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Pore Size Dependence on Growth Temperature Is a Common Characteristic of the Major Outer Membrane Protein OprF in Psychrotrophic and Mesophilic *Pseudomonas* Species

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***Pseudomonas* species adapt well to hostile environments, which are often subjected to rapid variations. In these bacteria, the outer membrane plays an important role in the sensing of environmental conditions such as temperature. In previous studies, it has been shown that in the psychrotrophic strain *P. fluorescens* MF0, the major porin OprF changes its channel size according to the growth conditions and could affect outer membrane permeability. Studies of the channel-forming properties of OprFs from *P. putida* 01G3 and *P. aeruginosa* PAO1 in planar lipid bilayers generated similar results. The presence of a cysteine- or proline-rich cluster in the central linker region is not essential for channel size modulations. These findings suggest that OprF could adopt two alternative conformations in the outer membrane and that folding is thermoregulated. In contrast, no difference according to growth temperature was observed for structurally different outer membrane proteins, such as OprE3 from the *Pseudomonas* OprD family of specific porins. Our results are consistent with the fact that the decrease in channel size observed at low growth temperature is a particular feature of the OprF porin in various psychrotrophic and mesophilic *Pseudomonas* species isolated from diverse ecological niches. The ability to reduce outer membrane permeability at low growth temperature could provide these bacteria with adaptive advantages.**

The gram-negative psychrotrophic bacterium *Pseudomonas fluorescens* grows at a wide range of temperatures, 0 to 32°C, and even 37°C for some potentially pathogenic strains isolated from patients (11). The physiological and biochemical behavior of this species is adapted by means of thermoregulated domains. Such regulation is observed for the extracellular production of enzymes such as proteases and lipases, which is clearly temperature dependent (4, 15). Different sets of proteins are also maximally synthesized at low, medium, or optimal growth temperatures in the psychrotrophic bacterium *Pseudomonas fragi* (18). Regulation by growth temperature has been reported in several bacteria, both at the transcriptional level (26) and at the translational level (41).

Random insertional mutagenesis with the *luxAB* reporter gene has shown that 40% of the genes of *Pseudomonas fluorescens* strain MF0 are thermoregulated, and at least three classes of genes with different optimal temperatures of expression can be described (35). The ability of the cell to thrive in stressful conditions depends on its ability to sense environmental changes and respond to them via highly integrated adaptive networks. This ability to adapt, a key characteristic of the *Pseudomonas* genus (38), involves a number of modifications that affect the outer membrane in particular. This membrane is the most external adaptive barrier protecting the cell against the environment. The permeability of the external membrane depends primarily on a set of proteins that form aqueous channels, the porins (30).

In the psychrotrophic *P. fluorescens* MF0 strain, adaptation to low growth temperature includes a decrease in outer membrane permeability that affects β-lactam resistance (32). The major outer membrane protein, OprF, has been shown to be involved in this phenomenon. In vitro channel activity experiments with this porin revealed that channel size in bacteria grown at low temperature (8°C) was only one-third of that at the optimal temperature (28°C), suggesting that OprF pore size is temperature-dependent (7). Similar results were obtained with OprF from another psychrotrophic *P. fluorescens* strain, OE 28.3, isolated from the wheat rhizosphere (7).

This pattern of behavior may reflect a specific feature of these two closely related *P. fluorescens* strains (6). However, it may instead reflect a much broader mechanism of cold adaptation designed to reduce outer membrane permeability in hostile environments. We tested these hypotheses by investigating the ionophore properties of other porins isolated from bacteria adapted to several environments.

We studied OprFs from *Pseudomonas* species of two thermal groups, psychrotrophic *P. putida* 01G3, isolated from polluted soil, and the mesophilic human pathogen *P. aeruginosa* PAO1. Finally, we studied a structurally and functionally different porin isolated from *P. fluorescens* MF0, identified as the OprE3 protein.

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MATERIALS AND METHODS

Bacterial strains and growth conditions. The psychrotrophic *P. fluorescens* strains MF0 (27) and OE28.3 (8) and *P. putida* 01G3 (5) were grown in nutrient broth (Difco), with vigorous shaking, at 28°C (optimal growth temperature) and

TABLE 1. Comparison of protein sequences deduced from the nucleotide sequences of the *oprF* genes

