



Lipid A structural characterization from the LPS of the Siberian psychro-tolerant *Psychrobacter arcticus* 273-4 grown at low temperature

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Abstract

Psychrobacter arcticus 273-4 is a Gram-negative bacterium isolated from a 20,000-to-30,000-year-old continuously frozen permafrost in the Kolyma region in Siberia. The survival strategies adopted to live at subzero temperatures include all the outer membrane molecules. A strategic involvement in the well-known enhancement of cellular membrane fluidity is attributable to the lipopolysaccharides (LPSs). These molecules covering about the 75% of cellular surface contribute to cold adaptation through structural modifications in their portions. In this work, we elucidated the exact structure of lipid A moiety obtained from the lipopolysaccharide of *P. arcticus* grown at 4 °C, to mimic the response to the real environment temperatures. The lipid A was obtained from the LPS by mild acid hydrolysis. The lipid A and its partially deacylated derivatives were exhaustively characterized by chemical analysis and by means of ESI Q-Orbitrap mass spectrometry. Moreover, biological assays indicated that *P. arcticus* 273-4 lipid A may behave as a weak TLR4 agonist.

Keywords *Psychrobacter arcticus* 273-4 · Lipid A · Mass spectrometry · Psychrotolerant · Permafrost

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Introduction

Much of life on Earth has evolved to colonize environments where the temperatures play a critical role in the selection and survival of a variety of organisms such as bacteria, archaea, yeasts, algae, insects, fishes and plants. These cold-adapted organisms are generally referred to as psychrophiles (Chattopadhyay et al. 2014).

Cold adaptation mechanisms are the result of intrinsic genome-wide features that facilitate the growth at low temperatures. Comparative studies of cold-adapted microorganisms are beginning to reveal which adaptations are common to all the psychrophiles and which are specific to the particular environment each psychrophile inhabits. Cold marine environments are distinctly different from cold terrestrial environments like permafrost: marine environments tend to have high thermal stability as well as stable solute concentrations, while terrestrial environments do not. Consequently, genomic analysis of microorganisms isolated from marine environments likely reflects adaptations to stable low temperatures, while that from terrestrial environments may reveal unique mechanism of cold adaptation due to

the extreme temperatures fluctuations (Ayala-del-Río et al. 2010).

It has long been known that one of the most significant impacts of low temperature is about the membrane fluidity, essential for its structural integrity and for the cellular functionality (Beales 2004; Chattopadhyay 2006; D'Amico et al. 2006). In fact, recent transcriptome analyses have shown that exposure to cold temperatures induces a rapid up-regulation of genes involved in membrane biogenesis, such as fatty acids and LPS biosynthesis, peptidoglycan and outer membrane proteins biosynthesis (De Maayer et al. 2014). The lipid composition governs the physical properties of the membranes and hence it is not surprising that this varies with the thermal habitat of the microorganism. In general, a reduction in temperature leads to a higher content of unsaturated, polyunsaturated and methyl-branched fatty acids, and/or a shorter acyl-chain length. This altered composition is thought to have a key role in increasing membrane fluidity by introducing steric constraints that change the packing order to reduce the number of interaction in the membrane.

Lipopolysaccharides (LPSs), heat-stable complex amphiphilic macromolecules, are the major constituent of the bacteria outer membranes that form a protective barrier around the cell. The LPS is composed of three different domains: a hydrophilic O-specific polysaccharide, and a core oligosaccharide that is covalently linked to the third glycolipid domain, the lipid A. Lipid A possesses a quite conservative structure usually consisting of a β -D-GlcN-(1 \rightarrow 6)- α -D-GlcN (GlcN, 2-amino-2-deoxy-glucopyranose) disaccharide backbone that bears 3-hydroxy fatty acid residues, linked as ester at 3 and 3' positions, and amide-linked at 2 and 2' positions, which are indicated as primary fatty acid residues. The 3-OH, in turn, can be esterified by secondary fatty acids. Generally, the α -anomeric position of the reducing GlcNI (proximal GlcN) residue and the hydroxyl at position 4' of the non-reducing GlcNII (distal GlcN) residue are linked to polar heads, phosphate groups. Despite this general structure, lipid A could present chemical differences that are responsible of the variation among bacterial species and among the same species; this variability has been linked to a variety of biological functions, including the survival under hostile conditions. The presence of phosphate groups and charged residues in the core oligosaccharides allowed the binding of divalent cations, which probably help in stabilizing the outer leaflet of the membrane. The alteration in the outer membrane property and related compositional change in LPS is well known to be related to the lipid A portion. At lower temperatures, in bacteria that are not naturally adapted to cold (mesophiles such as *E. coli* and *B. subtilis*), cold shock induces homeoviscous adaptation of phospholipid acyl structure in order to preserve membrane integrity. This response is related to the increased incorporation of unsaturated, short, and/or branched fatty acids to

maintain membrane fluidity as part of a set of physiological actions necessary to survival in cold conditions (Carty et al. 1999; Graumann and Marahiel 1999).

