Influence of Growth Temperature on Lipid and Phosphate Contents of Surface Polysaccharides from the Antarctic Bacterium *Pseudoalteromonas haloplanktis* TAC 125

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The chemical structural variations induced by different growth temperatures in the lipooligosaccharide and exopolysaccharide components extracted from the Antarctic bacterium *Pseudoalteromonas haloplanktis* TAC 125 are described. The increase in phosphorylation with the increase in growth temperature seems to be general, because it happens not only for the lipooligosaccharide but also for the exopolysaccharide. Structural variations in the lipid components of lipid A also occur. In addition, free lipid A is found at both 25 and 4°C but not at 15°C, which is the optimal growth temperature, suggesting an incomplete biosynthesis of the lipooligosaccharide component under the first two temperature conditions.

Lipopolysaccharides (LPSs) are amphiphilic molecules contained in the outer leaflet of the external membrane of gram-negative bacteria. They are anchored in the membrane by the lipid part (lipid A), which is covalently linked to an oligosaccharide fragment (core) that, in turn, is bonded to a polysaccharide part (O antigen, or O side chain). Due to their outward location, the LPSs are involved in mechanisms of interaction with the surroundings. Despite the fact that gram-negative bacteria colonize very different organisms and environments, LPSs show a common architectural structure (17). This suggests that the molecular structures of the LPS components can play an important role in host or environment specificity. In this context, the structures of LPSs of extremophilic bacteria evoke much interest owing to the extreme conditions under which they live (14). The cold adaptation of psychrophilic bacteria, which enables them to thrive in environments below 5°C, necessitates the acquisition of unique structural features for membrane components, so that membrane fluidity and effective transport of nutrients under cold conditions are guaranteed. The exopolysaccharides (EPSs) that many bacteria are able to produce may also be involved in interaction with the environment, in addition to having rheological properties of potential economical interest (6, 19, 21).

Recently we have been interested in structural elucidation of both the saccharide backbones (5) and the lipid A moieties (4) of the LPS components of *Pseudoalteromonas haloplanktis* TAC 125, a cold-adapted bacterium isolated from Antarctic seawater (1) and grown at 15°C. In the first paper (5), Corsaro et al. showed that the LPS fraction consists of two lipooligosaccharides (LOSs); that is, it lacks the O chains. The major component possesses the following sugar backbone structure: α -D-ManpNHAc-(1→3)- β -D-Galp-(1→4)- α -L-glycero-D-

manno-Hepp-(1→5)- α -D-Kdo4OPO₃H₂-(2→6)- β -D-GlcpNH₂4OPO₃H₂-(1→6)- α -D-GlcpNH₂-(1→OPO₃H₂). The latter two units are both acylated at positions 2 and 3, with 3-hydroxydodecanoyl residues (3-OH-12:0) linked both as esters and as amides (4). The hydroxyl of the (3-OH-12:0) residue linked at position 3 of the nonreducing glucosamine is esterified by a dodecanoyl residue (12:0). Here we describe the variations that occur in the LOS structures when the bacterium is grown at two temperatures other than 15°C: one lower (4°C) and one higher (25°C). Since this bacterium is also able to produce an EPS fraction, the EPS structures obtained at 4, 15, and 25°C were determined and compared to each other.

MATERIALS AND METHODS

General methods. Nuclear magnetic resonance (NMR) spectra were recorded on an INOVA 500 spectrometer by using a 5-mm multinuclear inverse Z-gradient probe. ¹³C and ¹H chemical shifts were measured in D₂O by using acetone (δ 31.4) and sodium 3-trimethylsilylpropionate-2,2,3,3-²H₄ (δ 0.00), respectively, as internal standards. Electrospray mass spectra were determined on a Micromass ZQ instrument (Waters). The sample (100 pmol) was deionized on Dowex H+ resin (Fluka), dissolved in 2% triethylamine in 50% acetonitrile, and injected into the ion source at a flow rate of 5 μ l min⁻¹. The spectrum was acquired in negative mode.