Protein[a]	N-terminal homology[b] (%)	Alignment of central linker region[c]	C-terminal homology (%)
P. fluorescens MF0 OprF (AAA85840)		GGNAGAVA**P**A**P**T**P**A**P**A**P**E**P**T**P**E**P**EA**P**----------VAQVVRVELDVKFDFK	
P. fluorescens OE28.3 OprF (AAD45981)	98.10	GGNAGAAA**P**A**P**T**P**A**P**A**P**E**P**T**P**E**P**EA**P**----------VAQVVRVELDVKFDFK	99.01
P. putida 01G3 OprF (AJ621778)	79.24	GGGS-KPAPAPVPAP-EV**C**SDSDNDGV**C**DNVDK**C**PDTPANVTVDADG**C**PAVTEVVRVELDVKFDFDK	75.00
P. aeruginosa PAO1 OprF (AAG05166)	74.07	-GGS-KAAPAPEPV-ADV**C**SDSDNDGV**C**DNVDK**C**PDTPANVTVDANG**C**PAVAEVVRVQLDVKFDFDK	88.23

[a] Medline identification numbers are given for each protein.
[b] The similarities in the N- and C-terminal domains were compared with the corresponding domains of OprF from *P. fluorescens* MF0 and calculated with the Blast program (http://www.infobiogen.fr). Values are percent homology.
[c] The proline-rich pattern is indicated in bold type. Conserved cysteines from the cysteine- rich pattern are indicated in bold underlined type.

8°C. To obtain corresponding physiological states, mesophilic *P. aeruginosa* PAO1 (39) was grown at 37 and 17°C, which gave similar generation times as for the psychrotrophic strains. The ability of these strains to grow at 4 or 40°C was used to confirm that they belonged to the psychrotrophic or mesophilic class. Cells were harvested in the late exponential phase by centrifugation at 8,000 × *g* for 10 min at 4°C.

Outer membrane protein purification and characterization. Outer membranes were extracted by the spheroplast procedure described by Mizuno and Kageyama (28), as modified by Dé et al. (6), and solubilized at 4°C. The protein composition of the outer membrane fractions was analyzed by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) in a 7 to 12% discontinuous gel system (25), which was then stained with Coomassie blue. Outer membrane porins were purified from the gel by excision of the protein bands followed by electroelution (Bio-Rad System) in 0.192 M glycine–25 mM Trizma base–0.25% Triton X-100. We checked the purity and the heat modifiability of the preparations by SDS-PAGE and silver staining. The concentration of the purified proteins was determined by the bicinchoninic acid method (Sigma). The amount of associated lipopolysaccharide was evaluated by means of the 2-keto-D-octonate (KDO) assay, as described by Karkhanis et al. (22). Purified porins were blotted onto a polyvinylidene difluoride strip (Immobilon; Millipore) and subjected to Edman degradation in an Applied Biosystems 492 automated protein sequencer. The sequences obtained were analyzed with the BlastP program (http://www.infobiogen.fr).

Trypsin digestion. The outer membrane protein OprE3 was purified by preparative SDS-PAGE followed by electroelution in 2% (wt/vol) SDS. The purified protein was concentrated by trichloroacetic acid-acetone (9:10, vol/vol) precipitation, and the resulting precipitate was dissolved in 0.25 M Tris-HCl–6 M guanidine. The protein was reduced by incubation with 10 mM dithiothreitol and alkylated in 50 mM iodoacetamide, and the samples were then blotted onto a polyvinylidene difluoride disk (Prosorb; Applied Biosystems), which was washed three times with water. Trypsin digestion (final protease-protein ratio of 1:5) was performed overnight at 37°C. The peptide mixture was dried and then redissolved in trifluoroacetic acid-acetonitrile-water (1:20:79, vol/vol/vol) and separated by capillary reversed-phase high-pressure liquid chromatography.

Reconstitution in planar lipid bilayers. Virtually solvent-free planar lipid bilayers were formed by the Montal and Mueller technique (29), by the apposition of two monolayers on a 100-μm-diameter hole in a thin Teflon film (10 μm) treated with hexadecane-hexane (1:40, vol/vol). Membrane currents under applied voltage were measured with a BLM 120 amplifier (Biologic) and Ag/AgCl electrodes. Current fluctuations were stored on a DTR 1202 (Biologic) and transferred to a computer for various calculations (current and amplitude histogram) performed with software from Intracell. A mixture of palmitoyloleoylphosphatidylcholine and dioleoylphosphoethanolamine (POPC and DOPE, 7:3, wt/wt) (Lipid Products) was used to supply the lipids; the electrolyte solution was 1 M NaCl–10 mM HEPES (pH 7.4), and the bulk concentration of the reincorporated proteins was about 10^{-9} M.