Psychrobacter arcticus 273-4 is a Gram-negative bacterium isolated from Kolyma region of Siberian permafrost core (Vishnivetskaya et al. 2000). *Psychrobacter arcticus* 273-4 is considered a psychro-tolerant microorganism as it can grow at low, even subzero temperatures and even more than 20 °C (Gilichinsky et al. 2007). The preliminary lipid A structure from *P. arcticus* grown at 24 °C has been already reported (Korneev et al. 2014). In this work, we evaluated the lipid A structure from the same bacterium grown at 4 °C. The lipid A was obtained from dried cells, extracted by PCP method and purified from the LPS (Casillo et al. 2015) after mild acidic conditions. The lipid A and its partially deacylated derivatives were completely characterized by MS and MS/MS Orbitrap mass spectrometry.

Experimental section

Isolation of the LPS

Psychrobacter arcticus 273-4 was isolated from permafrost soil located in Siberia and grown as previously reported (Casillo et al. 2015). Shake flask cultivation was performed in Luria–Bertani broth at 4 °C in aerobic condition. When the liquid cultures reached late exponential phase (about 90 h, OD_{600 nm} 4), cells were collected by centrifugation for 15 min at 7000 rpm at 4 °C. The doubling time of *P. arcticus* was found to be 17 h. LPS was extracted from dried bacteria cells (3.2 g) by hot phenol/water method (Westphal and Jann 1965) and the crude extract was subjected to enzymatic treatment (Casillo et al. 2015), obtaining 92 mg of sample (LPS_w, yield 2.9% w/w of dried cells). The LPS sample was suspended in chloroform/methanol (1:2, v/v) to a concentration of 10 mg/mL to remove phospholipids. The LPS was recovered from the pellet, whereas the phospholipids from the supernatant.

Mild acid hydrolysis of the LPS

The LPS (50 mg) was incubated with 5% aqueous CH₃COOH (7.6 mL) for 3 h at 100 °C. The sample was then centrifuged at 7000 rpm for 30 min and two fractions were recovered: the pellet (6.7 mg), constituted by lipid A, and the saccharidic portion (15.6 mg).

Chemical analysis

To a lipid A sample (0.5 mg), 1 mL of 1.25 M HCl/CH₃OH was added and the methanolysis reaction was carried out at 80 °C for 20 h. The crude mixture was extracted twice with

hexane. The methanol layer was dried and acetylated with acetic anhydride (50 μ L), in the presence of pyridine (50 μ L), at 100 °C for 30 min, and treated as already reported (Casillo et al. 2017a). The acetylated methyl glycosides were analyzed by Gas Chromatography–Mass Spectrometry (GC–MS). The hexane layer, containing the fatty acids as methyl esters derivatives, was analyzed by GC–MS. The analyses were performed on a Agilent Technologies gas chromatograph 6850A equipped with a mass selective detector 5973N and a Zebron ZB-5 capillary column (Phenomenex, 30 m \times 0.25 mm i.d., flow rate 1 mL/min, He as carrier gas). The following temperature programs were used: for acetylated methyl glycosides, 140 °C for 3 min, 140 °C \rightarrow 240 °C at 3 °C/min; for lipid analysis, 110 °C for 3 min, 140 °C \rightarrow 280 °C at 10 °C/min.

NH₄OH hydrolysis of lipid A

Lipid A (0.5 mg) was incubated with conc. NH₄OH (200 μ L) as reported (Silipo et al. 2002). The sample was dried and analyzed by ESI mass spectrometry.

Mass spectrometry

For MS and MS/MS analyses isolated, lipid A and NH₄OH-treated lipid A were analyzed with a Q-Exactive Plus Orbitrap (Thermo, Bremen, Germany) using a Triversa Nanomate (Advion, Ithaca, USA) ion source with a spray voltage set to -1.1 kV. The resolution was set to 280,000 full width at half maximum defined at m/z 200.

For negative ion mode, samples (~ 10 ng/mL) were dissolved in propan-2-ol/water/Et₃N (50:50:0.001, v/v/v). The obtained mass spectra were charge deconvoluted and the given mass numbers refer to the monoisotopic mass of the neutral molecules.

For the positive ion mode, samples were dissolved in propan-2-ol/water/30 mm ammonium acetate (50:50:4, v/v/v) adjusted with acetic acid to pH 4.5. For MS/MS analyses, the normalized collision energy (NCE) was set to either 13 or 14 NCE and the precursor selection was performed with unit resolution to isolate the monoisotopic signals.

Biological activity assay in murine Raw Blue 264.7 cells

Murine RAW Blue 264.7 cells (Invivogen) were cultured in Dulbecco's modified Eagle's medium, supplemented with 10% fetal calf serum (FCS), L-glutamine (2 mM) and sodium pyruvate (0.4 mM), in the presence of selection antibiotic Zeocin (200 μ g/mL). Lipid A was reconstituted in DMSO to provide 1 mg/mL stock solutions. Further dilutions were made in cell culture medium so that the final amount of DMSO in the cell culture did not exceed 0.01%. 5×10^4 Raw

Blue 264.7 cells were stimulated in 200 μ L total volume with *P. arcticus* 273-4 Lipid A in the presence or absence of *E. coli* O111:B4 LPS (Invivogen) at the indicated concentrations. Cells were incubated for 24 h at 37 °C and 5% CO₂, and the supernatants were analyzed for TNF production by ELISA (BD Biosciences).

