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Involvement of the C-terminal part of *Pseudomonas fluorescens* OprF in the modulation of its pore-forming properties

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Abstract

The major outer-membrane protein, OprF, from the psychrotrophic bacterium *Pseudomonas fluorescens* undergoes a reduction of its conductance value (from 250 pS to 80 pS) when the growth temperature is shifted from 28°C to 8°C. The involvement of changes in tertiary or quaternary structure in this behaviour, was implied by enzymatic digestion experiments in which OprFs purified from 8°C and 28°C cultures showed different accessibility to pronase. Resistant proteolytic fragments of 19 kDa, obtained from both OprF preparations, were identified as the N-terminal half of the native protein. These 19 kDa fragments induced ion channels in planar lipid bilayers with similar conductance values of 65–75 pS in 1 M NaCl, in contrast to the native proteins. Thus, the C-terminal part of the protein is required for the growth temperature-dependent modulation of OprF channel-forming properties. LPS was not detected on the proteolytic fragments while it was found in similar amounts on the native OprFs. These results suggest the LPS/porin association occurs through the C-terminal part of the porin. Radiolabelling experiments showed different phosphorylation levels of LPS for 8°C and 28°C cultures. Thus, in response to growth temperature, the structural modification of the LPS could be associated to the modulation of OprF pore size.

Keywords: Lipopolysaccharide; Ion channel; Lipid bilayer; Phosphorylation

1. Introduction

The outer membrane (OM) of Gram-negative bacteria has a molecular sieve function which regulates the uptake of nutrients, the excretion of waste products, the penetration of antibiotics and the exclusion of molecules that might damage the cells. This permeability is mainly due to pore-forming proteins embedded in the OM called porins [1]. The major *Pseudomonas aeruginosa* constitutive porin, OprF [2], is non-specific and hydrophilic compounds pass through the porin channel by passive diffusion. OprF has also been studied in the psychrotrophic *Pseudomonas fluorescens* strain MF0 and the sequence of the *oprF* gene has been determined [3].

Adaptation of psychrotrophic bacteria like *P. fluorescens* MF0 to low growth temperature involves a number of modifications [4,5], including a decrease in

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OM permeability that influences β-lactam resistance [6]. Moreover, upon reincorporation in planar lipid bilayers, *P. fluorescens* OprF displays differences in channel-forming properties depending on whether it was purified from cultures grown at low (8°C) or high (28°C) temperature [7]. The threefold reduction in the major conductance values (250 pS for OprF28°C to 80 pS for OprF8°C in 1 M NaCl) suggests that growth temperature modulates the pore structure of OprF. The identification of one single *oprF* gene for both proteins and their identical molecular masses suggest no modifications of OprF primary structure. However, Dé et al., after enzymatic digestion experiments performed on both proteins, showed a modification of accessibility of the cleavage sites to trypsin [7]. Structural modifications involving interactions between porin and lipopolysaccharide (LPS) were proposed to explain this functional change in the size of channels. Indeed, LPS, a major component of the OM, is known to be strongly associated with the bacterial porins of both *P. aeruginosa* and *Escherichia coli* [8–10]. It is involved in the insertion [11–14], trimerisation [12,15,16], and channel activity of some porins [17,18].

In this paper, the tertiary/quaternary structures of OprF purified from cultures grown at 8°C and 28°C were investigated to determine the region of OprF that modulates channel size in response to growth temperature. Proteolytic fragments obtained by enzymatic digestion were purified and characterised by functional analyses. The presence of LPS on these proteolytic fragments and on native OprFs was investigated in order to determine its potential role in modulating channel size in response to growth temperature.

2. Materials and methods

2.1. Bacterial strains and growth conditions

P. fluorescens strain MF0 is a psychrotrophic bacterium with an optimal growth temperature of 28°C. Bacteria were grown in Nutrient Broth (Difco). After vigorous shaking at 28°C overnight, or at 8°C for 48 h, cells were harvested in late exponential phase by centrifugation at $8000 \times g$ for 10 min at 4°C (Sorvall RC5B, rotor GSA).