RESULTS

Purification according to growth temperature and characterization of the porins. Outer membrane proteins were extracted from cultures grown at low or high temperature and then separated by SDS-PAGE. Heating the outer membrane proteins at 100°C for 10 min before loading made it possible to detect the major outer membrane porin as a heat-modifiable form with a different mobility than forms that had not been heated (16). The porins were then purified by electroelution in 0.25% Triton X-100 medium, as described in Materials and Methods, and subjected to N-terminal sequencing and BlastP searches, which led to their clear identification as OprFs.

Another outer membrane protein from *P. fluorescens* MF0 with an apparent molecular mass of 43 kDa, for which heating had no effect on the migration, was also purified. Its N-terminal sequence, NDQDQSKGFIEDSH, gave high similarity scores with *P. aeruginosa* OprD family proteins, supposed to act as specific porins (17). We carried out trypsin digestions followed by N-terminal sequencing of some fragments as a means of definitively identifying this protein. One of the sequences obtained (NAGGIADGGN) was 80% identical to the NAGGIGDGGT sequence, which is specific to the OprE3 protein among members of the OprD family in *P. aeruginosa* PAO1 (an alignment of these proteins is available at http://www.cmdr.ubc.ca/bobh/omps).

We analyzed the sequences of the OprFs used in this study and the known sequence of OprE3 from *P. aeruginosa* PAO1 (31) to identify structural differences potentially involved in the temperature-dependent channel-forming properties of OprF.

The amino acid sequences were aligned with ClustalW software (http://www.infobiogen.fr). All the OprF proteins studied here were well conserved in their N- and C-terminal sequences (Table 1), whereas the OprE3 protein from *P. aeruginosa* did not display any significant homologies. A dichotomy in the OprF central region has been described previously (2). This region forms a surface-exposed segment that links the N- and C-terminal parts of the protein: it presents two different highly conserved patterns (Table 1), a proline-rich stretch (with repeats of a 9P-X motif), as for *P. fluorescens* MF0 and OE28.3 (6), or a proline-rich loop region containing four cysteines forming two disulfide bonds (8, 12, 40) and six (*P. aeruginosa* PAO1) or seven (*P. putida* 01G3) prolines.

We thus had available several porins from different families (OprFs and OprE3), purified from bacteria belonging to different thermal groups (mesophilic and psychrotrophic), species (*P. fluorescens, P. putida, and P. aeruginosa*), and phylogenetic clusters (proline-rich and cysteine-rich patterns), isolated

TABLE 2. Single-channel conductance values in 1 M NaCl obtained with porins purified from cultures grown at the optimal or low temperature[a]

Strain	Conductance (pS)		Reference(s)
	Optimal growth temp	Low growth temp	
P. fluorescens MF0 OprF	250 ± 7	80 ± 3	7
P. fluorescens OE28.3 OprF	270 ± 7	90 ± 3	7
P. putida 01G3 OprF	260 ± 6	85 ± 4	This work
P. aeruginosa PAO1 OprF	240 ± 10	95 ± 5	6, 37 This work
P. fluorescens MF0 OprE3	30 ± 4	35 ± 4	This work

[a] Mean conductance values were obtained with a Gaussian fitting of the amplitude histograms. Several single-channel experiments (n = 10 to 15) were carried out and used to calculate average conductance values and standard deviations.

from various ecological niches. We compared the pore-forming properties of these porins as a function of the growth temperature.

Channel-forming properties in planar lipid bilayers. Triton X-100-solubilized OprFs from *P. putida* and *P. aeruginosa* purified from cultures grown at low temperature were reincorporated into POPC-DOPE lipid bilayers at room temperature. The proteins, added to the *cis* compartment of a measurement cell, induced small current fluctuations in response to the application of a constant potential. Amplitude histograms associated with the recordings made it possible to obtain mean major conductance values from several experiments (n ranging from 10 to 15) of about 85 pS and 95 pS for OprF from *P. putida* and *P. aeruginosa*, respectively (Fig. 1A and B). Under the same experimental reconstitution conditions, the mean conductance value obtained for *P. putida* OprF grown at 28°C was 260 pS. Table 2 summarizes the conductance values obtained for OprFs from different organisms according to the growth temperature. It clearly shows that the reported difference in the ionophore behavior of *P. fluorescens* OprF (7, 10) is a feature shared by other OprFs from various psychrotrophic and mesophilic *Pseudomonas* species: a large diameter with a high conductance value (240 to 270 pS in 1 M NaCl) at the optimum growth temperature and a smaller diameter (about one-third that at the optimum temperature, 80 to 95 pS) for growth at the low temperature.

