J Ind Microbiol Biotechnol (2007) 34:49–54
DOI 10.1007/s10295-006-0160-x

ORIGINAL PAPER

Growth temperature and OprF porin affect cell surface physicochemical properties and adhesive capacities of *Pseudomonas fluorescens* MF37

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Received: 15 March 2006 / Accepted: 5 July 2006 / Published online: 24 August 2006

Abstract Pseudomonads adapt to various ecological niches by forming biofilms, which first requires bacterial adhesion on surfaces. We studied the influence of growth temperature on surface physicochemical properties of *Pseudomonas fluorescens* MF37 and on its adhesive capacities onto inert surfaces. It presented a global hydrophilic character, measured by microbial adhesion to solvent (MATS), and showed a cell surface more hydrophilic at 8 and 28°C than at 17°C. Moreover, *P. fluorescens* MF37 was more adhesive at 17°C. This critical temperature thus should be carefully taken into account in food safety. Adhesion onto inert surfaces is thus influenced by the growth temperature, which modifies the bacteria cell wall properties through changes in the outer membrane components. Therefore, we studied the effect of the loss of OprF, the major outer membrane protein, known to act as an adhesin (root, and endothelial cells). The OprF-deficient mutant was able to adhere to surfaces, but showed the same physicochemical and adhesion properties on abiotic surfaces whatever the growth temperature. OprF is thus not essential in this adhesion process. However, we suggest that OprF is involved in the bacterial environmental temperature sensing by *P. fluorescens*.

Keywords *Pseudomonas fluorescens* · Surface physicochemical properties · Adhesion · Temperature · OprF

This work was supported by a grant from the Région Bretagne (Doctoral fellowship to G.H.).

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Introduction

Numerous food products, mainly conserved by refrigeration, are contaminated and altered by *Pseudomonas* species. These Gram-negative bacteria are found in various environments such as soils, rhizosphere or drinking water. They are able to grow over a wide range of temperatures (0–32°C), and some emerging strains are able to cross the temperature frontier, as demonstrated by the increasing involvement of *P. fluorescens* in nosocomial diseases [23]. The striking adaptation abilities of *Pseudomonas* species, which are at least in part explained by their genome content in regulatory genes, are a key for their survival in hostile environments where rapid variations of temperature can occur [28]. The first step of the surface colonization, adhesion, depends on physicochemical factors such as cell surface hydrophobicity, surface charges, van der Walls interactions or Lewis-acid–base properties [3], but also on biotic or abiotic

surface properties. The structure–function of the cell surface components must also be taken into account because the composition in lipids, lipopolysaccharides, and outer membrane proteins can change to allow the bacteria to adapt to their micro-environmental conditions [8, 13]. Furthermore, the major outer membrane protein of *Pseudomonas*, pleiotropic OprF, the principal function of which is to form a nonspecific constitutive hydrophilic channel, has been reported to act as an adhesin on biotic surfaces [9, 10, 25]. Bodilis et al. [6] suggested, moreover, an involvement of the proline-rich motif of this protein in the aspecific adhesion on polystyrene of several environmental *Pseudomonas* spp. isolates. An important question is thus what is the contribution of this porin in the adhesion process on inert surfaces.

In order to answer to this question, two strains of *P. fluorescens* were studied: MF37 and its OprF-deficient mutant (MF373). We analysed (1) the physicochemical properties of their cell surface, and (2) the adhesion abilities on two abiotic surfaces (one hydrophilic and one hydrophobic), with regards to several growth temperatures, chosen according to the temperatures used in food industries to prevent contaminations and limit food spoiling by *Pseudomonas* species.

Materials and methods

Bacterial strains and growth conditions

Pseudomonas fluorescens MF37, a rifampicin-resistant mutant derived from *P. fluorescens* MF0, isolated from raw milk, was used in this study [14]. We had several OprF deficient mutants of *P. fluorescens* MF37, all of them showing numerous modifications in the protein content of the outer membrane, in addition to the absence of OprF [7]. Repeated construction procedures allowed us to obtain 54 new mutants, including the MF373 strain, which did not show any other protein alteration in the outer membrane than the loss of OprF when tested by SDS-PAGE. Bacterial strains were grown in 25 ml of M17 medium (Difco) containing 0.5% glucose (GM17) at 28, 17, or 8°C, on a rotary shaker (180 rpm) until stationary phase was reached, i.e. 12 generations for each strain at each temperature.