2.2. Isolation of OprF proteins and proteolytic fragments

OMs were isolated by the method of Mizuno and Kageyama [19] with the modifications described by Dé et al. [3]. OprF purification from 8°C and 28°C cultures was performed by preparative electrophoresis followed by electroelution in the presence of sodium dodecyl sulfate (SDS). This method was also used for the purification of $OprFs_{177}$ obtained by pronase digestion. Five hundred μg of 8°C or 28°C OprF was digested with pronase from *Streptomyces griseus* (Fluka) at 200 μg ml^{-1}. The homogeneity of the samples was confirmed by analytical SDS–polyacrylamide gel electrophoresis (PAGE) (7/15%) with silver staining. The concentration of the electroeluted proteins was determined by the ESL protein assay kit (Boehringer Mannheim) or estimated after SDS–PAGE by staining with Coomassie Brilliant blue G250 using increasing BSA concentrations as a standard.

2.3. Mass spectrometry MALDI-TOF (matrix-assisted-induced desorption and ionisation time of flight)

Mass spectra were obtained with a time of flight mass spectrometer (Voyager Elite XL, Perseptive Biosystems, Framingham, MA). All spectra were acquired in the positive-ion mode and the acceleration voltage was set to 20 000 V. Aliquots of 0.5 μl of the protein solution and 0.5 μl of 2.5 dihydroxybenzoic acid dissolved in a 50% (v/v) of acetonitrile/aqueous 0.1% TFA solution were mixed on the stainless plate and dried prior to analysis. External calibration was performed with bovine serum albumin (*m/z* 66431).

The N-terminal sequence analysis of $OprF_{177}$ was determined by automated Edman degradation (477A Protein Sequencer, Applied Biosystems) after electroblotting on polyvinylidene difluoride membrane (Millipore).

2.4. Enzymatic kinetics measurements

Capillary electrophoresis was performed on a P/ACE 5510 (Beckman Instruments, Fullerton, CA). Separation was performed using a coated capillary with 100 μm i.d., 47 cm total length (eCAP SDS

14–200 kit, Beckman), run at 14.1 kV at 25°C and monitored at 214 nm. OprF protein was incubated at 4°C with different concentrations of pronase and samples were injected over a period of 30 s by low pressure every 25 min. Accessibility to pronase was determined by measurement of the decrease of the OprF peak area (μUnit Absorbance) at different times. The data were analysed by system Gold software (Beckman Instruments).

OprF concentration decrease was expressed by the following equation: $dS/dt = -Ke_aS$, where S is the concentration of porin at time t, e_a the active enzyme concentration, and k the degradation constant.

The disappearance of OprF is accompanied by the appearance of proteolytic fragments (S_1,S_2,...) that were also degraded by pronase (data not shown). So, we supposed that the active enzyme (e_a) is statically proportional to the amount of OprF remaining, thus:

$$e_a = e_0S/(S + S_1 + S_2 + ...) = e_0S/S_0$$

where S_0 is the concentration of porin at time zero, and e_0 is the enzyme concentration at time zero. So, the curves could be fitted by the equation: $S = S_0/(1+Ke_0t)$. This allowed us to calculate the degradation constant k.

2.5. Reconstitution in planar lipid bilayers

Virtually solvent-free planar lipid bilayers were formed by the method of Montal and Mueller [20] as modified by Saint et al. [21]. Diphytanoylphosphatidylocholine (DPhPC, Avanti, Birmingham, USA) was used as the lipid, and the electrolyte solution was 1 M NaCl, 10 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (Hepes) (pH 7.4). The proteolytic fragments (2 ng in octyl-POE 0.3%) from both grown temperatures were added to the measurement compartments in a symmetric manner. For the selectivity experiments, a NaCl gradient was created across the lipid bilayer: from 0.1 M on the *cis* side to 1 M on the *trans* side. The zero-current potential was corrected by deducing the asymmetric potential due to the salt gradient.

2.6. Purification and phosphorylation study of LPS

P. fluorescens LPS (8°C and 28°C) was purified as described by Darveau and Hancock [22] with the following slight modifications. (i) Before sonication, three phosphate-buffered saline (PBS) washes were performed to remove exopolysaccharide produced by the bacteria at 28°C. (ii) A final protein digestion was added at the end of LPS purification; samples were incubated for 8 h and 200 μg ml^{-1} of pronase (Sigma) was added each hour.

The concentration of LPS was evaluated by 2-keto-D-octonate (KDO) assay using the method of Karkhanis et al. [23].