Gas chromatography-mass spectrometry (GC-MS) analyses were performed on an Agilent Technologies 5973N MS instrument equipped with a 6850A GC and an RTX-5 capillary column (Restek; height, 30 m; inner diameter, 0.25 mm; flow rate, 1 ml min⁻¹; carrier gas, He). Acetylated methyl glycoside analysis was performed with the following temperature program: 150°C for 5 min, an increase from 150 to 250°C at 3°C min⁻¹, and 250°C for 10 min. The alditol acetate mixture was analyzed with the following temperature program: 150°C for 5 min, followed by an increase from 150 to 300°C at 3°C min⁻¹. The partially methylated alditol acetates were analyzed with the following program: 80°C for 2 min, an increase from 80 to 240°C at 8°C min⁻¹, and 240°C for 10 min. Methyl esters of fatty acids were analyzed with the following program: 130°C for 5 min, an increase from 150 to 280°C at 10°C min⁻¹, and 280°C for 10 min. Acetylated octyl glycosides were analyzed with the following program: 150°C for 5 min, an increase from 150 to 260°C at 6°C min⁻¹, and 260°C for 15 min.

Phosphate determination was performed according to the work of Kaca et al. (8).

Bacterial strain and growth conditions. Isolation of *P. haloplanktis* TAC 125 from Antarctic seawater has been reported previously (1). The strain was grown aerobically at 4, 15, and 25°C in TYP medium (16 g of Bacto Tryptone/liter, 16 g

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of yeast extract/liter [both from Difco], 16 g of sea salt/liter [pH 7.5]), as reported previously (5). Analyses reported in this paper were performed on cell pellets and corresponding culture medium collected from mid- to late-exponential phase.

LOS purification. LOSs were extracted from lyophilized cells according to the PCP method (7). LOS yields were 1.1, 0.9, and 0.2% of dried cells grown at 4, 15, and 25°C, respectively.

Quantitative determination of LOS. To 1 mg of each LOS fraction sample, an aqueous solution of inositol (5 mg/ml) was added in the same quantity (10 μ l) as the internal standard. The sample was dried over P_2O_5 overnight. After this time the sample was treated with 1 M HCl-CH₃OH (1 ml) at 80°C for 20 h and neutralized with Ag₂CO₃. The methyl glycosides obtained were acetylated with pyridine (200 μ l) and acetic anhydride (100 μ l) for 30 min at 100°C. The solvents were evaporated, and the residue was treated with chloroform-water (1:1). The organic layer was washed with water three times and then evaporated under a gentle stream of air at room temperature. The residue was dissolved in acetone and analyzed by GC-MS (5).

In order to evaluate the relative ratios between lipid A and LOS, the heptose monosaccharide was used as a marker for the LOS content. Considering that *P. haloplanktis* TAC 125 grown at 15°C produces only the LOS fraction, the value of the ratio of the area of the acetylated methyl glycoside heptose peak to the area of the inositol acetate peak indicates the largest amount of LOS (100%) that can be present in our sample (1 mg). If the sample (1 mg) also contains lipid A, the value of this ratio will be smaller. Therefore, the difference between the values of the ratio for samples of the bacterium grown at 4 and 25°C, and comparison to the value at 15°C (100%), indicates the relative amounts of free lipid A at different temperatures. The relative amount of free lipid A was 77% for 25°C samples and 30% for 4°C samples.

EPS purification. After centrifugation to eliminate the cells, the filtrates from the cultures of three temperatures (4, 15, and 25°C) were dialyzed (Spectrapore membranes; cutoff, 3,500 Da) against tap water and lyophilized (1.86 g/liter for 4°C, 1.79 g/liter for 15°C, and 1.53 g/liter for 25°C). The crude EPS samples were then chromatographed on a Sephacryl S 300 column (height, 44 cm; inner diameter, 1.5 cm; Pharmacia) by elution with 50 mM NH₄HCO₃ at a flow rate of 15 ml/h. The collected fractions (3 ml) were assayed for protein content (280 nm) and for sugars (phenol test; 490 nm). For each sample, a pure mannan fraction was separated from a protein-enriched mannan fraction.

Glycosyl analysis of EPS. The mannan nature of the two EPSs was established by alditol acetate derivatization of the sugars, following hydrolysis of the EPS fractions, and GC-MS analysis as described previously (3). The molar ratios of alditol acetates were evaluated by using inositol as an internal standard. The absolute configuration of the sugars was determined by treating the sugars with (+)-2-octanol as described elsewhere (3).

Quantitative analysis of mannan. To determine mannan yields, quantitative analysis was performed on dialyzed culture filtrates of the three samples as follows. Ten microliters of a 5-mg/ml inositol solution was added to 2 mg of each sample and derivatized as alditol acetates, as reported for glycosyl analysis. The yield was calculated as the ratio of the area of the mannitol acetate peak to the area of the inositol acetate peak; it was 12.5% for both 4 and 25°C and 19.9% for 15°C.