Characterization of physicochemical surface properties of strains by MATS method

Stationary phase cells were harvested by centrifugation for 10 min at 7,000*g* at 4°C, washed twice, and resuspended in 0.9% NaCl. The microbial-adhesion-to-solvents partitioning method was previously described by Bellon-Fontaine et al. [2]. The following pairs of solvents were selected: (1) chloroform and hexadecane (Prolabo), and (2) ethyl acetate and decane (Carlo Erba).

A suspension containing 10^8 CFU in 2.4 ml of 0.9% NaCl (corresponding to an OD_{400} between 0.8 and 0.9 = $[A_0]$) was mixed for 65 s (10 s by inversion, 45 s with vortex, and 10 s by inversion) with 0.4 ml of one of the four solvents indicated above. The mixture was held for 15 min to ensure complete separation of the two phases before the OD of the aqueous phase was measured at 400 nm (A). The percentage of cells in each solvent was subsequently calculated using the following equation: percent affinity = $[1 - (A/A_0)] \times 100$. Each experiment was performed in triplicate on two independent cultures.

Measurement of cell adhesion on abiotic surfaces

The surfaces used for adhesion tests were polystyrene or glass. Stationary cells were harvested by centrifugation for 10 min at 7,000*g*, washed twice, and resuspended in 0.9% NaCl to obtain an OD_{400} between 0.6 and 0.7. Twenty milliliters of the bacterial suspension were poured into a Petri dish (polystyrene adhesion test) or on a glass slide (glass adhesion test) and stored at 20°C. After 2 h of contact with planktonic cells, each adhesion surface was washed three times for 5 min with sterile demineralized water on an orbital shaking table to remove non-adherent cells. Cell adhesion was evaluated by determining the percentage of contaminated surface as follows. Adherent cells were stained for 20 min with a solution of 0.2% crystal violet, and washed twice for 1 min with demineralized water. The adhesion surfaces were then air dried before analysis with an Axioplan 2E microscope (magnification 157.5×) (Carl Zeiss, Iena, Germany) coupled to a camera. Images were analyzed with Image Tools software as grey-scale interpretations on the screen. The area covered by the adhered bacteria was converted into a percentage of the total area. For each experiment, two supports were analyzed, and ten fields were observed per supports.

Statistical analyses

Affinities values for solvents and percentages of colonized surface were analyzed with Student's t tests.

Results and discussion

Influence of growth temperature on the cell surface physicochemical properties of *P. fluorescens* MF37

Three temperatures were used in this study: 28°C which is the optimal growth temperature, and 8 and 17°C which are used for food conservation and processing, respectively. Moreover, the temperature of 17°C is considered as critical [15] because it corresponds to the junction of two separate physiological growth domains of *P. fluorescens* MF37, the cold (0–17°C) and the sub-optimal ones (17–28°C). The microbial-adhesion-to-solvents (MATS) partitioning method is based on the comparison of the microbial cell affinities for monopolar and nonpolar solvents which exhibit similar van der Waals surface tension components [2]. The following pairs of solvents were selected to study the Lewis-base and the Lewis-acid characters of the bacterial cell surface: chloroform and hexadecane, and ethyl acetate and decane, respectively. Moreover, the affinities with nonpolar solvents, such as hexadecane or decane, are directly correlated with the cell surface hydrophobicity.

The affinity values of *P. fluorescens* MF37 for hexadecane were less than 50% for the three temperatures tested showing a general hydrophilic character of the cell surface (Table 1). However, whereas the percentage affinity to hexadecane was about 15% for cells grown at 8 and 28°C, it rose to 46.7% when cells were cultured at 17°C. The surface of *P. fluorescens* MF37 was thus highly hydrophilic when bacteria were grown at low or optimal temperatures, whereas its hydrophilic property was greatly reduced at 17°C. Hydrophilic characteristics have already been reported for other *Pseudomonas* species [11, 16, 27], but this is the first time that it has been investigated at temperatures used in food industries.