To study the phosphorylation of the LPS, 20 μl of [^{32}P]orthophosphate (370 MBq ml^{-1}, Amersham) was added to 10 ml of late exponential phase cultures of *P. fluorescens* strain MF0 to a final activity of 20 μCi ml^{-1} [5]. After 20 min for a culture grown at 28°C or 4 h for a culture grown at 8°C, cells were harvested and LPS purified [5,22]. Ten μg of LPS-purified samples were subjected to SDS–PAGE and autoradiography for 30 min.

3. Results and discussion

For the psychrotrophic bacterium *P. fluorescens*, adaptation to a drop in growth temperature is mediated by a decrease in OM permeability [6]. This phenomenon could involve the major OM protein, OprF, for which a porin function has already been demonstrated [3] and which displays different channel-forming properties depending on the growth temperature [7]. Previous results showed that primary or secondary structure was not involved. In contrast, preliminary tryptic digestion experiments implied a difference in OprF folding, or structural modification of the LPS, a component that interacts with the porin [7]. In order to assess the role of the N-terminal and C-terminal parts of the native porin in the modulation of pore sizes in response to growth temperature, enzymatic digestions were performed with OprF8°C and OprF28°C.

3.1. Enzymatic digestion of P. fluorescens OprFs

Enzymatic digestion of *Escherichia coli* OmpA with pronase yields a unique 19 kDa protease-resistant fragment, corresponding to the N-terminal part of the porin [24]. Similarly, the two forms of OprF

