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The Major Outer Membrane Protein Oprf is Required for Rhamnolipid Production in *Pseudomonas aeruginosa*

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Summary

The OprF porin is the major outer membrane protein of bacteria belonging to the *Pseudomonas* genus, and is partially exposed on the cellular surface. A study based on the comparison between *P. aeruginosa* H103 and its *oprF*-deficient mutant led to the finding that the absence of OprF abolished swarming but not swimming and twitching motilities. These phenotypes were explained at least in part by the inability of the *oprF* mutant to produce biosurfactant rhamnolipids. The levels of mRNAs encoding the rhamnolipid biosynthetic enzymes RhlA and RhlB were strongly decreased in the absence of OprF, indicating that rhamnolipid production was impaired at the transcriptional level. We suggest that the presence of OprF in the outer membrane of *P. aeruginosa* is required for environments colonization, making thus OprF a serious target for limiting *P. aeruginosa* spreading in case of cystic fibrosis.

Keywords: OprF; Porin; Rhamnolipid; Motility; *Pseudomonas*

Abbreviations: LC/MS: Liquid chromatography coupled to Mass Spectrometry

Introduction

Pseudomonas aeruginosa tis an ubiquitous germ known as an important opportunistic pathogen of humans, causing a variety of infections among which chronic lung infections in cystic fibrosis patients [1-3]. *Pseudomonas* members are also described for their striking ability to adapt to various ecological niches [4]. This versatility requires a particularly well developed ability to adapt to changes of environmental conditions, to which proteins of the outer membrane may contribute, due to their partial exposition at the cell surface. Among them, OprF is one of the very few general porins [5], allowing non-specific diffusion of ionic species and of small polar nutrients [6]. OprF is also a major structural protein, anchoring the outer membrane to the peptidoglycan layer [7,8]. It is necessary for adaptation to various environments since it allows growth in low-osmolarity conditions [8], is over-produced in high salinity condition [9] and in ASM medium, which mimics the lung environment during cystic fibrosis [10]. It allows also the bacteria to respond to temperature variations by modulating the outer membrane permeability through a change in channel size [11]. OprF has been furthermore involved in adhesion to eukaryotic cells [12], and in biofilm formation under anaerobic conditions [13] enabling microcolonies formation [10]. Finally, we have recently shown that OprF is required for full virulence expression [14].

P. aeruginosa displays three types of motility: swimming in liquid or at low agar concentrations, twitching on solid surfaces, and swarming on semisolid media. Swimming and twitching result from the polar flagellum of *P. aeruginosa* and type IV pili, respectively, whereas swarming depends on both appendages and of rhamnolipids [15-17]. Rhamnolipids are biosurfactants composed of mono- or di-rhamnose linked to the lipid components 3-(3-hydroxyalkanoyloxy) alkanoic acids (HAAs) [18,19]. These glycolipids play a central role in swarming motility by acting as surface-modifying agents [20]. They can enhance cell surface hydrophobicity, by inducing LPS release from the outer membrane [21] and by adsorbing onto the cell surface [22,23], which can in turn modify the bacterium-substratum interactions. They affect biofilm formation through microcolonies formation, motility [24], maintaining fluid channels between mushroom-like structures [25], and mediating cell detachment from biofilms [26]. Recently, rhamnolipids have been furthermore suggested to act as protective agents of *P. aeruginosa* against polymorphonuclear leukocytes, functioning as a biofilm shield *in vivo* [27,28].

Since bacterial motility plays a key role in the bacterial adaptation to environments, especially in surfaces colonization, we further investigated the function of OprF in *P. aeruginosa* motility. In this study, we show that an *oprF* knock out leads to impaired swarming, but not swimming or twitching motilities, at least partly through a deep alteration in rhamnolipid production.

Materials and Methods

Bacterial strains and growth conditions

The strains were *P. aeruginosa* H103 (PAO1 prototroph), its *oprF* mutant H636 obtained by homologous recombination with an *oprF* fragment containing a streptomycin cassette [29], and H636O, which corresponds to H636 complemented by plasmid pRW5 (encoding carbenicillin resistance) consisting in the functional *oprF* gene from *P. aeruginosa* H103 cloned into pUCP19 [14,30]. Cultures were inoculated at an initial OD_{600} of 0.07, and bacteria were grown at 37°C on a rotary shaker (180 rpm) in Luria Bertani (LB) broth. In complement, 500 µg streptomycin mL^{-1} only or with 300µg carbenicillin mL^{-1} were added in H636 and H636O cultures, respectively.

