Low-Temperature-Induced Changes in Composition and Fluidity of Lipopolysaccharides in the Antarctic Psychrotrophic Bacterium *Pseudomonas syringae*

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The Antarctic psychrotrophic bacterium *Pseudomonas syringae* was more sensitive to polymyxin B at a lower (4°C) temperature of growth than at a higher (22°C) temperature. The amount of hydroxy fatty acids in the lipopolysaccharides (LPS) also increased at the lower temperature. These changes correlated with the increase in fluidity of the hydrophobic phase of lipopolysaccharide aggregates in vitro.

The outer membrane (OM) of gram-negative bacteria is asymmetric due to the presence of lipopolysaccharides (LPS) exclusively in the outer leaflet and phospholipids in the inner leaflet of the bilayer membrane (17). Accordingly, the hydrophobic interior of the OM is mostly made up of penta- or hexa-acyl chains of lipid A from LPS and the diacyl chains of phospholipids. The packing density of the hydrocarbon chains in the LPS is higher than that of phospholipids (28). Chemically, LPS molecule generally contains, apart from the hydrophobic lipid A, two distinct carbohydrate components: an inner “core oligosaccharide” linked to the lipid A and an outer “O-antigenic chain” of carbohydrate repeat units that is linked to the “core” region (19). The phosphate groups present in the core oligosaccharides and lipid A of LPS bind the divalent cations, such as Ca^{2+} and Mg^{2+} , which probably help in stabilizing the outer leaflet of the membrane. It is also known that the LPS layer plays a major role in preventing diffusion of molecules through the OM. The permeation of molecules through the membrane is affected when the LPS leaflet is perturbed by antibiotics such as polymyxin B or by mutations that alters LPS structure (17, 18, 19). The porin channels located in the OM also regulate the entry of solutes by molecular sieving in bacteria (7, 17, 19), the exclusion limit being <600 to 700 Da as seen in *Escherichia coli*. While these are very important, little is known about the nature and importance of the hydrophobic phase of LPS in regulation of the OM function, especially at a lower temperature of growth.

The cytoplasmic membrane of bacteria tends to maintain a “homeoviscous state” for functioning at a lower temperature (27). Three kinds of alterations, namely, increase in the level of unsaturated fatty acids, decrease in the fatty acid chain length, and increase in branching of the chains (24, 25), are mainly known to maintain the “fluidity” or liquid crystalline state of the inner cytoplasmic membrane at lower temperatures. Although similar investigations on the change in OM at a low

temperature have been conducted with some mesophilic bacteria (11, 16, 22, 23, 31, 36), such studies on cold-adapted bacteria do not appear to exist. For this reason, we have initiated some studies with the Antarctic psychrotrophic bacterium *Pseudomonas syringae*, which has the ability to grow at 0 to 4°C (20, 21, 26). In this report we examine the alteration in OM property and related compositional change in the LPS of low (4°C)- and high (22°C)-temperature-grown cells of *P. syringae* and correlate them with the fluidity of the hydrophobic core of LPS aggregates in vitro.

The Antarctic bacterium *P. syringae* Lz4w was maintained and grown in Antarctic bacterial medium, which contains Bacto Peptone (0.5%) and yeast extract (0.2%), at low and high temperatures (4° and 22°C, respectively) as reported earlier (26). The growth was monitored, when required, by measuring the turbidity of the culture (optical density at 600 nm [OD_{600}]) on a Hitachi spectrophotometer (model no. 150-20). Unless otherwise mentioned, the experiments were carried out with the cells in the log phase of growth (OD_{600} , ~0.6).

Changes in the OM property of *P. syringae*. To ascertain whether there is a change in the OM property during growth at a lower temperature (4°C), we measured the susceptibility of *P. syringae* to polymyxin B. This cationic antibiotic is known to interact with LPS and disrupt the OM by a self-promoted uptake mechanism (16, 18, 29). We observed that the MIC of polymyxin B (1 $\mu\text{g}/\text{ml}$) at 4°C increased to 4 $\mu\text{g}/\text{ml}$ when *P. syringae* was grown at 22°C, indicating a change in the OM. The change was also monitored by measuring the fluorescent intensity of the probe *N*-phenyl-1-naphthylamine (NPN) in the presence of polymyxin B (14). The excitation and emission wavelengths for NPN were fixed at 356 and 410 nm, respectively, and the spectra were recorded at room temperature (22°C) in a Hitachi fluorescence spectrophotometer (model no. F-4010). The data (Fig. 1) indicate that NPN exhibited a higher fluorescent intensity with different concentrations of polymyxin B when the cells were grown at room temperature (4°C). A similar result was also observed in an independent assay method (8), in which the lysozyme-mediated lysis of cells was monitored by a drop in cellular turbidity at OD_{600} . For example, under identical conditions in the presence of polymyxin B (6 $\mu\text{g}/\text{ml}$) and lysozyme (50 $\mu\text{g}/\text{ml}$), the initial OD_{600} (0.6) of the suspensions of 4°C- and 22°C-grown cells dropped