Methylation analysis. EPSs were methylated according to the procedure of Ciucanu and Kerek (2). Briefly, a polysaccharide sample (1 mg) was dried for one night over P_2O_5 and then dissolved in anhydrous dimethyl sulfoxide (500 μ l). A small quantity of NaOH powder was added, and the sample was left for 1 h at room temperature under stirring. CH₃I (300 μ l) was then added, and the sample was left for 1 h more. Water (500 μ l) was added to the sample, and extraction with chloroform (three times) was performed. The organic layers were collected and dried. Partially methylated alditol acetates were obtained after hydrolysis of permethylated mannans, reduction, and acetylation as reported for glycosyl analysis and were injected into the GC-MS.

RESULTS AND DISCUSSION

Although *P. haloplanktis* TAC 125 was originally isolated from Antarctic seawater (where the temperature is generally around 0°C), it grows in a temperature range between 4 and 30°C. In this paper we analyze culture filtrates and cells grown at three different temperatures (4, 15, and 25°C) in order to describe the variation, if any, in the structures of surface polysaccharides (LOSs) and extracellular polysaccharides (EPSs).

After extraction of LOS fractions by the PCP method (7),

FIG. 1. Transformed ESI-MS spectra of LOS fractions from *P. haloplanktis* TAC 125 grown at 4°C (a), 15°C (b), and 25°C (c). In panel b, the baseline in the transformed spectrum between m/z 1,200 and 1,800 did not appear. Also note that the relative intensities of the two clusters of peaks centered at 2,134 and 2,332 m/z could vary due to partial alkaline hydrolysis in certain experiments; for example, this happens when the triethylamine concentration is higher (10%), as described in reference 5.

LOS yields were 1.1, 0.9, and 0.2% for growth at 4, 15, and 25°C, respectively. Glycosyl analysis of the three samples showed the presence of the same sugars: GlcN, ManN, Gal, L,D-Hep, and 3-deoxy-D-manno-octulosonic acid (Kdo). Electro-Spray MS (ESI-MS) spectra (Fig. 1) showed significant differences among the three samples. The spectrum at 15°C (Fig. 1b) showed two clusters of peaks, centered at $2,332.63 \pm 0.18 m/z$ and $2,134.38 \pm 0.12 m/z$, which had been attributed to triphosphorylated penta- and tetraacyl forms, respectively, the latter containing four primary 3-hydroxydodecanoyl residues and the former containing an additional secondary dodecanoyl unit (5). At 25°C the ESI-MS spectrum (Fig. 1c) of the LOS fraction appeared much more complex in that, besides the clusters at $2,333.13 \pm 0.24 m/z$ and $2,135.00 \pm 0.20 m/z$, it showed a cluster with a mass 80 atomic mass units (AMU) higher, at $2,413.13 \pm 0.28 m/z$, suggesting an additional phosphate group. Moreover, in this spectrum, two clusters of signals at $1,475.25 \pm 0.06 m/z$ and $1,277.00 \pm 0.22 m/z$ occurred, indicating the presence of the pentaacyl and tetraacyl forms of lipid A (4) without core structure, which were completely absent in the spectrum at 15°C. The spectrum at 4°C showed two clusters of signals, one centered at $2,319.00 \pm 0.02 m/z$ and the other centered at $1,475.25 \pm 0.37 m/z$. (Fig. 1a), assignable to triphosphorylated pentaacyl LOS and only pentaacyl lipid A species, respectively. Interestingly, the relative intensities of cluster peaks were different from those of the spectra shown in Fig. 1b and c, suggesting a different distribution of homologous acyl chains. In particular, the cluster at $2,319.00 m/z$ occurred at a mass 14 AMU lower than the corresponding species found at 15 and 25°C. This suggested that at 4°C a higher number of acyl chains with one less methylene was present. This finding was supported by fatty acid analyses (see below).

The cluster centered at $2,413.13 \pm 0.28 m/z$ in the ESI-MS spectrum of the LOS fraction at 25°C (Fig. 1c) suggested the presence of a tetraphosphorylated pentaacyl species which was missing at both 4 and 15°C. A quantitative analysis performed on 4°C and 15°C LOS samples revealed that in both cases the phosphate content was lower than that of the 25°C sample by a factor of 4. This finding is in agreement with the suggested increase in LPS kinase activity at higher temperatures, as found for the Antarctic psychrotroph *Pseudomonas syringae* (16). More recently, in the case of a gram-positive psychrophilic bacterium which lacks LPSs, the amount of phosphoglycerol was found to be greater at 24°C than at 4°C (13).