Results

Isolation of lipid A and compositional analysis

The extraction of *Psychrobacter arcticus* 273-4 dried cells by hot phenol-water method (Westphal and Jann 1965) yielded about 2.9% of LPS (Casillo et al. 2015). The LPS was subjected to 5% acetic acid hydrolysis to obtain the lipid A fraction. The lipid A was recovered as pellet after centrifugation of the crude reaction and washed twice with water and freeze dried. The compositional analysis of the lipid A sample was performed through a methanolysis reaction, after which the methanol layer was extracted with hexane, acetylated and analyzed by GC–MS (Fig. S1). The chromatogram indicated the glucosamine as the only monosaccharide constituting the glycolipid. The fatty acids methyl esters derivatives were recovered from the hexane layer and analyzed by GC–MS (Fig. S2). The fatty acids analysis showed the presence of mainly 3-hydroxy-dodecanoic acid [C12:0(3-OH)], together with a minor amount of 3-hydroxy-undecadecanoic acid [C11:0(3-OH)], 3-hydroxy-tridecanoic acid [C13:0(3-OH)], and 3-hydroxy-tetradecanoic acid [C14:0(3-OH)], as hydroxylated species. The analysis also revealed the occurrence of decanoic (C10:0), dodecanoic (C12:0), tetradecanoic (C14:0), and pentadecanoic (C15:0) acids. The abundance of the signals attributable to hexadecanoic (C16:0), hexadecenoic (C16:1), eptadecanoic (C17:0), together with the octadecanoic (C18:0) and octadecenoic (C18:1) acids, suggested the presence of membrane phospholipids in the lipid A sample. Phospholipids' contaminants were removed from LPS by extraction with a chloroform–methanol mixture, and a very low contamination due to phospholipids was revealed by GC–MS (Fig. S3). The presence of phosphate groups was deduced from NMR spectra of the core oligosaccharide (Casillo et al. 2015) and confirmed by mass spectrometry.

Mass spectrometry analysis

The charge-deconvoluted ESI mass spectrum obtained in negative ion mode (Fig. 1) for the lipid A from *P. arcticus* revealed a complex pattern of four main signals clusters around **M1–M4**, representing tri-, tetra-, penta-, and hexa-acylated lipid A glycoforms (Table 1). The heterogeneity within each cluster is caused by variation of the fatty