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FIG. 1. OM permeability of *P. syringae* cells as measured by NPN fluorescence assay. The fluorescence measurements were carried out at room temperature in the suspension of 4°C- and 22°C-grown cells ($OD_{600} = 0.5$) containing 10 μ M NPN and in the presence of a variable amount of polymyxin B. The degree of changes in the OM has been shown as a function of fluorescence intensity at 410 nm in arbitrary units (a.u.).

to 0.14 and 0.35, respectively, in 1 h at room temperature. Thus, it appeared that the *P. syringae* had a growth temperature-induced alteration of the OM property.

Changes in the acyl chain composition of the LPS. The LPS from low (4°C)- and high (22°C)-temperature-grown cells of *P. syringae* were isolated by aqueous phenol (35) as well as by the modified phenol-chloroform-light petroleum method of Brade and Galanos (1). The protease, DNase, and RNase digestions of LPS and the final purification by ultracentrifugation were carried out as described earlier (21). The two procedures (1, 35) yielded similar results showing both S- and R-type LPS (Fig. 2). The LPS isolated by a proteinase K digestion method (9) also produced similar profiles by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (data not shown). For subsequent analysis, the fatty acids were prepared by hydrolysis of LPS in 4 M HCl for 4 h, followed by a treatment in 1 M NaOH for 2 h, both at 100°C (6, 13). The methyl esters of the fatty acids were made (15) and were analyzed by gas chromatography on a Hewlett-Packard 5890 Series II plus instrument with a 30-m-long HP-5 column (fused 5% phenylmethyl silicone column with 0.32-mm inside diameter and 0.25- μ m film thickness) using nitrogen as the carrier gas. The initial and final temperatures of the column were fixed at 120 and 250°C, respectively, with a ramping temperature of 5°C.

Table 1 shows that, at the lower temperature, there was an increased amount of hydroxylated fatty acids compared to that of fatty acids of LPS from higher (22°C)-temperature-grown cells. The 3-OH $C_{10:0}$, 2-OH $C_{12:0}$, and 3-OH $C_{12:0}$ together represented about 72% of the acyl chains as opposed to the 45% that was observed in the LPS of 22°C-grown cells. Among the three hydroxy fatty acids, the 3-OH $C_{10:0}$ and 3-OH $C_{12:0}$ constituted the bulk amount and the amount of 2-OH $C_{12:0}$ remained relatively unaltered. The hydroxy groups of these fatty acids probably function in a manner analogous to that of the branched fatty acids in phospholipid membrane in causing steric perturbations in the hydrophobic core (5) and thereby

FIG. 2. LPS profile of *P. syringae* grown at 4°C (lane 1) and 22°C (lane 2). The LPS were isolated by the method of Brade and Galanos (1), separated on by sodium dodecyl sulfate–10% polyacrylamide gel electrophoresis, and stained with silver nitrate (30).

help maintain the homeoviscous state of the OM at lower temperature.

Interestingly, it is also evident from Table 1 that the amount of the unsaturated $C_{16:1}$ acyl chain was greater (23%) in 22°C-grown cells than that in the low-temperature (4°C)-grown cells (13%). This result is counterintuitive and in contrast to the data available for mesophilic *E. coli*, *Salmonella enterica* serovar Minnesota, and *Proteus mirabilis* (16, 23, 31, 36), where the amount of palmitoleic acid (i.e., *cis*- $\Delta^9 C_{16:1}$) in lipid A increases at the expense of $C_{16:0}$ (in *P. mirabilis*) and $C_{12:0}$ (in *E. coli* and *S. enterica* serovar Minnesota) at a lower temperature ($\leq 15^\circ\text{C}$) of growth. However, a decrease in the $C_{16:1}$ acyl chain

TABLE 1. Acyl chain composition of LPS from low (4°C)- and high (22°C)-temperature-grown cells of *P. syringae*^a

Acyl chain	Amt (% mol/mol) grown at:	
	4°C	22°C
3-OH $C_{10:0}$	31	17
$C_{12:0}$	4	4
2-OH $C_{12:0}$	25	21
3-OH $C_{12:0}$	16	7
$C_{16:0}$	6	21
$C_{16:1}$	13	23
$C_{18:1}$	4	3
Others (unknown)	1	3

^a The values (average of three measurements) have been rounded off to the nearest whole number.

