixture and purified by centrifugation at 105000 *g* for three times. The residue was lyophilized. The LPS of *P. syringae* was prepared as an LPS–protein complex by extracting the wet biomass with 0.85% NaCl [11]. The preparations of ELPS, LPS, lipid A, core oligosaccharide, and OPS were obtained as described earlier [9, 10]. The total content of carbohydrates, proteins, lipids, fatty acids, and the hydrophilic components of lipid A, as well as the degree of LPS phosphorylation, were determined as described elsewhere [10].

The ice nucleation activity of microbial cells and various preparations was assayed with a Selena chamber [2] and expressed as a percentage of frozen drops (N/N_o, %, where N is the number of frozen drops and N_o is the total number of drops).

RESULTS AND DISCUSSION

The ice nucleation activities of intact microbial cells and various preparations were determined under identical conditions with distilled water as the control. Drops of distilled water began to freeze at –9°C. The freezing of aqueous bacterial suspensions at temperatures above this point could be due to the ice nucleation activity of bacterial cells. As is evident from the data presented in Table 1, the intact cells of most of the strains studied exhibited low ice nucleation activity, except the cells of *P. syringae* pv. *aptata* IMV 185; *P. fluorescens* IMV 472 (biovar I); and, especially, *P. syringae* pv. *syringae* (*holci*) IMV 1951, which possessed high ice nucleation activity. The cells of the latter strain exhibited the highest ice nucleation activity; their aqueous suspensions began to freeze at –1°C (2% frozen drops) and completely froze at –3°C (100% frozen drops) (Table 1). In contrast, the aqueous cell suspensions of two strains (*P. fluorescens* IMV 1433 and IMV 2125) began to freeze at lower temperatures (–12°C) than did distilled water (–9°C), which suggests that the cells of these strains possess antifreeze activity. The ice nucleation activity of the bacterial strains studied did not show any notable correlation with their taxonomic status. It should, however, be noted that the high ice nucleation activity of strains IMV 185 and IMV 1951 of the phytopathogenic species *P. syringae* is in agreement with

Table 2. The ice nucleation activity of the ELPSs derived from various bacterial strains of the genus *Pseudomonas*

Strain	Percentage of frozen drops of ELPS suspensions at temperatures from –1 to –19°C																		
	–1	–2	–3	–4	–5	–6	–7	–8	–9	–10	–11	–12	–13	–14	–15	–16	–17	–18	–19
P. fluorescens IMV 4125 (biovar I)										2	4	4	22	74	94	100			
P. fluorescens IMV 1433 (biovar I)										2	8	12	34	36	42	74	92	100	
P. fluorescens IMV 1152 (biovar I)							4	8	16	34	40	62	86	100					
P. fluorescens IMV 247 (biovar II)									4	8	16	34	56	80	100				
P. fluorescens IMV 472 (biovar I)							6	18	24	32	38	70	88	100					
P. fragi IMV 4002							2	16	36	60	80	96	100						
P. pseudoalcaligenes IMV 4134					4	6	14	16	20	24	36	64	100						
P. syringae pv. *syringae* CPPB 281							2	6	100										
pv. *maculicola* IMV 381											4	8	12	18	26	50	80	100	
pv. *aptata* IMV 185							4	36	94	100									
pv. *syringae* (*holci*) IMV 1951							4	6	40	66	100								
Distilled water									2	6	10	16	22	30	38	74	86	94	100

the finding of Maki and Villougby [12] that most biogenic ice nuclei occurring on plant leaves represent bacterial cells. Some strains exhibited a stepwise character of freezing of their cell suspensions (Table 1). This agrees with the suggestion of Dubrovsky *et al.* [13] that the ability of microbial cells to initiate water freezing is due to the presence of different sites on the cell surface that possess different ice nucleation activity.