To explain further the hydrophilic properties of the cell surface, we studied the Lewis-base and Lewis-acid characteristics. When looking at the Lewis-base characteristics, the affinity of *P. fluorescens* MF37 was higher for chloroform, a polar electron-acceptor solvent, than for hexadecane, a nonpolar solvent, whatever the growth temperature (Table 1). These observations indicated the strong electron-donating nature (i.e. the basic surface properties) of *P. fluorescens* MF37. The Lewis-base character of the cell surface, determined by the difference of affinity between chloroform and hexadecane, was maximal at 8°C (55.7 ± 6.4) and decreased with higher growth temperatures (15.8 ± 5.6 at 17°C and 7.5 ± 10.7 at 28°C). Similarly, when looking at the Lewis-acid character, *P. fluorescens* MF37 presented a weaker affinity for the electron donor solvent (ethyl acetate) than for the associated nonpolar solvent (decane) at 17 and 28°C, whereas the values were not significantly different at 8°C. *P. fluorescens* MF37. This showed temperature-dependent surface properties, presenting an hydrophilic character at 28°C, linked to both Lewis-base and Lewis-acid properties; and an hydrophilic cell surface at 8°C, which was essentially due to the Lewis-base character. The cell surface was moderately hydrophilic at 17°C, linked to both Lewis-base and Lewis-acid properties. These results clearly show that the physicochemical surface properties of this strain were different at 8, 17, and 28°C, therefore suggesting that *P. fluorescens* MF37 strongly modifies its cell surface properties in response to the environmental temperature.

At 17°C, changes in various properties were observed in *P. fluorescens*: the production of secreted enzymes was maximal [14, 21], causing difficulties in extracting outer membranes of the bacteria. Moreover, the permeability of the outer membrane to the β-lactamine mezlocillin is modified around 17°C, via the channel size of major porin OprF [22]. The strong modification of cell surface properties observed in the present study is therefore an additional phenomenon occurring in *P. fluorescens* around 17°C.

Influence of growth temperature on nonspecific adhesion of *P. fluorescens* MF37

As the environmental temperature has a notable influence on the hydrophilicity of the bacterial external surface, we investigated its effect on the nonspecific adhesive behavior of *P. fluorescens* MF37. Percentages

Table 1 Percentage of affinity for solvents of *P. fluorescens* MF37, measured by MATS method

Growth temperature (°C)	% affinity (mean ± SD)[a]			
	Chloroform	Hexadecane	Decane	Ethyl acetate
8	70.2 ± 2.6	14.5 ± 3.8	15.3 ± 3.8	18.2 ± 2.8
17	62.5 ± 3	46.7 ± 2.6	46.7 ± 2.8	25.4 ± 1.9
28	24.3 ± 7.3	16.8 ± 3.4	29.7 ± 6.2	15.2 ± 1.3

[a] Each experiment was performed in triplicate on two independent cultures

of surface colonized by *P. fluorescens* MF37 on glass (a hydrophilic surface) and on polystyrene (a hydrophobic surface), at the different temperatures, are presented in Table 2. Whatever the surface tested, *P. fluorescens* MF37 was able to adhere, suggesting that hydrophobic or hydrophilic interactions between the support and the bacteria are important in the adhesion process, but that other interactions may be involved as well. Nevertheless, adhesion appears to be significantly stronger for cells grown at 17°C than at the other temperatures, as shown by Student's t test ($P < 0.05$). Indeed, 46.6% of the polystyrene surface was colonized after 2 h of incubation with cells cultivated at 17°C versus only 31 and 35.1% when the cells were grown at 8 and 28°C, respectively. Similarly, the percentage of colonized hydrophilic surface was higher when the growth temperature was 17°C. *P. fluorescens* MF37 was thus able to colonize both supports in a temperature-dependent fashion.