Changes in the acyl chain composition of LOS with temperature have been reported for mesophilic (9, 10) and psychrophilic (18) bacteria. In particular, growth at low temperatures is associated with increases in unsaturation, hydroxylation, and branching degree as well as with a decrease in the length of the acyl residues. We found (Fig. 2; Table 1) no regular trends in

FIG. 2. GC-MS chromatograms of fatty acid methyl esters of *P. haloplanktis* TAC 125 grown at 4°C (a), 15°C (b), and 25°C (c). Peaks in the profiles are reported as relative intensities. Peaks a, 3-OH $C_{10:0}$; peaks b, $C_{12:1}$; peaks c, $C_{12:0}$; peaks d, i-3-OH $C_{11:0}$; peaks e, a-3-OH $C_{11:0}$; peaks f, 3-OH $C_{11:0}$; peaks g, i- $C_{13:0}$; peaks h, a- $C_{13:0}$; peaks i, $C_{13:1}$; peaks l, $C_{13:0}$; peaks m, i-3-OH $C_{12:0}$; peaks n, 3-OH $C_{12:0}$; peaks o, $C_{14:0\Delta}$; peaks p, $C_{14:0}$; ●, phthalates (contaminant).

TABLE 1. Fatty acid composition of LOSs of *P. haloplanktis* TAC 125 grown at 4, 15, and 25°C^a

Fatty acid	% (mol/mol) for bacteria grown at:		
	4 °C	15 °C	25 °C
3-OH C _{10:0}	2.7		1.9
C _{12:1}	2.4		0.8
C _{12:0}	17.7	21.0	16.6
i-3-OH C _{11:0}	1.3	3.3	3.9
a-3-OH C _{11:0}	0.6	1.7	2.0
3-OH C _{11:0}	14.6	1.7	5.2
i-C _{13:0}		1.0	1.1
a-C _{13:0}		0.8	1.3
C _{13:1}	7.8	7.3	3.2
C _{13:0}	4.4	4.5	3.2
i-3-OH C _{12:0}	5.5	4.5	6.3
3-OH C _{12:0}	34.8	29.9	48.0
C _{14:0} Δ	6.1	4.5	4.0
C _{14:0}	1.7	9.7	2.6

^a Obtained from integrated areas of fatty acid methyl ester peaks shown in Fig. 2.

structural variations associated with varying the bacterial growth temperature among 4, 15, and 25°C; in some cases, the structural variations observed were contrary to expectations. For example, we observed the smallest amount of hydroxylated fatty acids at 15°C (41.1%) and the largest at 25°C (67.0%) instead of, as expected, at 4°C (59.5%). The degree of branching did not seem to change significantly (13.5, 15.8, and 18.6% at 4, 15, and 25°C, respectively). As for the length of the acyl chains, we found that the compounds with the shortest chains (C₁₀ plus C₁₁) were more abundant at both the lowest and the highest growth temperatures (19.2 and 13.0% at 4 and 25°C, respectively) than at 15°C (6.7%). The relative abundances of fatty acids with the longest chains (C₁₃ plus C₁₄) were 20.0, 27.8, and 15.4% at 4, 15, and 25°C, respectively. Finally, the increase in the degree of desaturation at lower temperatures is in agreement with findings reported for mesophilic bacteria but in contrast with those reported for a psychrotropic *Pseudomonas* sp. (11). Therefore, the above data indicate that the structural effects of varying the bacterial growth temperature on the acyl moiety of LOS are more complex than expected. For example, if only growth at 4 and 15°C is considered, the variations in hydroxylation, desaturation, and acyl chain length are in agreement with those expected for mesophilic bacteria. At 25°C unforeseen results are obtained. These might be explained by the fact that the bacterium grows with great difficulty at this temperature, and its metabolism could be very different from that at 4 and 15°C, which are the most convenient growth temperatures for this bacterium.

A more significant difference, appearing in the ESI-MS spectra, is the presence of free lipid A only for cells grown at 4 and 25°C. Amounts of lipid A were determined (see Materials and Methods) to be 30 and 77% at 4 and 25°C, respectively. The lack of a linear trend of lipid A yield with temperature variation seems to parallel the contradictory data reported for the variation in the length of LPS O-chain polysaccharides in mesophilic bacteria. Actually, both an increase (15, 22) and a decrease (20) in O-chain length with growth at lower temperatures have been described. The absence of lipid A at 15°C might be related to the fact that this temperature is