To understand what chemical components of the cell surface are responsible for the ice nucleation activity of bacteria, we studied the ice nucleation activity of their ELPSs and LPSs, which are the uppermost carbohydrate-containing components of the cell surface of gram-negative bacteria. It should be noted that LPSs (which are fixed in the outer membrane of bacterial cells) and ELPSs (which are excreted from the cells during their cultivation in liquid and on solid media) are similar in composition, structure, and biological properties [9, 14].

Experiments showed that the ELPSs of some bacterial strains possess antifreeze activity since they lowered the freezing temperature of drops by 1–2°C as compared to that of distilled water. A comparison of the data presented in Tables 1 and 2 showed that sometimes there was a correlation between the antifreeze activities of ELPSs and the cells from which they were isolated. At the same time, the ELPS of *P. syringae* pv. *maculicola* IMV 381 exhibited the highest antifreeze activity among the ELPSs studied, although the antifreeze activity of the cells of this strain was relatively low. In general, ELPSs might be responsible for the antifreeze activity of bacterial cells. Of interest in this regard is the observation of Hao Xu *et al.* [15] that the removal of more than half (56 mol %) of the carbohydrate component of a biopolymer from *Pseudomonas putida* GR12-2 had no significant influence on the antifreeze activity of this biopolymer, which was composed of protein, carbohydrate, and lipid components.

The maximum ice nucleation activity was observed for the ELPS of *P. pseudoalcaligenes* IMV 4134, which exceeded that of the cells of this strain (Tables 1, 2). The high activity of this ELPS correlated with the absence of 2-oxydodecanoic acid in its constituent lipid A and the prevalence of the R forms of LPS molecules, which are characterized by an elevated content of lipid components.

The structure of the O-specific chain of the ELPS molecule probably does not influence ice nucleation, as is evident from the fact that the O-specific chains of the ELPSs of *P. fluorescens* IMV 1433 and IMV 1152 have identical structure [10], whereas the ELPS of strain IMV 1433 has antifreeze activity and the ELPS of

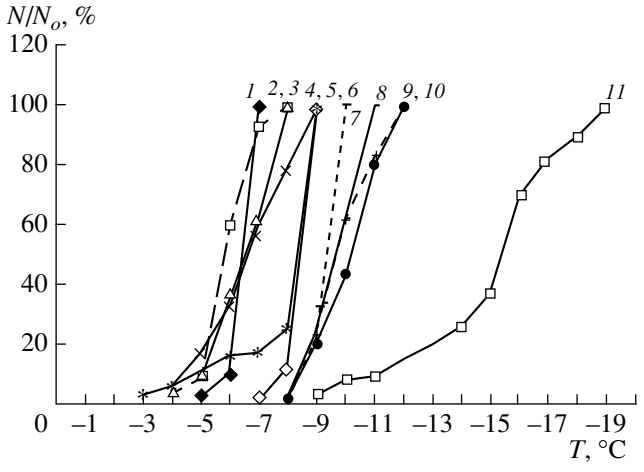

Fig. 1. The ice nucleation activity of the ELPS of *P. syringae* at different ELPS concentrations: (*1–3*) 0.4%; (*4–6*) 0.2%; (*7–10*) 0.1%; (*11*) the control.

