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Full Virulence of *Pseudomonas aeruginosa* Requires OprF▽

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OprF is a general outer membrane porin of *Pseudomonas aeruginosa*, a well-known human opportunistic pathogen associated with severe hospital-acquired sepsis and chronic lung infections of cystic fibrosis patients. A multiphenotypic approach, based on the comparative study of a wild-type strain of *P. aeruginosa*, its isogenic *oprF* mutant, and an *oprF*-complemented strain, showed that OprF is required for *P. aeruginosa* virulence. The absence of OprF results in impaired adhesion to animal cells, secretion of ExoT and ExoS toxins through the type III secretion system (T3SS), and production of the quorum-sensing-dependent virulence factors pyocyanin, elastase, lectin PA-1L, and exotoxin A. Accordingly, in the *oprF* mutant, production of the signal molecules *N*-(3-oxododecanoyl)-L-homoserine lactone and *N*-butanoyl-L-homoserine lactone was found to be reduced and delayed, respectively. *Pseudomonas* quinolone signal (PQS) production was decreased, while its precursor, 4-hydroxy-2-heptylquinoline (HHQ), accumulated in the cells. Taken together, these results show the involvement of OprF in *P. aeruginosa* virulence, at least partly through modulation of the quorum-sensing network. This is the first study showing a link between OprF, PQS synthesis, T3SS, and virulence factor production, providing novel insights into virulence expression.

Pseudomonas aeruginosa is a ubiquitous Gram-negative bacterium capable of surviving in a broad range of natural environments. It is best known as a human pathogen associated with hospital-acquired infections (sepsis, gut and pulmonary infections of immunocompromised patients) and as a leading cause of morbidity and mortality among individuals suffering from cystic fibrosis (8).

Bacteria regulate their metabolism by sensing extracellular signals in order to adapt to various environmental conditions (38). *P. aeruginosa* interacts with the external medium through its outer membrane (OM), the components of which may be involved in the virulence process of the bacterium. OprF is among the very few general porins of *P. aeruginosa* allowing nonspecific diffusion of ionic species and small polar nutrients, including polysaccharides with cutoff sizes below 1,519 Da (49). OprF is also described as a structural protein, anchoring the OM to the peptidoglycan layer (57, 69). Being partially exposed on the external surface of the bacteria, its functions in host-pathogen interactions have started to be unraveled in recent years. OprF is indeed involved in various processes, including adhesion to eukaryotic cells (2) and biofilm formation under anaerobic conditions (33, 78). Recently, the interaction of OprF with gamma interferon was shown to result in production of two bacterial virulence factors, the lectin PA-1L and the phenazine pyocyanin (72), suggesting that OprF could be a sensor of the host immune system (67).

In *P. aeruginosa*, virulence toward several animals and plants involves the quorum-sensing (QS) regulatory network, which implies that cell density-dependent processes of intercellular communication control the expression of extracellular virulence factors (61). Briefly, QS in *P. aeruginosa* consists of two *N*-acyl-L-homoserine lactone (AHL) systems (*las* and *rhl*) linked via a 4-quinolone-dependent system. In the *las* system, the *lasI* gene product directs the synthesis of the cell-released autoinducer *N*-(3-oxododecanoyl)-L-homoserine lactone (3O-C_{12}-HSL), which interacts with its transcriptional regulator LasR to activate target promoters. The *rhl* system functions similarly, with RhlI, *N*-butanoyl-L-homoserine lactone (C_4-HSL), and RhlR being the AHL synthetase, autoinducer, and transcriptional regulator, respectively (52, 61). The *las* and *rhl* systems are interconnected (19, 52) and have been found to regulate the timing and production of multiple virulence factors, including elastase, staphylolytic protease, exotoxin A, rhamnolipids, pyocyanin, lectins, and superoxide dismutases (6, 61). Finally, there is a family of non-AHL-signaling molecules, the 4-hydroxy-2-alkylquinolines (HAQs), which includes 3,4-dihydroxy-2-heptylquinoline, also known as the *Pseudomonas* quinolone signal (PQS), and its precursor, 4-hydroxy-2-heptylquinoline (HHQ) (21, 22). As ligands of the transcriptional regulator MvfR (73), PQS and HHQ affect the

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regulation of a number of biologically important functions, such as virulence and biofilm formation (see reference 21 for a recent review). HAQ- and AHL-dependent QS systems are intimately linked: AHL-mediated QS is involved in HAQ biosynthesis since, for instance, the complex LasR/3O-C_{12}-HSL is required for the normal expression of *mvfR*, which controls *pqsABCDE*, necessary for HHQ synthesis, and also *pqsH*, the product of which is essential for the conversion of HHQ into PQS (23).

Among the important QS-dependent virulence factors, one finds the elastase LasB, the phenazine compound pyocyanin, the lectin PA-1L, and exotoxin A. LasB is an extracellular elastolytic metalloproteinase involved in the degradation of the host cell membranes (77). Pyocyanin contributes to tissue damage (36) and is able to induce pulmonary pathophysiology in mice (14). Lectin PA-1L is a soluble specific galactose binding lectin, involved in *P. aeruginosa*-induced lung injury. Exotoxin A is a lethal toxin which inhibits protein synthesis in mammalian cells (70). The type III secretion system (T3SS) is responsible for the translocation of virulence factors into eukaryotic cells (18, 28) and is mildly QS controlled (7, 59). T3SS is of major importance in acute infection, by facilitating dissemination of *P. aeruginosa* through epithelia (27), and in cytotoxicity toward polymorphonuclear cells and macrophages (18).