Lastly, we investigated the effect of growth temperature on the channel-forming properties of another outer membrane protein from *P. fluorescens* MF0, which we identified as OprE3. This protein, purified from cultures grown at 8°C, induced small channels with major single conductance values of 35 pS. Representative single-channel recordings are shown in Fig. 1C. When purified from cultures grown at 28°C, this protein be-

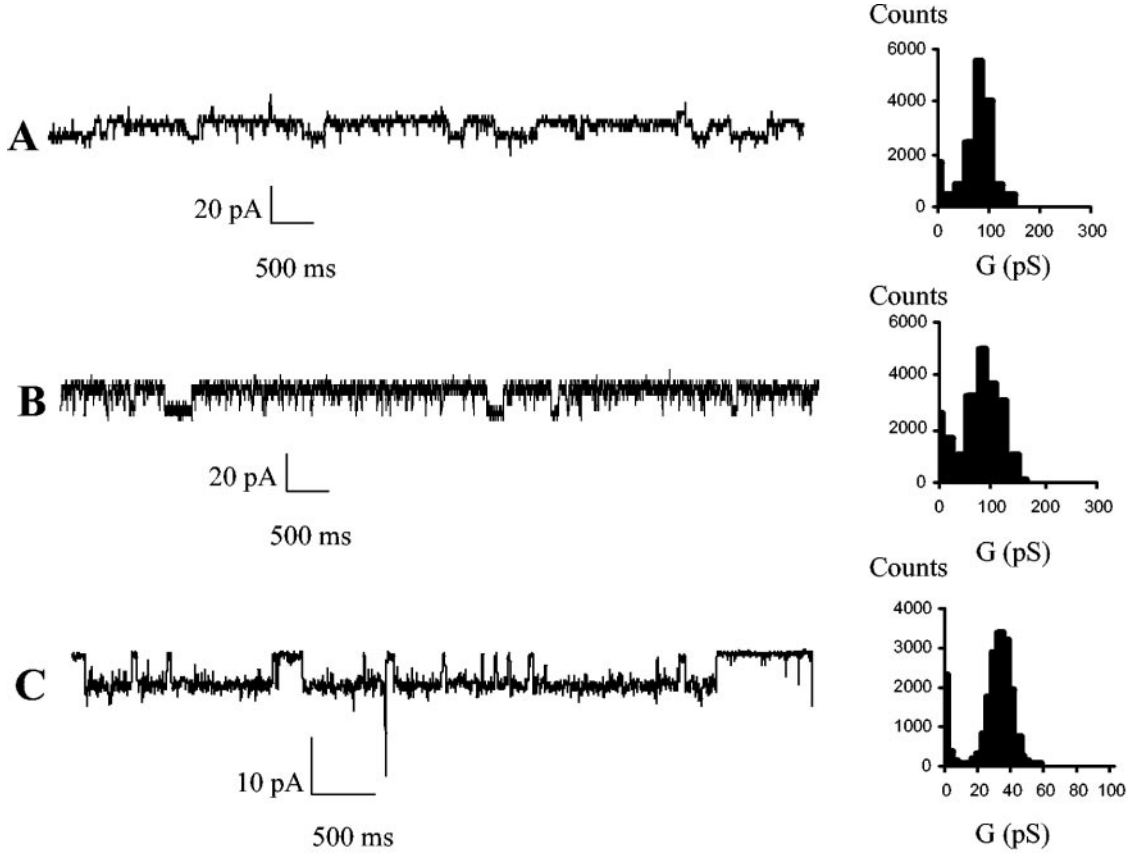

FIG. 1. Representative single-channel recordings and associated amplitude histogram of porins purified from cultures grown at the low temperature and reincorporated into planar lipid bilayers. Recordings were made in 1 M NaCl–10 mM HEPES, pH 7.4. Digitization rate, 3,000 Hz; filter, 300 Hz. (A) OprF (8°C) from *P. putida* 01G3 at +90 mV. (B) OprF (17°C) from *P. aeruginosa* PAO1 at +110 mV. (C) OprE3 (8°C) from *P. fluorescens* MF0 at −150 mV.

haved similarly, forming channels with single conductance values of 30 pS (Table 2). Our conductance values are consistent with those obtained with the structurally similar OprD2 protein of 20 pS in 1 M KCl (20) and 30 pS in 1 M NaCl (21).

Lipopolysaccharide quantification. As lipopolysaccharide is a major outer membrane compound and is closely bound to OprF (13), we investigated whether lipopolysaccharide was associated with the porins studied at the low and optimal bacterial growth temperatures in an assay based on KDO, a specific sugar found exclusively in lipopolysaccharide, as described previously (22). The studied porin OprFs were associated with similar amounts of KDO at both the low and optimal temperatures (data not shown). In *P. fluorescens* MF0, this amount of KDO has been shown to correspond to the binding of one molecule of lipopolysaccharide per OprF molecule (13). In contrast, no bound lipopolysaccharide was detected on the purified OprE3 proteins.