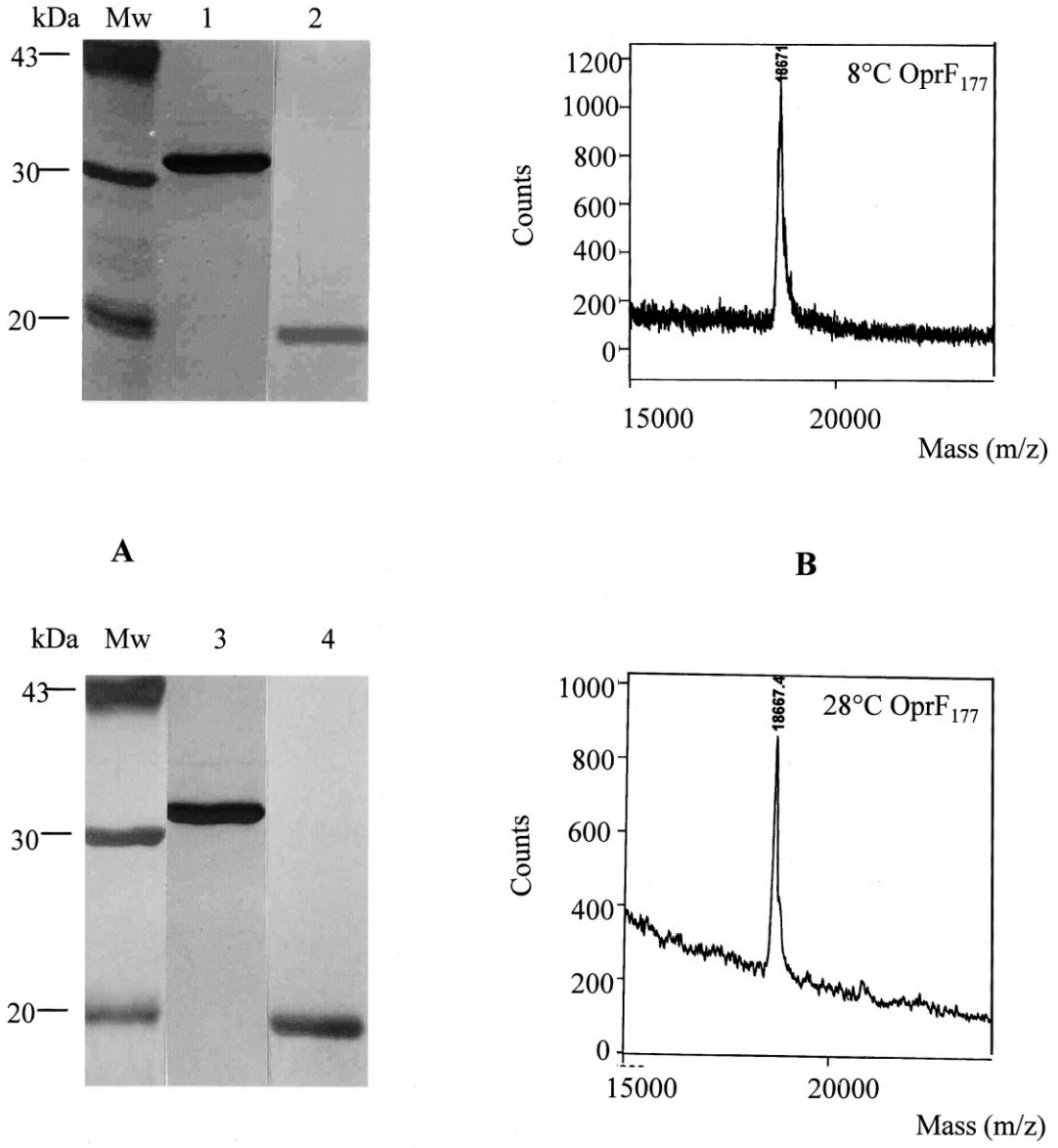

Fig. 1. SDS–PAGE of native OprFs and their purified proteolytic fragments. (A) Native OprF8°C (lane 1) and the corresponding $OprF_{177}$ fragment (lane 2), native OprF28°C (lane 3), and the corresponding $OprF_{177}$ fragment (lane 4). (B) Mass spectroscopy of purified $OprF_{177}$.

isolated from 28°C and 8°C cultures were treated with pronase (200 μg ml^{-1}) for 6 h and in both cases yielded a resistant 19 kDa proteolytic fragment. These fragments were purified by SDS–PAGE and electroelution (Fig. 1A). Their molecular masses, as determined by MALDI-TOF mass spectrometry, were 18 671 and 18 667 Da for the 8°C and 28°C proteins, respectively (Fig. 1B). The N-terminal sequences of these fragments were identical (QGQGAVEGELFYKKQ) and corresponded to the N-terminus of native OprF. Using the known amino acid sequence of OprF from strain MF0 [3] and the molecular mass of proteolytic fragments, it was possible to calculate that the proteolytic cleavage occurred at threonine 177 in the proline-rich region of OprF. Apart from this fragment ($OprF_{177}$), no other proteolytic peptides were detected, implying the C-terminal part was totally digested. As already described for *P. aeruginosa* OprF [25] and the related porin in *E. coli*, OmpA [26], the N-terminus consist of a

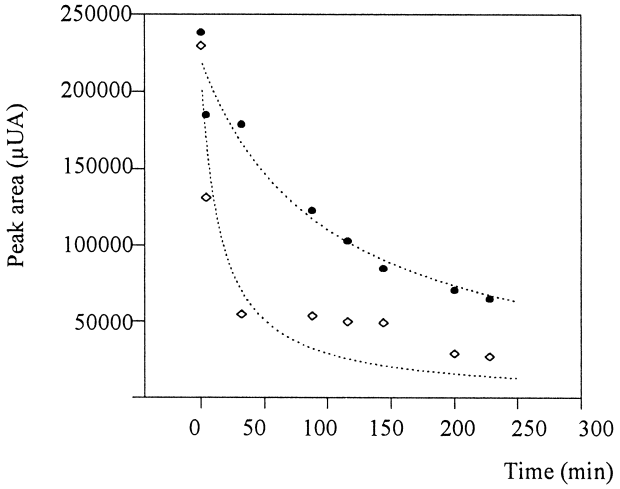

Fig. 2. Enzymatic digestion of OprF purified from cells grown at 8°C (□) and 28°C (●). Kinetics of proteolysis of both porin preparations solubilised in 0.025% SDS, 192 mM glycine and 25 mM Tris–HCl, and incubated with pronase.

β-barrel domain. A recent crystal structure of the N-terminal fragment 1–171 of *E. coli* OmpA ($OmpA_{171}$) showed unambiguously that the N-terminal part consists of an extended eight-stranded β-barrel [27]. In our case, the resistance of the *P. fluorescens* OprF N-terminus to protease treatments could be due to a domain structured with a high proportion of antiparallel β-strands.