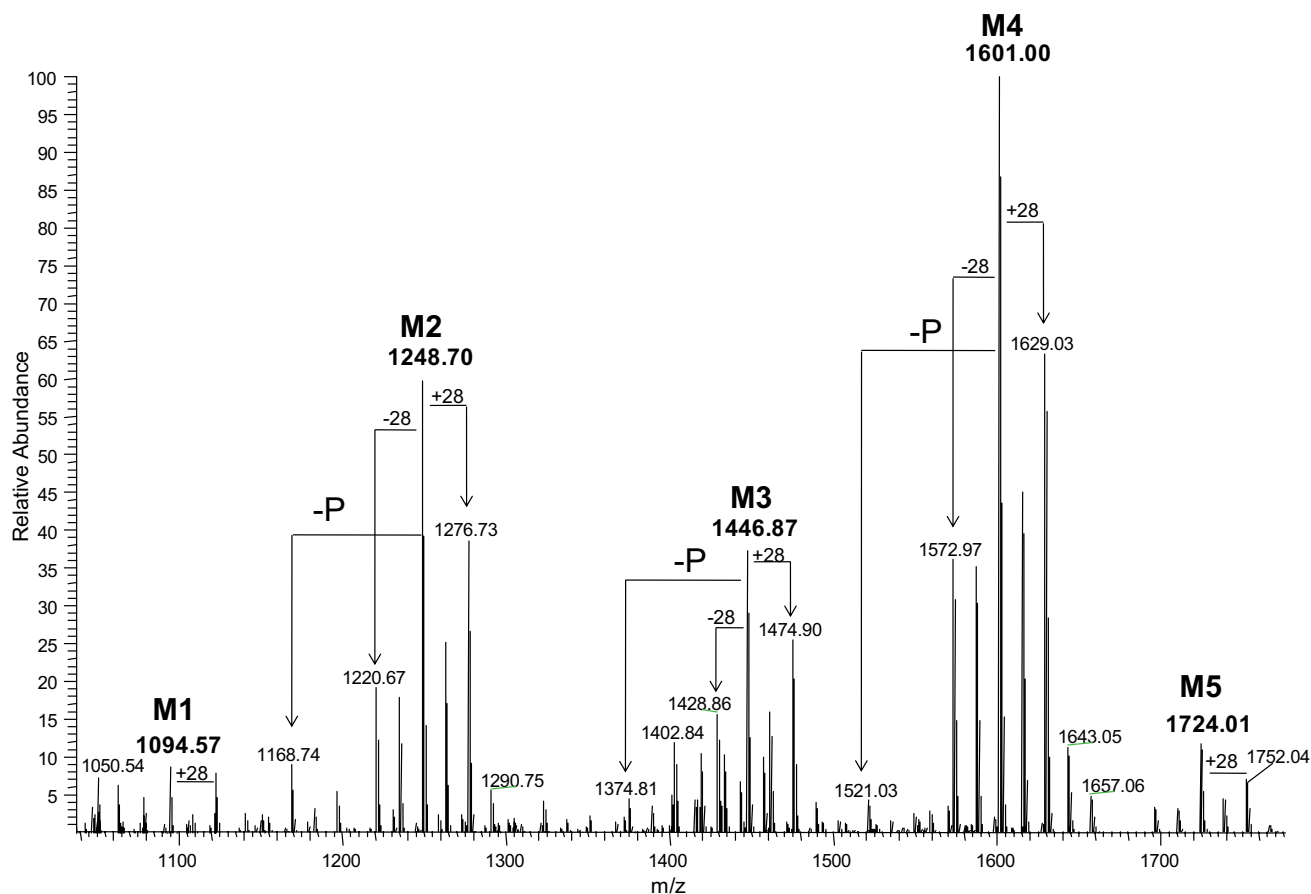


Fig. 1 Charge deconvoluted mass spectrum of the lipid A from *P. arcticus* 273-4. The spectrum was acquired in the negative ion mode and the mass numbers given refer to the monoisotopic peaks of the neutral molecules

Table 1 Composition of the main species present in the lipid A from *P. arcticus* 273-4 (see Fig. 1)

Species	M_{acc}	M_{obs}	Composition
M1	1094.567	1094.57	$\text{GlcN}_2\text{P}_2[\text{C12:0(3-OH)}]_3$
M2	1248.715	1248.70	$\text{GlcN}_2\text{P}_2[\text{C12:0(3-OH)}]_3(\text{C10:0})$
M3	1446.880	1446.87	$\text{GlcN}_2\text{P}_2[\text{C12:0(3-OH)}]_4(\text{C10:0})$
M4	1601.019	1601.00	$\text{GlcN}_2\text{P}_2[\text{C12:0(3-OH)}]_4(\text{C10:0})_2$
M5	1724.01	1724.01	$\text{GlcN}_2\text{P}_2\text{PEtN}[\text{C12:0(3-OH)}]_4(\text{C10:0})_2$

acids chain length, with the replacement of a C12:0(3-OH) with one or two C11:0(3-OH) (– 14.01 and – 28.03 Da, respectively) and by the substitution of a C12:0(3-OH) with a C13:0(3-OH) (+14 Da) or a C14:0(3-OH) (+ 28 Da). Further heterogeneity is caused by species with only one phosphate group (– 79.97 Da, labeled by – P) and by an additional phosphoethanolamine (+123.01 Da) (signal cluster around **M5**).

Chemical analysis and mass spectra results allowed to assign to the tri-acylated species **M1** (calculated molecular mass (1094.567 Da) the following composition:

$\text{GlcN}_2\text{P}_2[\text{C12:0(3-OH)}]_3$. The tetra-acylated species **M2** differs from **M1** by + 154.13 Da, indicating the presence of an additional decanoic acid (calculated molecular mass 1248.715 Da). The results of the main species are summarized in Table 1.

To understand the position of the secondary fatty acids, the lipid A from *P. arcticus* was treated with conc. NH_4OH , a procedure that allows to hydrolyze acyl and acyloxyacyl esters, leaving intact acyl and acyloxyacyl amides (Silipo et al. 2002).

The charge deconvoluted negative ions ESI mass spectrum of the lipid $\text{A}_{\text{NH}_4\text{OH}}$ (Fig. 2, Table 2) revealed the presence of four main signals, the most abundant of which, indicated as **K3**, displayed a measured mass of 1050.54 Da. **K3** signal corresponded to a molecular species, of which the composition was identified as $\text{GlcN}_2\text{P}_2[\text{C12:0(3-OH)}]_2(\text{C10:0})$ (calculated molecular mass 1050.542 Da), thus indicating that the decanoic acid was linked as acyloxyacyl amide. In addition, correlated to this species, signals differing of – 14 Da and both + 14 and + 28 Da were displayed, confirming that *P. arcticus* lipid A shows heterogeneity about the length of 3-OH fatty acids (Korneev et al. 2014).