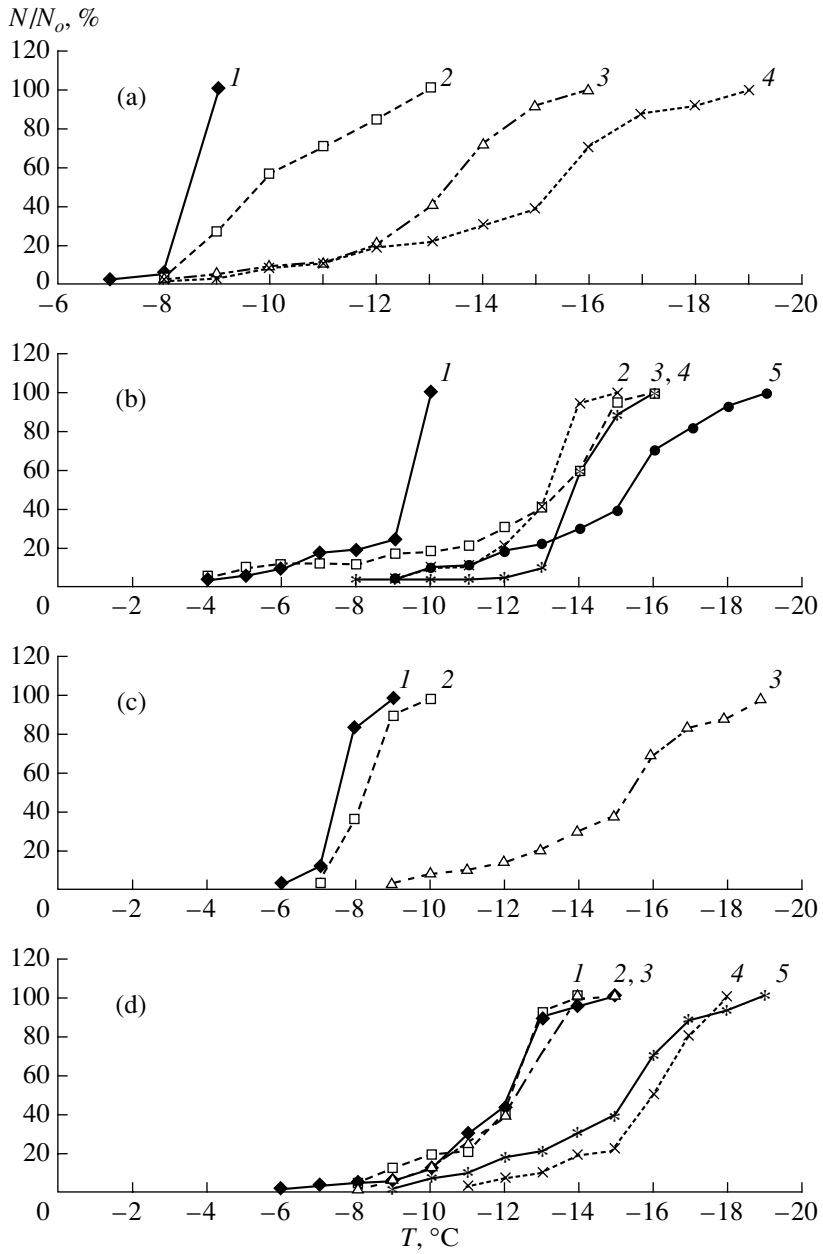

Fig. 2. The ice nucleation activity of the *P. syringae* ELPS, LPS, and their structural components. Panel (a) (strain IMV 281): (*1*) ELPS, (*2*) OPS, (*3*) LPS, (*4*) control. Panel (b) (strain IMV 1951): (*1*) ELPS, (*2*) LPS, (*3*) OPS, (*4*) core oligosaccharide, (*5*) control. Panel (c) (strain IMV 185): (*1*) ELPS, (*2*) LPS, (*3*) control. Panel (d) (strain IMV 381): (*1*) LPS, (*2*) OPS, (*3*) lipid A, (*4*) ELPS, (*5*) control.

strain IMV 1152 has ice nucleation activity (Table 2). As an aside, the ELPS of *P. syringae* pv. *maculicola* IMV 381 exhibited the maximum antifreeze activity.

Thus, ELPSs are unlikely to be directly involved in ice nucleation. The relatively high ice nucleation activity of some ELPS preparations may be due to an increased viscosity of these preparations. The aforementioned correlation between some structural features of the ELPS of strain IMV 4134 and its ice nucleation activity needs further studies on a wider range of strains.