FIG. 3. Fluidity in the LPS aggregates as measured by the fluorescence emission spectra of pyrene. The purified LPS from 4° and 22°C-grown cells were extensively dialyzed against Milli-Q water and were pelleted by ultracentrifugation ($100,000 \times g$, 4 h) to remove divalent cations. The lyophilized pellets of LPS were suspended in a buffer (5 mM HEPES, pH 7.4, and 150 mM NaCl), and the fluorescent probe pyrene (6.5 μ M) was added to the suspension for recording fluorescence spectra (excitation at 333 nm and emission at 360 to 520 nm). The amount of monomer and dimer (excimer) of the pyrene molecules in the LPS aggregates was calculated from the fluorescence intensities at 372 and 470 nm, respectively, and the ratios were plotted. All measurements were carried out at room temperature.

at the lower growth temperature was observed earlier in *Yersinia enterocolitica* (34; quoted in reference 12).

In order to investigate the nature of linkage of the acyl chains, the ester-bound fatty acids were released from LPS by treatment with 0.25 M CH_3ONa at 37°C for 15 h (13) and the fatty acid methyl esters were prepared as described above. The amide-linked fatty acids were cleaved by the silver oxide and silver trifluoromethane sulfonate method in a water-free petroleum ether at 40 to 60°C and were esterified in situ by methyl iodide as described earlier (36). The results indicated that the $\text{C}_{16:0}$ and $\text{C}_{16:1}$ fatty acids were N linked, whereas 3-OH $\text{C}_{10:0}$, $\text{C}_{12:0}$, and 2-OH $\text{C}_{12:0}$ were ester linked in the LPS of cells grown at both temperatures (4° and 22°C). However, most of the 3-OH $\text{C}_{12:0}$ was observed to be amide linked only in 4°C-grown cells. The peak related to the 3-OH $\text{C}_{12:0}$ fatty acid in 22°C-grown cells was barely visible in the gas chromatogram.

Fluidity in the hydrophobic phase of LPS aggregates. The LPS form macromolecular aggregates in vitro (2, 3, 33). The fluidity (order-disorder conformation) in the hydrocarbon chains of LPS aggregates was measured. The fluorescent dye pyrene, a probe for lipid fluidity (10, 32), was used. The pyrene monomers laterally diffuse in the membrane to form excimers, and the ease with which the excimers are formed from monomers reflects the fluidity of the hydrophobic phase, which can be assessed from the ratio of excimer to monomer. Figure 3

shows that the excimer/monomer ratio of pyrene is higher in LPS aggregates prepared from 4°C-grown cells than in aggregates from 22°C-grown cells. Thus, at the tested temperature (22°C), there is an indication that the LPS of low-temperature-grown cells have a more fluid environment in the hydrophobic interior than do those prepared from the high-temperature-grown cells.

Significance of changes in LPS at the low temperature of growth. The lower growth temperature is expected to increase the fluidity of the LPS in order to achieve the homeoviscous adaptation. Indeed, with mesophilic members of *Enterobacteriaceae* (16, 22, 23, 31, 36), growth at 10 to 15°C presumably increases fluidity by increasing the content of palmitoleic acid in LPS, apparently by the induction of a specific LpxP transferase (4). Our study described in this paper showed that, in a facultative psychrophile as well, the fluidity of the hydrophobic domain of LPS increased in 4°C-grown cells as shown by the pyrene excimer assay. However, unexpectedly, the LPS of these cells contained smaller amounts of the main unsaturated fatty acid ($\text{C}_{16:1}$) and increased amounts of hydroxylated fatty acids. The relationship of the presence of hydroxylated fatty acid residues and the fluidity remains a topic for further study. It was shown earlier that the same strain of Antarctic *P. syringae* produced, at a lower growth temperature, LPS with lower phosphate content in the core region (21). The possible interaction between the lower fluidity and the altered phosphate

content on the survival of bacteria, for example in the presence of antibacterial agents, again remains to be studied.

In conclusion, our data suggest that the increased hydroxylated fatty acids in LPS of *P. syringae* might have a role in the observed changes of the OM at 4°C. The understanding of the exact nature of structural changes in lipid A and other parts of the LPS in this bacterium might throw new light in the future on the importance of the OM in the physiology of highly cold-adapted bacteria from Antarctica.

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