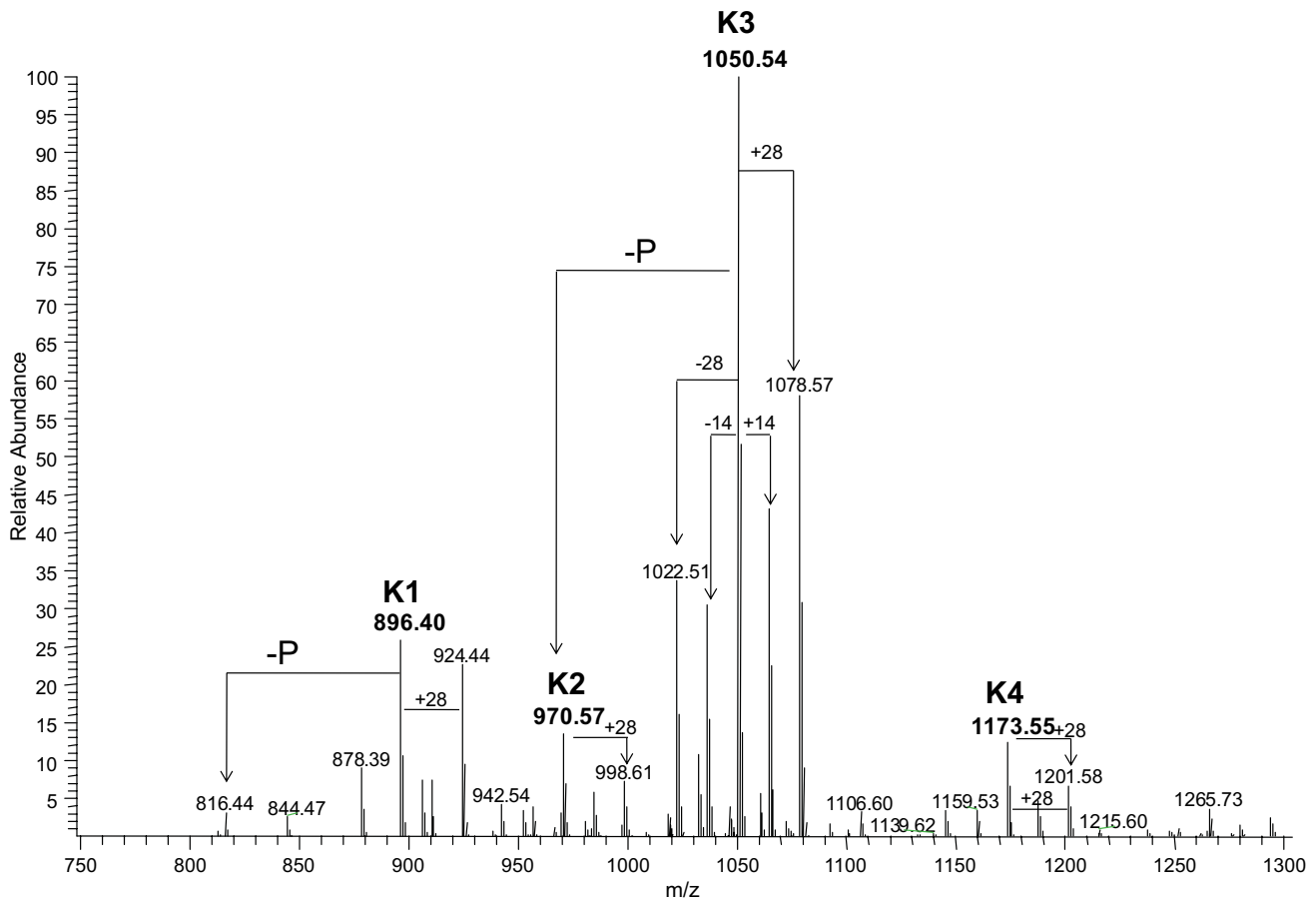


Fig. 2 Charge deconvoluted mass spectrum of the lipid A_{NH_4OH} from *P. arcticus* 273-4. The spectrum was acquired in the negative ion mode and the mass numbers given refer to the monoisotopic peaks of the neutral molecules

Table 2 Composition of the main species of the *P. arcticus* 273-4 lipid A_{NH_4OH} observed in the ESI mass spectrum

Species	$[M - H]^-$ calculated	$[M - H]^-$ observed	Composition
K1	896.403	896.40	$GlcN_2P_2[C12:0(3OH)]_2$
K2	970.575	970.57	$GlcN_2P[C12:0(3OH)]_2[C10:0]$
K3	1050.542	1050.54	$GlcN_2P_2[C12:0(3OH)]_2[C10:0]$
K4	1173.55	1173.55	$GlcN_2P_2PEtN[C12:0(3OH)]_2[C10:0]$

To obtain the exact distribution of fatty acids on each glucosamine residue, lipid A and lipid A_{NH_4OH} samples were analyzed by MS and MS/MS in the positive ion mode. Starting from lipid A_{NH_4OH} sample, the MS experiment indicated the presence of the same species revealed in the negative ion mode, as $[M + H]^+$ adducts.

The MS/MS of the $[M + H]^+$ ions leads to the diagnostic important fragment ions B^+ and the $(Y-P)^+$ allowing to determine the distribution of the N-linked primary and secondary fatty acids on both glucosamine residues (Table 3). In particular, oxonium ions B^+ indicated that the C12:0(3-OH) of GlcNII can be substituted by a C14:0(3-OH) (Supporting Information, Figs. S4 and S5), and that the decanoic

acid is always linked as an acyloxyacyl residue. Moreover, the $(Y-P)^+$ fragment ion indicated that the N-substitution of GlcNI is always due to a C12:0(3-OH).