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Motility assays

These assays were essentially performed as described by Rashid & Kornberg [31]. Briefly, LB plates containing 0.3 % agar were point inoculated with a toothpick and incubated for 24 hours at 37°C. Swimming was quantified by measuring the circular turbid zone. The swarming assay was identical, except that LB plates contained 0.5 % agar and were incubated for 48 h. For twitching assays, cells were stab inoculated to the bottom of the Petri dish through a thin (~ 3 mm) LB agar layer (1 % agar). After incubation at 37°C for 48 h, the agar was removed, the Petri dish was washed with a stream of tap water, and the cells attached to the polystyrene surface were stained with crystal violet (1 % w/v) solution. Each assay was made at least in triplicate.

Phage PO4 sensitivity assay

10 µL of lysates that contain 102 plates forming units (PFU) of phages was mixed with 107 colonies forming units (CFU) of P. aeruginosa cells grown to OD600 of 0.7 and resuspended in 100µL of LB. After 10 min of incubation, 3mL of top agar was added, and the mixture was plated. Numeration of plaques was made after 16 to 24 h of incubation at 37°C.

Rhamnolipid quantification

The drop-collapse test was performed as previously described [32]. Rhamnolipids were further extracted and analyzed by liquid chromatography coupled to mass spectrometry (LC/MS) as previously described [33].

Quantitative RT-PCR

Extraction of RNAs, synthesis of cDNAs and real time PCR were achieved as previously described [9] using primers described in Table 1. PCR reactions were performed in triplicate and the standard deviations were lower than 0.15 CT. The relative quantification of the mRNAs of interest was obtained by the comparative CT ($2^{-\Delta\Delta CT}$) method [34], using 16S rRNA as endogenous control [35]. ΔCT values were calculated by subtracting the 16S rRNA CT value from the CT value of an mRNA of interest from the same sample. ΔCT values were then obtained by calculating the difference between: i) the ΔCT value of a given mRNA resulting from *P. aeruginosa* H636 cells grown to a specific stage, and ii)

Gene	sequences (5'-3')	References
rhlA	F: GATCGAGCTGGACGACAAGTC R: GCTGATGGTTGCTGGCTTTC	[33] [33]
rhlB	F: GAACAGGCAGACCACGCC R: CGCATCTTCACCCAATGGAT	[33] [33]
rhlC	F: ACCGGATAGACATGGGCGT R: GATCGCTGTGCGGTGAGTT	[33] [33]
pilA	F: ATTGCCATTCCCCAGTATCAGA R: CGAAGCACCTTCCGAACG	This study This study
fliC	F: CTCGGAAAACGCTACCAACG R: GCGAAGTCGGTGTCCTTGAT	This study This study
estA	F: GGTTGGCCATGCCTTCCT R: ACGTCATTCCGTTGAACATCC	This study This study
16S	F: CAGGATTAGATACCCTGGTAGTCCAC R: GACTTAACCCAACATCTCACGACAC	[35] [35]

Table 1: Primers used in quantitative RT-PCR experiments.

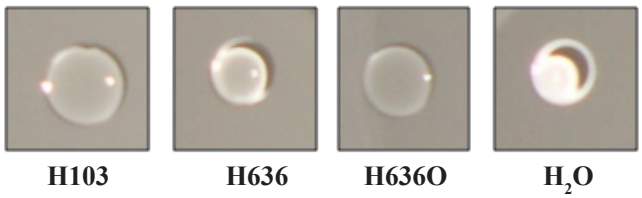

Figure 1: Drop-collapse test performed by adding 10 µL of culture supernatants of the indicated strains (48h) to water drops (20 µL) on glass slides.