The bacterial cell surfaces and their nonspecific adhesive behavior are significantly modified at the studied temperature of 17°C, and are probably implicated in the bacterial adaptation to its environment. It seems therefore that *P. fluorescens* has a more adhesive character at moderate temperatures rather than at the extremes. These moderate temperatures, narrow 17°C, can be associated to cold chain break down and must therefore be taken into account in food safety, at the levels of preparation, conservation and even transport of food products.

Effect of the loss of the OprF porin on the physicochemical properties of the cell surface and on the adhesion of *P. fluorescens* MF37

Physicochemical modifications of the cell surface suggest changes in the outer membrane composition in response to the growth temperature. Some outer membrane components have been put forward to explain how various surface properties were generated according to the environmental temperature, such as the lipid composition in particular in the fatty acids [20], the outer membrane fluidity [18, 19], or lipopolysaccharide phosphorylation [13, 24] and lipopolysaccharide length.

Since the major outer membrane protein OprF is thermo-sensitive [8, 17], exhibits extracellular domains, such as the variable loops exposed to the cell surface [4], and is described as an adhesin in the rhizosphere [9], we investigated its involvement in the physicochemical properties of the *P. fluorescens* cell surface and in its adhesive behavior. We constructed a stable OprF-lacking *P. fluorescens* mutant: strain MF373 [7]. The affinity of the strain MF373 to nonpolar solvents was surprisingly quite stable whatever the growth temperature (around 30%, Fig. 1), showing the hydrophilic properties of the cell surface of the mutant. The MF373 hydrophilicity appeared higher than that of the wildtype strain at 8 and 28°C. The loss of the major outer membrane protein might have been followed by a deep reorganization of the outer membrane, as suggested by the higher hydrophilic character of the cell surface.

When looking at the adhesion properties, the percentages of colonized surface by the OprF-deficient mutant were quite similar on both surfaces (just below 40% on glass and just above 40% on polystyrene), whatever the temperature tested (Fig. 2). At low and optimal temperatures, the adhesion on both supports of the OprF-deficient mutant was significantly higher than that of the wildtype (Fig. 2). These results are in agreement with the cell surface properties described above, thereby confirming the close relationship between the hydrophobic/hydrophilic nature of the bacterial surface and its adhesive capacities. Moreover, since the adhesive properties were conserved despite the absence of OprF, we must assume that OprF protein is not the major protein involved in this process, under our conditions. In Pseudomonads OprF has been shown to be involved in bacterial adherence to fibronectin [25], epithelial cells [1], or wheat roots [9], suggesting a role in a more specific way in adhesion to

Table 2 Percentage of colonized surface by *P. fluorescens* MF37 on abiotic surfaces at 8, 17 and 28°C

Growth temperature (°C)	% colonized surface (mean ± SD)[a]	
	Glass	Polystyrene
8	31.6 ± 1.4	31 ± 0.9
17	39.2 ± 0.3	46.6 ± 2.1
28	25.9 ± 0.2	35.1 ± 2.5

[a] Ten fields were observed per support with two independent cultures

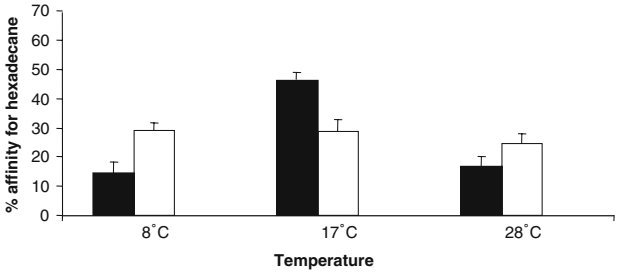

Fig. 1 Cell surface hydrophilicity measured by MAT method (hexadecane) in water containing NaCl 0.9% for *P. fluorescens* MF37 (*black bars*) and its OprF-deficient mutant *P. fluorescens* MF373 (*white bars*) at the temperatures indicated. Each experiment was performed in triplicate on two independent cultures