When MS/MS experiments were performed on the intact lipid A, the distribution of all the fatty acids on the two GlcN residues for the main species was obtained (Table 3). In general, the fragmentation leads to displaying both B1 and Y1 fragments, (Domon and Costello 1988) together with fragments due to the loss of fatty acids mainly as free acids instead of ketene. In particular, the $(Y-P)^+$ fragment ions analysis indicated the same substitution of GlcNII for tetra-acyl, penta-acyl, and hexa-acyl species (Table 3, Supporting Information Figs. S6–S8). Instead, a different acylation

Table 3 Positive fragment ions predicted and observed in the MS/MS spectra

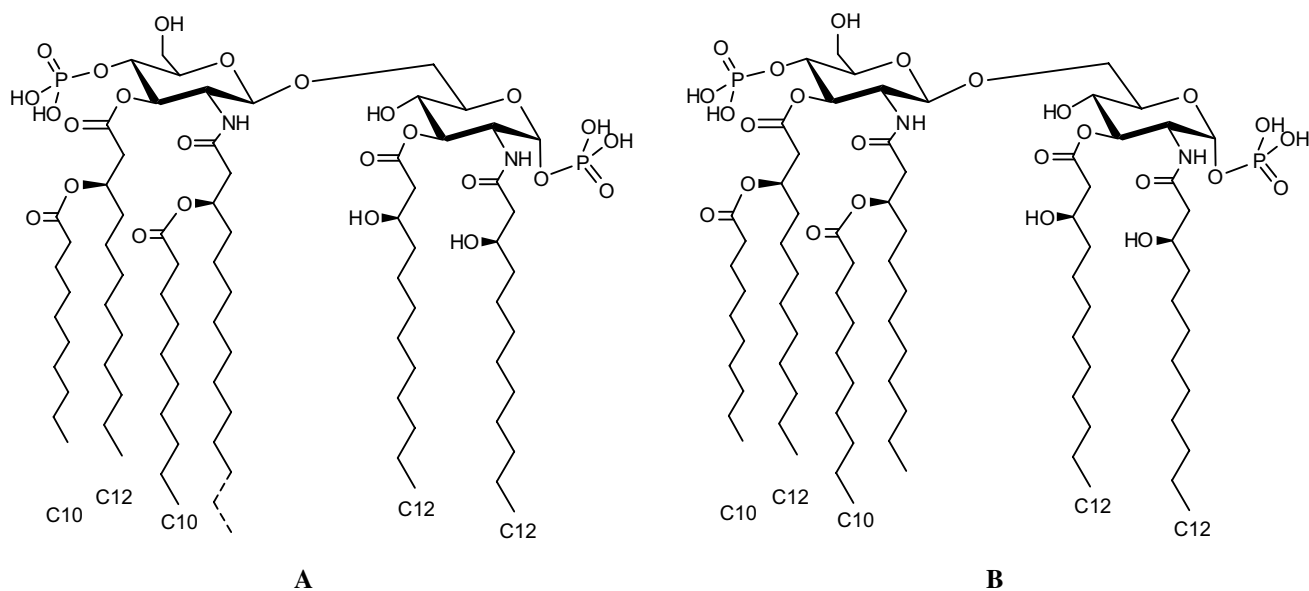
Species	Parent ion	B_{acc}	B_{oss}	Composition	$(Y-P)_{acc}$	$(Y-P)_{oss}$	Composition
<i>P. arcticus</i> NH_4OH	1051.58	594.347	594.34	GlcNP[C12:0(3OH)] [C10:0]	360.242	360.24	GlcN[C12:0(3OH)]
<i>P. arcticus</i> NH_4OH	1079.48	622.378	622.37	GlcNP[C14:0(3OH)] [C10:0]	360.242	360.24	GlcN[C12:0(3OH)]
<i>P. arcticus</i> tetra-acyl	1277.75	622.378	622.37	GlcNP[C14:0(3OH)] [C10:0]	558.407	558.40	GlcN[C12:0(3OH)] ₂
<i>P. arcticus</i> penta-acyl	1475.93	820.543	820.53	GlcNP[C12:0(3OH)] [C14:0(3OH)] [C10:0]	558.407	558.40	GlcN[C12:0(3OH)] ₂
<i>P. arcticus</i> hexa-acyl	1629.93	974.682	974.67	GlcNP[C12:0(3OH)] [C14:0(3OH)] [C10:0] ₂	558.407	558.40	GlcN[C12:0(3OH)] ₂

pattern was observed for GlcNI, as suggested by the B^+ ions composition. Moreover, in almost all the MS/MS experiments, a cluster of B^+ fragment ions was clearly visible, with difference of -14 and -28 u with respect to the main signal, suggesting that the same parent ion matches with a different fatty acids combination.