Kinetic measurements using capillary electrophoresis allowed us to calculate the difference in the proteolysis rates of the two forms of OprF. The optimal pronase concentration was 0.2 μg ml^{-1}; at higher concentrations, proteolytic degradation proceeded too fast for detection of full length OprF. As expected, only one fragment of about 19 kDa was obtained from electropherograms of both native proteins OprF8°C and OprF28°C (data not shown). Fig. 2 clearly shows a difference in degradation rate between the two OprF preparations as confirmed by the kinetic constant k, estimated to be 0.35 and 0.05 ml $(\mu g\ min)^{-1}$ for the 8°C and 28°C forms of OprF, respectively. The difference observed for kinetic constants between the two preparations could involve a modulation of cleavage site accessibility to pronase as supported by earlier OprF digestion experiments performed with trypsin [7]. This difference of accessibility could be due to protein conformational changes or a masking of cleavage sites by an accessory component (the LPS, for example), confirming the modulation of the tertiary and/or quaternary structure of this porin in response to growth temperature.

To determine if the deletion of C-terminal fragment had an effect on OprF porin activity, $OprF_{177}$ produced from both the 28°C and the 8°C forms of the native protein was reconstituted in planar lipid bilayers.

3.2. Functional characterisation of $OprFs_{177}$

Addition of 8°C $OprF_{177}$ into the electrolytic compartment induced, after few minutes, current fluctuations when voltage was applied (Fig. 3). The average single-channel conductance value was (65 ± 4) pS in 1 M NaCl, 10 mM Hepes (pH 7.4) (Fig. 3). The incorporation of the 28°C $OprF_{177}$ showed the same current fluctuations (Fig. 3) as those observed for 8°C $OprF_{177}$, corresponding to a (75 ± 3) pS conductance value in 1 M NaCl, 10 mM Hepes (pH 7.4) (Fig. 3). For both preparations of $OprF_{177}$, single-channel current was a linear function of voltage and no voltage gating (in the voltage range of ± 170 mV) was detected. Ion selectivity experiments using a gradient of NaCl concentration (0.1 M:1 M, *cis:trans*) were used to determine the P_{Na}/P_{Cl} ratio according to the Goldman–Hodgkin–Katz equation [28]. The ratio values, 1.2 for 8°C $OprF_{177}$ and 1.45

Table 1
Single-channel conductance and selectivity values of OprF and $OprF_{177}$ purified from cultures grown at 28°C and 8°C

	Culture at 28°C		Culture at 8°C	
	Single-channel conductance (pS)	Selectivity P_{Na}/P_{Cl}	Single-channel conductance (pS)	Selectivity P_{Na}/P_{Cl}
OprF [7]	250 ± 7	1.7	80 ± 3	2.8
	280 ± 10[a]	n.d.	95 ± 8[a]	n.d.
$OprF_{177}$	75 ± 3	1.4	65 ± 4	1.2

n.d., not determined.
[a]Controlled values obtained with full-length OprF purified by SDS–PAGE.