Figure 1 shows that the ice nucleation activity of the *P. syringae* LPS depends little on its concentration within 0.2–0.4% but is notably diminished at concentrations lower than 0.1%. This observation shows that the formation of ice nuclei requires a certain number of interacting macromolecules. This confirms the supposition of Zachariassen [16] that ice nucleation is a cooperative process. Sothworth *et al.* [17] showed that the ice nucleation protein they studied contained repeating sequences of 8, 16, and 48 amino acid residues, which could be responsible for the arrangement of water mol-

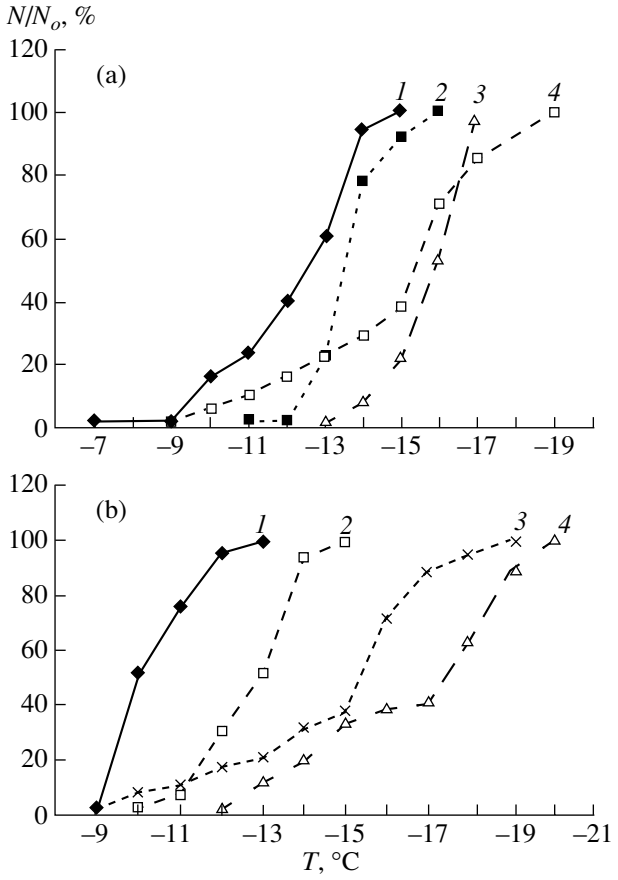

Fig. 3. The ice nucleation activity of the *P. fluorescens* ELPS, LPS, and OPS. Panel (a) (strain IMV 4125): (*1*) LPS, (*2*) ELPS, (*3*) OPS, (*4*) control. Panel (b) (strain IMV 1433): (*1*) OPS, (*2*) LPS, (*3*) control, (*4*) ELPS.