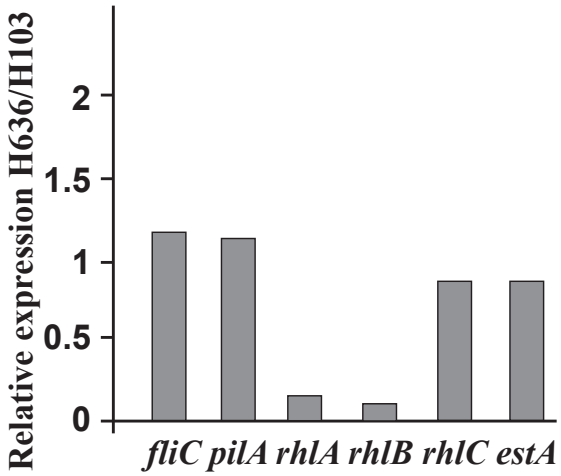

Figure 2: Expression levels of the indicated genes in the oprF mutant H636, relatively to P. aeruginosa H103. mRNAs were assayed by quantitative RT-PCR performed on RNA extracted from H636 and H103 strains. Values above and below 1 show a higher and a lower mRNA level in H636 than in H103, respectively. PCR reactions were performed in triplicate and the standard deviations were lower than 0.15 CT. The experiments were performed twice with independent bacterial cultures.

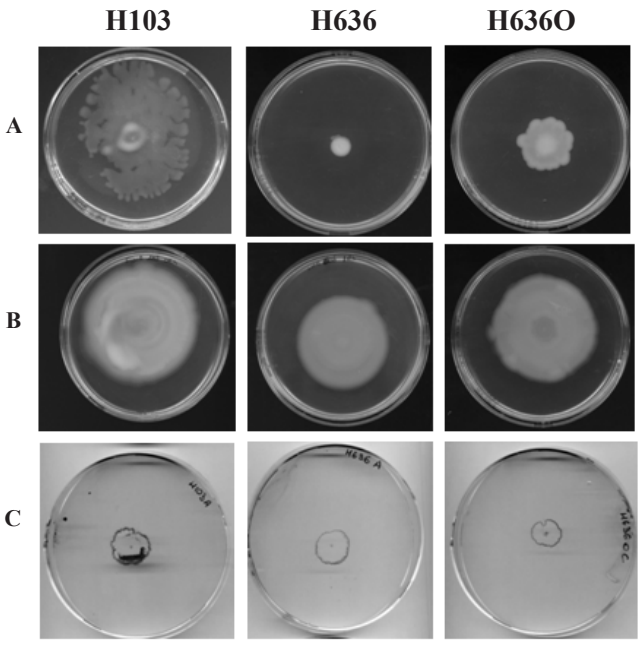

Figure 3: Swarming (A), swimming (B) and twitching (B) motilities of P. aeruginosa strains H103 (wild type), H636 (oprF mutant), and H636O (complemented oprF mutant).

the ΔCT value of the same mRNA from P. aeruginosa H103 (wild type) cells grown to the same stage. Relative mRNA level values are equal to $2^{-\Delta\Delta CT}$: values above and below 1 show a higher and a lower mRNA level in the *oprF* mutant H636 than in the wild type strain, respectively (eg a value of 0.5 indicates that the mRNA level was divided by 2 in H636).

Results

Three strains were compared in this study: the *P. aeruginosa* H103 wild type strain, its *oprF* mutant H636, and the *oprF*-complemented mutant strain H636O. The three growth curves obtained in LB medium at 37°C with shaking were similar, with a doubling time of 45 min [14].

Swarming is altered in the *oprF* mutant

The *oprF* mutant was unable to swarm, and the complementation of H636 with the *oprF* gene restores partially the swarming ability (Figure 1A). Swimming and twitching were slightly but not significantly altered (Figure 1B & Figure 1C), suggesting that both flagella and type IV pili were functional. To further dissect the swarming motility deficiency phenotype, quantitative RT-PCR experiments were performed to assay the transcription level of *fliC* and *pilA* genes, encoding the main flagellum and type IV pilus subunits, respectively. Figure 2 showed that their expression were not significantly down-regulated in the *oprF* mutant compared to H103 strain. The functionality of type IV pili was furthermore assayed by infecting the strains with the PO4 phage, which uses type IV pili as receptors [36]. The three strains were similarly sensitive to phage PO4 (the number of lysis plates being similar), indicating that type IV pili were present and functional in the H636 *oprF* mutant.