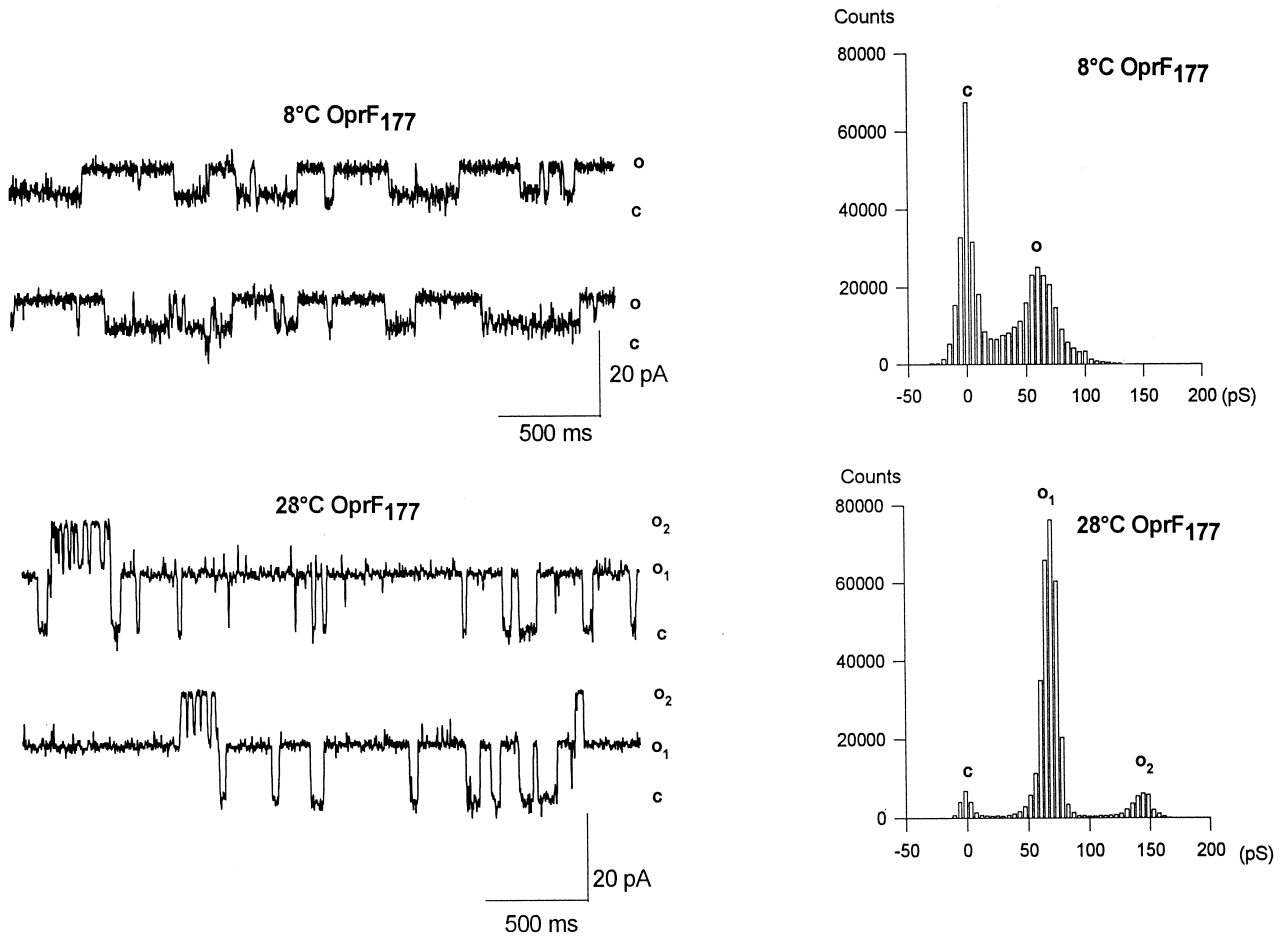

Fig. 3. Single-channel current fluctuations and associated amplitude histograms of 8°C and 28°C $OprF_{177}$. Conductance value: 65 pS for the 8°C $OprF_{177}$ (applied potential 100 mV) and 75 pS for the 28°C $OprF_{177}$ (potential 170 mV). The lipid was diphytanoylphosphatidylcholine and the aqueous phase contained 1 M NaCl, 10 mM Hepes, pH 7.4.

for the 28°C $OprF_{177}$, demonstrated the weak cationic selectivity of these fragments (Table 1).

Thus, reconstitution experiments showed that the $OprF_{177}$ fragment isolated from either form of native OprF was able to induce ion channels in planar lipid bilayers with similar conductance values (65 and 75 pS in 1 M NaCl). Interestingly, a similar conductance value was recently described for the N-terminal part of OmpA of *E. coli* [29]. In contrast, the conductance values of 8°C and 28°C full-length OprF-purified by SDS–PAGE under the same conditions or by OPOE-PAGE [7] are different (80–95 pS and 250–280 pS, respectively, Table 1). These results suggest the C-terminal domain is responsible for the modulation of OprF ionophore activity in response to growth temperature. The C-terminus of *P. aeruginosa* OprF has also been proposed to play a role in cell length, growth in low osmolarity media and peptidoglycan association [30]. As already suggested, different LPS–porin associations could modulate outer membrane permeability. The possible association of LPS with native OprFs and $OprFs_{177}$ derived from cells grown at 8°C and 28°C is therefore of particular interest.

3.3. LPS–porin associations

Two types of porin-associated LPS exist: loosely bound LPS that is released in the migration front during SDS–PAGE, and tightly bound LPS [31]. Owing to the method used to purify native porins (SDS–PAGE and electroelution), loosely bound LPS was not detected. In contrast, the quantity of tightly bound LPS could be determined using an as-

Table 2
Determination of KDO and LPS quantities for native OprF purified from cultures grown at 28°C and 8°C and from derived proteolytic fragments