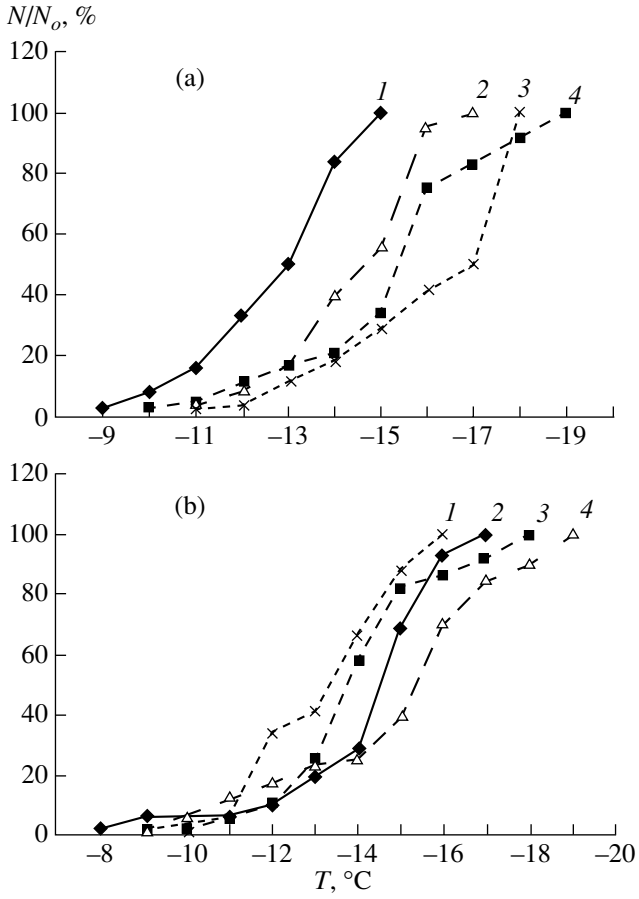

Fig. 4. The ice nucleation activity of the ELPS, LPS, their structural components, and intact cells of *P. fluorescens* IMV 247 (biovar I). Panel (a): (*1*) ELPS, (*2*) LPS, (*3*) cells, (*4*) control. Panel (b): (*1*) OPS, (*2*) lipid A, (*3*) core oligosaccharide, (*4*) control.

ecules into a lattice that acts as an ice nucleation template. Taking into account the fact that LPS molecules are composed of regular repeating units, one can suggest the same mechanism for the ice nucleation activity of LPSs.

A comparative analysis of the ice nucleation activity of the intact cells, ELPSs, LPSs, and LPS structural components (lipid A, core oligosaccharide, and O-specific polysaccharide) of various strains (Tables 1, 2; Figs. 2–4) showed that all of these preparations influenced the process of ice nucleation. The cells of strain IMV 1951 actively stimulated ice nucleation, so that their aqueous suspensions began to freeze at –1°C and completely froze at –3°C (Table 1), i.e., at temperatures at least 8°C higher than distilled water. Noteworthy is the fact that distilled water freezes between –9 and –19°C, whereas the suspension of IMV 1951 cells froze within a much narrower temperature range (between –1 and –3°C). The ELPS of strain IMV 281 also exhibited a very narrow range of freezing temperatures (Table 2).

All the preparations obtained from microbial cells influenced the process of ice nucleation. In most cases, however, the ice nucleation activity of the ELPSs, LPSs, and their structural components differed from that of the intact cells from which these preparations were obtained. This suggests that LPSs and their structural components may play a role in ice nucleation but this role is not crucial. The fraction of O-specific polysaccharide, which lacks a protein component and lipid A, also influenced the process of ice nucleation (Figs. 2–4), suggesting the involvement of the carbohydrate moiety of the LPS molecule in ice nucleation. However, the structure of the O-specific chain of the LPS molecule does not influence ice nucleation, as is evident from the fact that the O-specific chains of the LPSs of *P. fluorescens* IMV 1433 and IMV 1152 have identical structure [10], whereas the LPS of strain IMV 1433 has antifreeze activity and the LPS of strain IMV 1152 has ice nucleation activity. The ice nucleation activity of the intact cells of the bacterial strains studied did not show any correlation with their taxonomic status, although the maximum ice nucleation activity was observed for the phytopathogenic species *P. syringae*. Some strains exhibited a stepwise character of freezing

of their cell suspensions, suggesting that the ability of microbial cells to initiate water freezing may be due to the presence of different sites on the cell surface that possess different ice nucleation activity.

The maximum ice nucleation activity was observed for the ELPS of *P. pseudoalcaligenes* IMV 4134, which correlated with the absence of 2-oxydodecanoic acid in its constituent lipid A and the prevalence of the R forms of LPS molecules, enriched in lipid components.

Thus, LPSs and their structural components may play a role in ice nucleation, but this role is not crucial. It is unlikely that the structure of the O-specific chain of the LPS molecule can influence ice nucleation, as is evident from the different activities of the O-specific chains of the LPSs of *P. fluorescens* IMV 1433 and IMV 1152, which have identical structure. The specific dependence of the ice nucleation activity of LPSs on their concentration is consistent with the hypothesis postulating a cooperative mechanism of ice nucleation. The high ice nucleation activity of ELPSs suggests their important role in the protection and survival of microbial cells.

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