OprF is required for rhamnolipid production

Since swarming is depending on type IV pili, flagella and rhamnolipids [17], we next searched for the ability of the *oprF* mutant to produce these major biosurfactants. To achieve this rapidly, we first used the drop collapse test as previously described [32]. As shown on Figure 3, the drop spreading out in case of H103 and H636O suggested that biosurfactants were present. This was not the case for H636, suggesting a lack or a reduction in the biosurfactants amount. In supernatants of stationary phase cultures of the wild type strain, ten different ionic species, corresponding up to twelve rhamnolipids species, were identified by LC/MS (Table 2). Extra-cellular rhamnolipid production by the oprF mutant was nearly abolished: only three ionic species (*m/z* 675, 677, and 705) were detected in H636 supernatants, but in 25 to 50-fold lower amounts than in H103 supernatants (Table 2). Furthermore, the non detection of the ionic species *m/z* 649 in H636 supernatants indicated that Rha-Rha-C10-C10 production dropped more than 500 fold in the absence of OprF. We observed similar results when assaying rhamnolipids from supernatants of 48 and 72 h cultures (data not shown), thereby ascertaining that production of extra-cellular rhamnolipids by the *oprF* mutant is dramatically impaired, and not only delayed. The complementation of the mutation restored an efficient extra-cellular production of all rhamnolipid species, since their amounts were only 1.1 to 2.6-fold lower than in H103 supernatants (Table 2).

rhlAB expression is altered in the absence of OprF

To investigate whether the production of extra-cellular rhamnolipids by the *oprF* mutant is impaired at the biosynthesis or at the secretion level, we assayed rhamnolipids from bacterial pellets. A defect in rhamnolipid secretion was expected to lead to higher rhamnolipid accumulation in H636 OprF-negative cells than in H103 cells. This was however not the case (Table 2, intra cellular amounts), suggesting that the main defect occurred at the biosynthesis level. Mono-rhamnolipid biosynthesis specifically requires the successive actions of the enzymes RhlA and RhlB, encoded by the operon *rhlAB* [37,38]. Di-rhamnolipids are synthesized from mono-rhamnolipids by a third enzyme, the rhamnosyltransferase 2 RhlC [39]. Rhamnolipid production furthermore requires the autotransporter esterase EstA, the role of which remains unknown [40]. We therefore examined by quantitative RT-PCR whether the expression of these genes was affected in the *oprF* mutant. Whereas *rhlC* and *estA* mRNA levels were not significantly reduced in H636 mutant compared to H103 wild-type strain, the *rhlA* and rhlB mRNA levels were respectively 4.6 and 9-fold lower than in H103 (Figure 2). The down-regulation of the *rhlAB* operon seems strong enough to explain the rhamnolipid biosynthesis defect of the oprF mutant.

Discussion

OprF is the major outer membrane protein, partially exposed to the cell surface. Our study enabled us to find evidences for the involvement of OprF in swarming, but not in swimming and twitching motilities. Transcription of *fliC* and *pilA* genes are not affected, and the sensitivity to phage PO_4 indicates that the retraction function required for phage infection is maintained on the *oprF* mutant cell surface [16]. Taken together, these results suggest that flagella and type IV pili are expressed and functional in the oprF mutant. Since rhamnolipids are known to play a central role in swarming through their surfactant properties [20,41,42], we then focused on their production. Consistently, we found

Rhamnolipid	Ionic species* (*m/z*)	Extra cellular amount† (10^4 Area /OD_{600})			Intra cellular amount‡ (10^4 Area /OD_{600})		
		H103	H636	H636O	H103	H636	H636O
Rha-C10-C10	503	12.3 ± 1.1	ND	11.3 ± 1.9	ND	ND	ND
Rha-C10-C12:1	529	3.9 ± 0.2	ND	2.2 ± 0.3	ND	ND	ND
Rha-C12-C10 Rha-Rha-C8-C10	531	2.9 ± 0.1	ND	2 ± 0.2	ND	ND	ND
Rha-Rha-C10-C8	621	32.5 ± 7.4	ND	24.9 ± 5.2	ND	ND	ND
Rha-Rha-C8-C12:1	647	3.1 ± 0.7	ND	1.9 ± 0.1	ND	ND	ND
Rha-Rha-C10-C10	649	502.4 ± 14.5	ND	321.7 ± 55.4	13.2 ± 6.3	5.1 ± 2.2	41.5 ± 12.8
Rha-Rha-C10-C12:1	675	138.1 ± 18.1	3.3 ± 0.3	113.8 ± 13.7	5.5 ± 2.2	1.5 ± 0.7	16.1 ± 5.5
Rha-Rha-C10-C12, Rha-Rha-C12-C10	677	662.9 ± 3.6	13.2 ± 1.5	257.5 ± 16.6	15.4 ± 6.3	5.6 ± 2.8	56.9 ± 19.4
Rha-Rha-C12-C12:1	703	15.6 ± 3.3	ND	12.7 ± 1.2	0.9 ± 0.5	0.8 ± 0.1	3.7 ± 1.4
Rha-Rha-C12-C12	705	42.9 ± 25.2	1.7 ± 0.2	18.7 ± 1.5	2.9± 0.2	1.7 ± 0.1	5.3 ± 1.7
Total amount of rhamnolipids		1416.7 ± 74.2	18.2 ± 2	766.8 ± 1.5	37.9 ± 15.5	14.7 ± 5.9	123.5 ± 40.8