FIG. 3. ¹H NMR spectra of mannose anomeric region of EPS fractions in D₂O at 500 MHz for *P. syringae* pv. ciccaronei (a) and for *P. haloplanktis* TAC 125 grown at 4°C (b), 15°C (c), and 25°C (d). Assignment of anomeric protons: 5.45 ppm, 1-P-mannose (peak 1); 5.31 ppm, 2-linked mannose (peak 2); 5.14 and 5.12 ppm, 3-linked mannose (peaks 3); 5.10 and 5.08 ppm, 2,6-linked mannose (peaks 4); 5.06 ppm, terminal mannose (peak 5); 4.91 ppm, 6-linked mannose (peak 6). A detailed characterization of the mannose structure of *P. syringae* pv. ciccaronei has been given in reference 3.

the best one for bacterial growth; thus, complete biosynthesis of LOS is obtained under this condition. In fact, the finding of free lipid A without capping is very unusual, because a lack of capping prevents the survival of enteric bacteria (23). The only previous occurrence of free lipid A in membranes without capping, as seen in *P. haloplanktis* TAC 125, has been reported for the nonenteric bacterium *Neisseria meningitidis* (23).

The EPSs from the culture broths of *P. haloplanktis* TAC 125 grown at the three different temperatures contained proteins and carbohydrates, which were found at about 40 and 10%, respectively. Only minor differences in the quantitative

FIG. 4. ^{13}C NMR spectra of EPS fractions in D_2O at 125 MHz for *P. syringae* pv. *ciccaronei* (a) and for *P. haloplanktis* TAC 125 grown at 4°C (b), 15°C (c), and 25°C (d). Assignment of anomeric carbons: 103.5 ppm, terminal and 3-linked mannose (peak 1); 101.6 ppm, 2-linked mannose (peak 2); 100.6 ppm, 6-linked mannose (peak 3); 99.5 ppm, 2,6-linked mannose (peak 4); 97.1 ppm, 1-P-mannose (peak 5).

yields of EPS were seen among bacteria grown at the three temperatures. The bacterium produced little more of the EPS fraction at 15°C (19.9%) than at 4 and 25°C (12.5%). For all three purified samples, the glycosyl composition, determined by analysis of alditol acetates, indicated a monosaccharide composition consisting of mannose with traces of glucose. Methylation analysis showed the presence of terminal mannose, 2,6-linked mannose, 2-linked mannose, 3-linked mannose, 6-linked mannose, and terminal glucose. All sugars were in pyranose form and had an α anomeric configuration, except for glucose, which has a β configuration, as deduced by ^1H and ^{13}C NMR chemical shifts. The D configuration for all the sugars was established by GC analysis of their 2-octyl glycoside acetates (12). In addition, the presence of phosphate groups linked to the anomeric position of mannose units was suggested by the triplet occurring at 5.45 ppm in the ^1H NMR

spectra (Fig. 3). The presence of phosphate was also confirmed by direct quantitative evaluation, which found 1.5, 1.1, and 4.2% phosphate in samples grown at 4, 15, and 25°C, respectively. These results suggested that the EPSs of *P. haloplanktis* TAC 125, obtained at the three temperatures, had a phosphomannan structure made up of a backbone of 6-linked mannose residues, which is highly branched at C-2, with mono-, di- and trisaccharide side chains containing 2- and 3-linked mannose units. Some of these arms end with β -glucose residues. Structural details were deduced by the close similarity between the ^1H and ^{13}C NMR spectra (Fig. 3 and 4) of mannans with those of *Pseudomonas syringae* pv. *ciccaronei* (3). Slight differences appeared among the EPS structures obtained at the three different temperatures. In particular, the sample obtained at 15°C seemed to be less branched, as suggested by its ^1H and ^{13}C NMR spectra (Fig. 3c and 4c), which show relatively more intense signals of the anomeric proton and carbon of the 6-linked mannose residue (4.91 and 100.6 ppm, respectively) than are found in the spectra of samples at 4 and 25°C (Fig. 3d and 4d). In addition, a minor content of 2-linked mannose in the arms was suggested by the decrease in the anomeric ^1H and ^{13}C signals at 5.31 and 101.6 ppm, respectively.

In conclusion, changing the growth temperature of *P. haloplanktis* TAC 125 resulted in two significant LOS structural variations. The first is the finding of free lipid A, suggesting incomplete LOS biosynthesis under nonoptimal growth conditions; the second is a higher phosphate content with an increase in the growth temperature. Interestingly, the latter effect seems to occur also for the EPS structure, suggesting that the influence of temperature on kinase activity is general and applicable to all surface polysaccharides.

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