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* is 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 the bacteria to respond to temperature variations by modulating the outer membrane permeability through a change in channel size [11]. OprF has furthermore been implicated 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-hydroxylkanoxyloxy) 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 knockout leads to impaired swimming, 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 OD600 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-∆∆CT) method [34], using 16S rRNA as endogenous control [35]. ∆CT values were calculated by subtracting the 16S rRNA CT value from the CT value of an mRNA of interest from the same sample. ∆CT values were then obtained by calculating the difference between: i) the ∆CT 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 <br> R: GCTGATGGTTGCTGGCTTTC | [33] <br> [33] |
| rhlB | F: GACACGGGAGCACCAAGCC <br> R: CGCATCTCCACCACAGGAT | [33] <br> [33] |
| rhlC | F: ACCGGATAGACATGGGCGT <br> R: GATCGCTGTGCGGTGAGTT | [33] <br> [33] |
| pilA | F: ATTCGGATCTCCACGATATCAGA <br> R: GAAAGCACCCTTTACGAAACG | This study <br> This study |
| fliC | F: CTCGAAAACGCTACCAACG <br> R: GCGAAGTGGTGTCCTTGAT | This study <br> This study |
| estA | F: GGTTGGCCATGCCTTCCT | This study <br> This study |
| 16S | F: CAGGATTAGATACCCTGGTAGTCCAC <br> R: GACTTAACCCAACATCTACGACAC | [35] <br> [35] |

Table 1: Primers used in quantitative RT-PCR experiments.
the ACT 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^ΔΔCT:\ 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 assessed 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 oprF mutant.

**OpF 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 rhamnolipid species, were identified by LC/MS (Table 2). Extra-cellular rhamnolipid production by the oprF mutant was nearly abolished: only three ionic species, were identified by LC/MS (Table 2). Extra cellular amount†

**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 rhlA operon seems strong enough to explain the rhamnolipid biosynthesis defect of the oprf mutant.

**Discussion**

OpF 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 PO4 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† (10⁴ Area/OD₆₅₀) | Intra cellular amount† (10⁴ Area/OD₆₅₀) |
|------------|---------------------|------------------------------------------|----------------------------------------|
|            |                     | H103 | H636 | H636O | H103 | H636 | H636O |
| Rha-C₁₀-C₁₀ | 502                 | 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 | 531                 | 2.9 ± 0.1 | ND | 2 ± 0.2 | ND | ND | ND |
| Rha-Rha-C₈-C₁₀ | 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-C₈ | 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 | 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 | 677                | 662.9 ± 3.6 | 13.2 ± 1.5 | 257.5 ± 16.6 | 15.4 ± 0.3 | 5.6 ± 2.8 | 56.9 ± 19.4 |
| Rha-Rha-C₁₂-C₈ | 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 | 2.92 ± 0.2 | 1.7 ± 0.1 | 5.3 ± 1.7 |
| Total amount of rhamnolipids | 14167.7 ± 74.2 | 183 ± 2 | 7664 ± 1.5 | 37.9 ± 15.5 | 14.7 ± 5.9 | 123 ± 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 10⁴ Area/OD₆₅₀. *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 OD₆₅₀ 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 swimming motility defect. Rhamnolipid biosynthetic genes were shown to be essential (rhlA) or important (rhlB) for _P. aeruginosa_ swimming [15,20,24,37]. The roles of HAAs (3-3-hydroxyalkanoyloxy alkanoic acids), mono-rhamnolipids and di-rhamnolipids, synthetized 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 swim. However, restoration of the swimming phenotype was incomplete in the complemented mutant whereas rhamnolipid production reached near wild type levels. This indicated that the lack of oprF might not be the only cause of the swimming defect in the oprF mutant. Alternatively, swimming 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-RhlQ quorum sensing (QS) system and of its cognate autoinducer molecule, N-butyryl-L-homoserine lactone (C4-HSL) [44-47] which is itself regulated by the LasR-LasI system, encoding the group III (3-oxododecanoyl)-L-homoserine lactone (C12-HSL) [48] and of its cognate autoinducer, N-butyl-L-homoserine lactone auto inducer, (3OC6-HSL) [49,49], and the MvrF-PQS (Pseudomonas Quinolone Signal) /HHQ (4-hydrox-2-heptylquinoline) system [48]. We showed recently that QS molecule production was altered in the H636 oprf mutant since the amounts of 3OC6-HSL and C8-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 rhamnolipid might not be the only cause of the swimming defect in the oprf mutant. Alternatively, swimming might require a rhamnolipid overproduction, which might not be achieved properly in the complemented oprf mutant.

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