Table 2: Rhamnolipid amounts produced by *P. aeruginosa* H103, H636 and H636O grown to stationary phase in LB medium. ND: values not detected. Threshold: <0.8 x 10^4 Area/OD_{600}. *Ionic species leading to LC/MS peaks.† Rhamnolipids assayed from culture supernatants. ‡ Rhamnolipids assayed from cell pellets. †,‡ The LC/MS peak surface areas were divided by the OD_{600} values of the cultures. Each value is the average of three independent experiments.

that the rhamnolipid production is nearly abolished in the *oprF* mutant, which likely explains the swarming motility defect. Rhamnolipid biosynthetic genes were shown to be essential (*rhlA*) or important (*rhlB*) for *P. aeruginosa* swarming [15,20,24,37]. The roles of HAAs (3-3-hydroxyalkanoyloxy alkanoic acids), mono-rhamnolipids and di-rhamnolipids, synthesized by RhlA, RhlB and RhlC, respectively, were dissected: di-rhamnolipids and HAAs serve as attractant and repellents, respectively, while mono-rhamnolipids act as wetting agents [42]. The impairment in rhamnolipid production of the *oprF* mutant could therefore explain its inability to swarm. However, restoration of the swarming phenotype was incomplete in the complemented mutant whereas rhamnolipid production reached near wild type levels. This indicated that the lack of rhamnolipid might not be the only cause of the swarming defect in the *oprF* mutant. Alternatively, swarming might require a rhamnolipid overproduction, which might not be achieved properly in the complemented *oprF* mutant.

In the *oprF* mutant, rhamnolipids did not accumulate intracellularly at higher levels than in the wild type strain, indicating that the production is impaired at the biosynthesis level rather than at the secretion level. This impairment can be at least in part explained by lower levels of *rhlAB* mRNAs, suggesting an involvement of OprF in the *rhlAB* expression, which is already known to depend on a complex regulatory network [43]. Transcription of the *rhlAB* operon is under the direct control of the RhlR-RhlI quorum sensing (QS) system and of its cognate autoinducer molecule, N-butyryl-L-homoserine lactone (C_4-HSL) [44-47] which is itself regulated by the LasR-LasI system, encoding the *N*-(3-oxododecanoyl)-L-homoserine lactone auto inducer, ($3OC_{12}$-HSL) [48,49], and the MvfR-PQS (*Pseudomonas* Quinolone Signal) /HHQ (4-hydroxy-2-heptylquinoline) system [48]. We showed recently that QS molecule production was altered in the H636 *oprF* mutant since the amounts of $3OC_{12}$-HSL and C_4-HSL were reduced or delayed, respectively, while that of HHQ was increased [14]. It is thus possible that these alterations in QS molecule production contribute to the observed decrease in *rhlAB* transcription.

It is unclear whether OprF plays a direct and/or indirect role in the observed phenotypes. The importance of OprF in stabilizing the outer membrane and maintaining the integrity of the cell wall of *P. aeruginosa* has been previously described [7,8,14,29]. A possibility is that the lack of OprF can change the cell surface and the composition of the outer membrane. Based on these considerations, the alteration of the QS and rhamnolipid production could be due to major modifications of the cell wall. The alternative possibility that the absence of OprF impairs the observed phenotypes by other means than outer membrane disorganization cannot be disregarded, considering the major role of OprF in binding interferon γ and activation of the QS network [50]. OprF has been indeed suggested as an environmental outer membrane sensor, perceiving variations of its micro environment, and transmitting or transducing an unknown signal leading to QS activation [14,51]. Whatever the mechanisms involved linking OprF and rhamnolipid production, it is clear that the presence of OprF in the outer membrane of *P. aeruginosa* is required for environments colonization, making thus OprF a serious target for limiting *P. aeruginosa* spreading in case of cystic fibrosis.

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