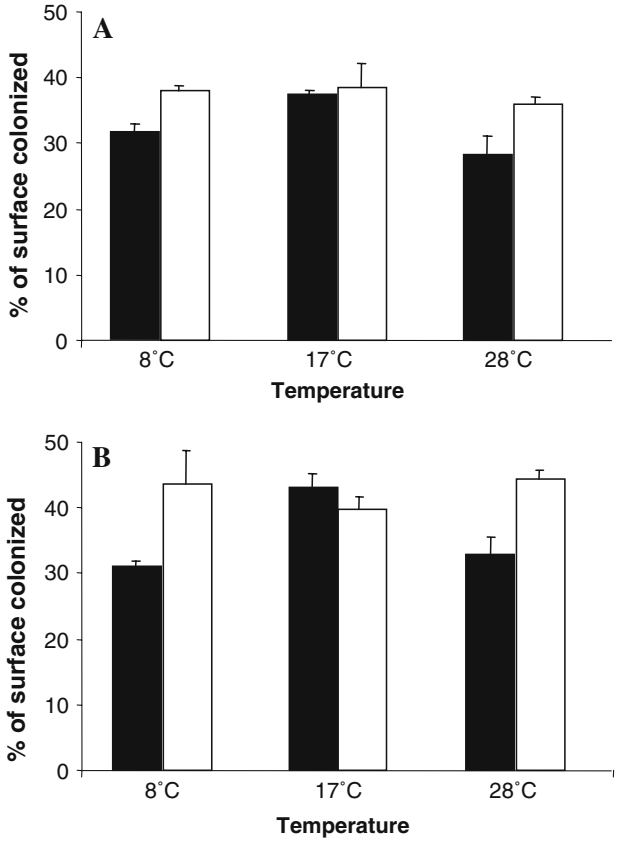

Fig. 2 Percentage of surface colonized by *P. fluorescens* MF37 (*black bars*) and its OprF-deficient mutant *P. fluorescens* MF373 (*white bars*) at the temperatures indicated. The surfaces tested were glass (**a**) and polystyrene (**b**). Ten fields were analyzed per support with two independent cultures

eukaryote cells, but not in abiotic surfaces. Nevertheless, Bodilis et al. [6] described a putative correlation between the OprF structure and adhesion to polystyrene. Our results suggest furthermore that OprF in involved in sensing environment temperature modifications. We hypothesize that OprF is the first event of a signaling pathway, conferring on the bacteria a way to sense and adapt on environmental changes. This assumption is strengthened by results of a recent study showing that the OprF porin of *P. aeruginosa* is implicated in host immune state sensing [29] by binding the interferon gamma cytokine. This allows bacteria to sense and response to the host immune system [30]. The absence of this major porin could disturb the *P. fluorescens* ability to respond to an environmental modification such as temperature.

Conclusion

Pseudomonas fluorescens strains are in general psychrotrophic, and able to colonize ecological niches which are subjected to rapid thermal variations [28]. To colonize a specific environment, the bacteria have to interact closely with it via an adhesion process [12], which is dependent of the physicochemical surface properties of the cell and the biotic or abiotic supports [3, 5, 26]. Taken together, our results demonstrate that the physicochemical surface properties of this strain and its adhesion capacities are closely linked, suggesting that hydrophobic/hydrophilic interactions could play a role in the attachment process of *P. fluorescens* to an inert surface. We also showed that the critical temperature of 17°C leads to a clear modification in the physicochemical properties of the *P. florescens* MF37 cell surface, correlated with its adhesive properties on nonspecific surfaces. Consequently, we suggest that particular care should be taken in the food industries, where this range of temperature is commonly used for food transformation and transport in order to limit contamination.

Finally, we showed that the major outer membrane protein, OprF, was not directly involved in the studied process of nonspecific adhesion. However, we demonstrated that the cell surface properties and adhesion ability were not anymore temperature-dependant in an OprF-deficient strain. We propose that OprF is one of the cell components able to sense the environmental temperature and able to transmit this signal inside the cell through a mechanism not yet described.

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