DISCUSSION

In the psychrotrophic *P. fluorescens* MF0 strain, adaptation to low growth temperature includes a decrease in outer membrane permeability, which affects β-lactam resistance (32). It has been suggested that the major outer membrane protein, the porin OprF, may be responsible for some nonspecific outer membrane permeability properties, as in *P. aeruginosa* (1). Moreover, OprF has the unusual feature of being able to modulate its channel size as a function of the growth temperature (7). Repeated conductance experiments in this study demonstrated that this property is not restricted to two isolated *P. fluorescens* strains but is instead common in the three *Pseudomonas* species studied. Moreover, we also found that this property was not specific to a particular cold-adapted thermal group, as it occurred in both psychrotrophic and mesophilic bacteria.

Sequence analysis of these OprFs showed that they had high similarity scores for their N- and C-terminal ends but were highly variable in the central region, corresponding to a surface-exposed segment (9, 12, 40). This region is likely to impose different three-dimensional constraints, depending on whether it consists of a proline-rich segment (*P. fluorescens*) or a cysteine-rich one (*P. putida* and *P. aeruginosa*). Similar results were obtained for all the OprFs tested, demonstrating that this region is not involved in channel size modulation.

On the contrary, no effect of growth temperature on pore size of OprE3 from *P. fluorescens* MF0 could be observed. The topological model proposed for this protein is very different from that for OprFs (19, 31). Thus, these results suggest that the decrease in outer membrane permeability at low temperature is mainly linked to the ability of OprF to decrease its pore size, and the intrinsic structure of OprF seems to be responsible for its channel modulation. Reincorporation of the *P. fluorescens* MF0 OprF N-terminal domain (excluding the proline-rich loop) extracted from bacteria cultured at low and high growth temperatures led to the formation of only the smallest size of channel (10). Similar results were obtained for the N-terminal domain of the *P. aeruginosa* OprF (3, 37), suggesting that the C-terminal part of the protein is required for the temperature-dependent formation of wider channels. Moreover, the C-terminal part of OprF is known to interact closely with several membrane components, such as peptidoglycan (33), but also with lipopolysaccharide (10, 13).

Growth temperature is known to affect several outer membrane components, including lipopolysaccharide in particular, in *Pseudomonas* species such as *P. aeruginosa* (23) and *P. syringae* (24). The phosphorylation of lipopolysaccharide is also temperature dependent; this molecule is more highly phosphorylated at high temperature than at low temperature in *P. syringae* (34) and *P. fluorescens* MF0 (10, 13). Lipopolysaccharide has also been shown to bind strongly to *P. fluorescens* MF0 OprF even after protein extraction and purification (10, 13).

In this study, we found that lipopolysaccharide bound to all the thermosensitive OprFs but not to OprE3. As the primary structure of the OprFs remains the same whatever the temperature (7), it has been suggested that lipopolysaccharide acts as a thermosensor, modifying its interactions with the C-terminal domain of OprF according to the degree to which it is phosphorylated. These interactions would result in conformational modifications, facilitating a decrease in pore size at low culture temperatures. Moreover, trypsin digestion kinetics determined in previous papers showed a different reactivity for OprF depending on the growth temperature (7, 10). These results suggest that the three-dimensional folding is maintained in a state that may correspond to the physiological one despite the freezing stages used in their preparation.

Low temperature is an important environmental factor that could affect the cell in numerous physical and biochemical parameters, inducing a decrease in the bacterial growth rate. As major outer membrane proteins, OprFs are important components of the interface between the bacterium and its surrounding environment. The behavior of these proteins in planar lipid bilayers suggests that they are temperature-responsive proteins, creating large pores in vivo at physiological temperature, mediating the transfer of hydrophilic molecules. The response of several *Pseudomonas* species from various thermal groups to temperatures below the optimal range for growth may be used to prevent the entry of toxic molecules, such as xenobiotics or toxins, that could affect more sensitive cells and to retain vital cell metabolites. This effect may be counterbalanced by the overproduction at low temperatures of extracellular enzymes for degrading macromolecules into smaller units to provide carbon and energy sources (14).

Bacteria from the *Pseudomonas* genus survive in all major natural environments, including hostile habitats subject to rapid variations, such as temperature shifts, suggesting that they have high physiological and genetic adaptability (38). Modulating the channel size of the major outer membrane protein in response to environmental temperature may provide the bacteria with advantages enabling them to adapt and evolve by colonizing very different ecological niches.

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