Starting from the parent ion at m/z 1277.75 corresponding to the tetra-acylated species (Fig. S6), B^+ ion suggested that position 3' of GlcNII was not acylated, as the presence of two C12:0(3OH) residues was deduced for the $(Y-P)^+$ fragment. The MS/MS spectrum of the penta-acylated form with parent ion at m/z 1475.93 showed an additional C12:0(3OH) localized at the position 3' of the distal GlcN (Fig. S7) when compared with that of the above-described tetra-acylated species (Fig. S6), as revealed from the increased value of the B^+ ion fragment at m/z 820.53. Finally, the hexa-acylated lipid A species

with parent ion at 1629.93 gave an MS/MS spectrum suggesting that the distal GlcN had the additional decanoic acid linked as acyloxyacyl ester at position 3'. Indeed, in this spectrum a mass shift of $+154$ u for the B^+ ion was observed (Fig. S8).

All together, these results led to define the detailed acyl distribution on each glucosamine residue on the lipid A of *Psychrobacter arcticus* 273-4 grown at 4 °C, as reported in Fig. 3a. A comparison with the lipid A isolated from the same bacterium grown at 24 °C (Fig. 3b) revealed that the main difference in the acylation pattern is the appearance of 3-hydroxyl-tetradecanoic acid in place of the 3-hydroxyl-dodecanoic one on the distal GlcN, reported for all the species described at 24 °C (Korneev et al. 2014). Moreover, the ESI mass spectrum (Fig. 1) indicated an additional phosphoethanolamine in the lipid A structure at 4 °C, the presence of which has not been reported at 24 °C (Korneev et al. 2014).

**Fig. 3** Structures of lipid A compounds extracted from *Psychrobacter arcticus* 273-4 grown at 4 °C (a) and 24 °C (b)

Evaluation of the biological activity: *P. arcticus* 273-4 lipid A induces TNF production in murine macrophages

To characterize the effect of *P. arcticus* 273-4 lipid A on TNF production, we used the murine macrophages RAW Blue 264.7 cell line expressing TLR4 as well as MD-2 and CD14, which are necessary for the transfer of monomeric LPS to the TLR4 cell surface receptor and further downstream signaling (Park et al. 2009). As seen in Fig. 4, *P. arcticus* 273-4 lipid A induced TNF production when used at high concentrations. However, *P. arcticus* 273-4 lipid A was much less effective than *E. coli* O111:B4 LPS (0–100 ng/mL) as much higher concentrations were needed to induce TNF (Fig. 4). *P. arcticus* 273-4 lipid A slightly enhanced the TNF-inducing effect of suboptimal LPS concentrations, but did not further increase the effect of high LPS concentrations. Together, these data suggest that *P. arcticus* 273-4 lipid A may behave as a weak TLR4 agonist.

Discussion

Microbial life in cold environments is possible, even at sub-zero temperatures, as the bacteria have developed the proper strategies to survive these habitats. These strategies include the up-regulation of the cell envelope components, such as peptidoglycan, proteins and lipopolysaccharides (De Maayer et al. 2014). In addition, in order to avoid the cells freezing and maintaining a membrane fluidity, phospholipids of outer membrane display an increased number of unsaturated and/or shorter fatty acids chains. Since the lipopolysaccharides

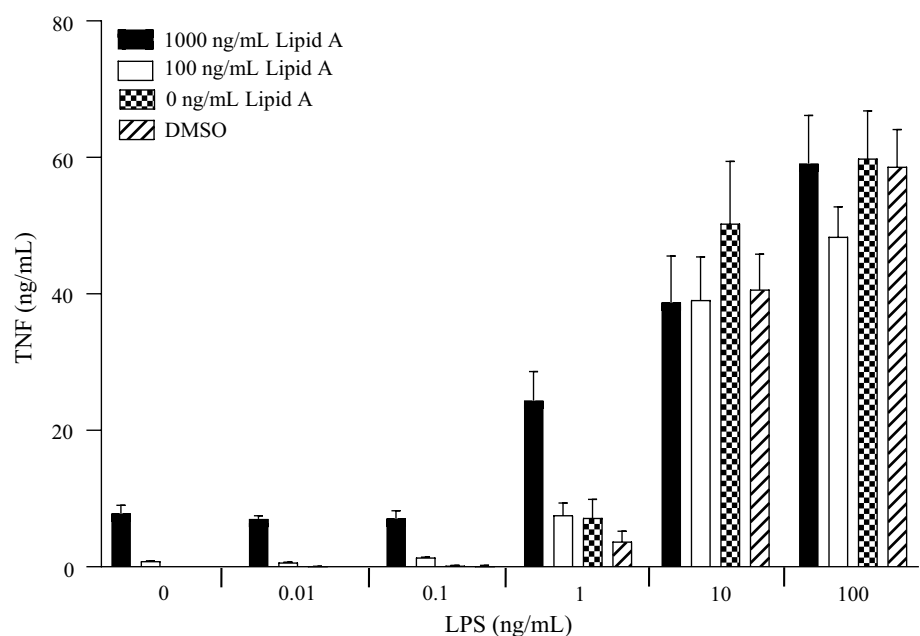
are the main components of the outer leaflet of Gram-negative bacteria, it is reasonable to hypothesize that some structural variations should also be present in the lipid A portion isolated from psychrophilic LPSs.