Protein	KDO quantity (μg of KDO/μg of protein)	LPS quantity (μg of LPS/μg of protein)
8°C OprF	$(4.0 \pm 0.4) \times 10^{-3}$	0.100 ± 0.010
28°C OprF	$(5.3 \pm 0.5) \times 10^{-3}$	0.133 ± 0.013
8°C $OprF_{177}$	$< 0.5 \times 10^{-3}$	< 0.012
28°C $OprF_{177}$	$< 0.5 \times 10^{-3}$	< 0.012

say based on KDO, a specific sugar exclusively found in LPS. Table 2 shows the amount of KDO in LPS 8°C and LPS 28°C was very similar (4.0×10^{-3} and 5.3×10^{-3} μg per μg of LPS, respectively). The same amount of LPS was bound to both native porins (0.10 and 0.13 μg of LPS/μg of protein, respectively); however, $OprF_{177}$ proteolytic fragments did not show tightly bound LPS (values < 0.01 μg of LPS/μg of protein). These results strongly suggest (i) the tight association of LPS molecules to OprF requires the C-terminal part of the porin, and (ii) the quantity of tightly bound LPS could not explain the modulation of pore-forming properties since the LPS/OprF molar ratio was equal and approximated to 1 for both native OprF8°C and Opr28°C. Therefore, we considered the possible involvement of structural modifications to the tightly bound LPS, such as differences in phosphorylation.

The phosphorylation of *P. fluorescens* LPS was studied at both growth temperatures. *P. fluorescens* cultures were grown at 8°C and 28°C in the presence of ^{32}P-orthophosphate prior to the purification of LPS, and identical amounts (10 μg) of purified LPS were analysed by SDS–PAGE and autoradiography (Fig. 4). The LPS was clearly more highly phosphorylated in cells grown at 28°C (Fig. 4, lane 2) than at 8°C (Fig. 4, lane 1) after the same number of generations. Interestingly, changes in LPS phosphorylation as a function of growth temperature have also been reported in another psychrotrophic bacterium, *P. syringae* [5]. Thus changes in the level of LPS phosphorylation could modulate the channel size of the OprF porin in response to temperature.

In conclusion, the pore-forming properties of the OprF28°C and OprF8°C are different whereas the N-terminal fragments ($OprF_{177}$) behaved identically when reincorporated in lipid bilayers. As showed by LPS–KDO determinations, LPS is mainly associated with the C-terminal part of OprF. From these data, we can conclude that the C-terminal part of the porin modulates the OprF pore and there is association between the pore size and LPS phosphorylation. Although other structural modifications to the LPS could account for this phenomenon, such as

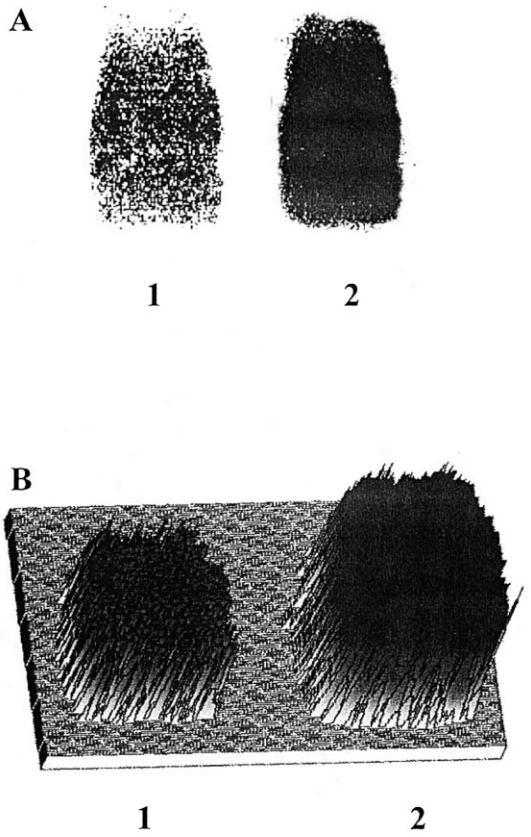

Fig. 4. Autoradiography of LPS purified from *P. fluorescens* after labelling with P-orthophosphate. (A) Cultures grown at 8°C (1) and 28°C (2). (B) Associated densitometric measurement using NIH program. Ten μg of purified LPS was applied in each slot before SDS–PAGE. Similar results were obtained from independent experiments.

changes in the length or degree of saturation of the lipid chains [4,31], the different levels of phosphorylation observed here could explain the modulation of OprF pore-forming properties in response to growth temperature.

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