The lipid A structures from cold-adapted bacteria, reported up to now, have been isolated from bacteria belonging to *Psychrobacter* (Korneev et al. 2014; Sweet et al. 2015), *Colwellia* (Casillo et al. 2017b; Sweet et al. 2015), *Psychromonas* (Corsaro et al. 2008; Sweet et al. 2014), and *Pseudoalteromonas* (Carillo et al. 2011; Corsaro et al. 2002) genera. All these structures share short chains, e.g., C10–C14, while only some of them display unsaturated chains too. Another typical difference with respect to their mesophilic counterpart is the large heterogeneity of their glycoforms. Nonetheless, only few studies about the differences in lipid As when the same bacterium is grown at different temperatures have been so far reported (Corsaro et al. 2004, 2008; Sweet et al. 2015).

Herein, we described the complete structure of the lipid A isolated from *Psychrobacter arcticus* 273-4 grown at 4 °C, and compared it with that obtained from the bacterium grown at 24 °C. In addition, a comparison with the lipid A from *Psychrobacter cryohalolentis* K5, a Siberian permafrost isolate, has been reported, too.

The main hexa-acyl cluster of lipid A characterized in the present work displays in the mass spectrum at least five structures, varying from each other by single methylene units (CH₂). Two less abundant additional clusters of penta-acyl and tetra-acyl forms were also displayed. Finally, the lowest intensity cluster of hexa-acyl species showed an additional PEtN substituent. All these glycoforms are generated by the bacterial utilization of a

Fig. 4 Effect of *P. arcticus* 273-4 lipid A on LPS-induced TNF production in murine Raw Blue 264.7 cells. Cells were incubated with the indicated amounts of *P. arcticus* 273-4 lipid in the presence or absence of different concentrations of *E. coli* O111:B4 LPS, as indicated. DMSO was used as a vehicle control. Error bars represent the standard deviation of the mean of biological triplicates. Graph is representative of at least three independent experiments



wide number of acyl chains, ranging from C11:0(3OH)-C14:0(3OH) as primary fatty acids, and C10:0–C15:0 as secondary ones. This variability has already been observed for lipid A structures from other cold-adapted bacteria (Carillo et al. 2011; Corsaro et al. 2004), and could be connected to the homeoviscous adaptation (Zhang and Rock 2008) in response to temperature fluctuations.

A comparison of the here-reported lipid A structure with that of *P. arcticus* grown at 24 °C (Korneev et al. 2014) revealed that the main molecular species show a significant higher abundance, and an additional phosphoethanolamine appeared when the bacterium is grown at 4 °C. This was unexpected, as in the case of *Pseudoalteromonas haloplanktis* TAC 125 and *Psychromonas arctica* an increased content of phosphate was revealed in the bacterium grown at higher temperature (Corsaro et al. 2004, 2008). Unfortunately, no spectra were reported for *P. arcticus* grown at 25 °C, thus hampering a more precise comparison.

Psychrobacter cryohalolentis has been reported to produce hexa-acylated species only, which were similar to those of *P. arcticus*. This was not surprising, as the lipid A structure has been found to be conserved at least for the primary acyl chains in the LPSs belonging to bacteria of the same genus. In addition, changes in the relative abundance of the hexa-acyl species have been reported for *P. cryohalolentis* grown at 4, 15, and 25 °C (Sweet et al. 2015). A comparison between *P. cryohalolentis* and *P. arcticus* grown at 4 °C revealed that *P. arcticus* species produces hexa-acyl species with shorter fatty acids chains. In contrast, even if a shift toward a shorter overall average of the acyl units at cold temperature was reported for *P. cryohalolentis* species, no differences towards shorter chains, e.g., C10:0 and C13:0, were observed in its fatty acids analyses at lower temperatures (Sweet et al. 2015). For the *P. arcticus* species grown at 4 °C, we found the primary 3-hydroxy acyl chain length on the GlcN residues spanning from undecanoyl to tetradecanoyl, whereas at 24 °C only a minor species containing a 3-hydroxy decanoyl residue instead of a 3-hydroxy dodecanoyl one was reported (Korneev et al. 2014), thus confirming that also *P. arcticus* adjust its membrane fluidity by altering the fatty acids length. Finally, no differences in the phosphate content were reported for *P. cryohalolentis* when grown at 4, 15, and 25 °C (Sweet et al. 2015).

In conclusion, in this study, we described the lipid A structure from *P. arcticus* 273-4 grown at 4 °C, demonstrating the importance to punctually characterize the structure of the species revealed from mass spectra in order to deduce differences among growths at different temperatures. A fine structural characterization of LPS molecules could act as a springboard for studying the relevance of outer membrane features into cold adaptation (Benforte et al. 2018). Finally, biological activity assays indicated that *P. arcticus* lipid A induced low TNF production on murine macrophages when

compared to *E. coli* LPS, suggesting a weak TLR4 agonist behavior.

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