In this study, we show that the QS network is disorganized and that the production of the above-described major virulence factors is impaired in the absence of OprF. These new data emphasize an important role for OprF in infectious processes.

MATERIALS AND METHODS

Bacterial strains and growth conditions. The strains were *P. aeruginosa* H103 (PAO1 wild-type prototroph); H636 (H103 *oprF*::Ω), its *oprF* mutant obtained by homologous recombination with an *oprF* fragment containing a streptomycin (Sm) cassette (68); and H636O (H103 *oprF*::Ω/pOprF), which corresponds to H636 complemented by plasmid pRW5 (encoding carbenicillin [Cb] resistance), consisting of the functional *oprF* gene from *P. aeruginosa* H103 cloned into pUCP19 (69). H636O overexpresses *oprF*, since quantitative reverse transcription-PCR and Western blots showed that the levels of its mRNA and its product are 2- to 5-fold and about 2-fold higher than those for H103, respectively (data not shown). For the functionality of the T3SS assay, we used as a control the *P. aeruginosa* strain CHA, a bronchopulmonary isolate from a cystic fibrosis patient which produces large amounts of the T3SS effectors ExoS and ExoT (66). Bacteria were grown at 37°C in a rotary shaker (180 rpm) in 25 ml of LB broth (containing 171 mM NaCl) in 250-ml Erlenmeyer flasks; appropriate aeration is critical for the similar growth of the H103 and H636 strains. For precultures, H636 and H636O were grown in the presence of Sm (500 μg ml^{-1}) and of Sm and Cb (300 μg ml^{-1}), respectively. Cultures were inoculated at an initial optical density at 600 nm (OD_{600}) of 0.07. For HAQ and *pqsH* expression-related experiments, conjugation was performed with *Escherichia coli* strain SM10 carrying a mini-CTX plasmid (5) encoding tetracycline (Tc) resistance and containing a *pqsH-lacZ* fusion (M. G. Lamarche and E. Déziel, unpublished data). The transconjugants were selected on *Pseudomonas* isolation agar (PIA) containing Tc (125 and 75 μg ml^{-1} for solid and liquid media, respectively) and Sm and/or Cb, when required, as described above. In this case, to verify that the chromosomal insertion has no effect on bacterial growth, we monitored the biomass production by assaying proteins within cell pellets after culture centrifugation using the Bradford assay (9).

***Caenorhabditis elegans* infection model.** *C. elegans* wild-type Bristol strain N2 worms, kindly provided by the *Caenorhabditis* Genetics Center (Minneapolis, MN), were grown at 22°C on nematode growth medium (NGM) agar plates using *E. coli* OP50 as the nutrient. Synchronous worm cultures were obtained by exposure to a sodium hypochlorite-sodium hydroxide solution, as previously described (63). The resulting eggs were then incubated at 22°C for 48 h, i.e., until the worms had reached the L4 life stage (confirmed by light microscopy). To test the virulence activity of the bacterial strains, the microorganisms were grown as described above in LB broth overnight and harvested, and 10^9 bacteria were spread onto NGM solidified agar plates and incubated at 37°C overnight. The plates were cooled to room temperature for 4 h, and 20 to 30 L4-synchronized worms were placed on the plates and incubated at 22°C. Worm survival was scored daily for 12 days using an Axiovert S100 optical microscope (Zeiss, Oberkochen, Germany) equipped with a digital camera (DXM 1200F; Nikon Instruments, Melville, NY). A worm was considered dead when it remained static without grinder movements for more than 20 s. An assay consisted of three independent replicates (plates). The virulence value of each bacterial strain was the mean of three independent assays. For killing assay, nematode survival was calculated by the Kaplan-Meier method, and the significance of survival differences was tested using the log rank test (Prism software, version 4.0; GraphPad Software, San Diego, CA). The growth and the viability of strains H103, H636, and H636O were tested as follows. A total of 10^9 bacteria, previously grown on LB medium, were spread onto NGM agar plates and incubated at 37°C overnight, allowing the development of a homogeneous lawn that was visually similar for each strain. The plates were then cooled to room temperature for 4 h and incubated at 22°C (as was the case for the worm tests). After 0, 5, 7, and 11 days of incubation at 22°C, a 1-cm^2 piece of agar was extracted, placed in 1 ml of LB medium, and vigorously mixed to allow resuspension of the bacteria. Dilutions were plated out on agar-solidified LB plates, and bacteria were allowed to grow at 37°C overnight before being numerated. For each strain, three agar plates were used and the experiment was repeated three times.

Chicory leaf model of infection. *P. aeruginosa* strains were grown overnight at 37°C in LB medium until they reached the stationary phase. After centrifugation, bacterial cells were washed in 10 mM $MgSO_4$ and diluted to 10^8 CFU ml^{-1}, and 10 μl of this suspension was injected with a syringe into the midrib of chicory (*Cichorium intybus*) leaves that had previously been washed with 0.1% bleach. The leaves were placed on dishes containing a Whatman filter impregnated with sterilized water. The plates were kept in a growth chamber at 37°C, and the leaves were monitored for symptoms of rot for 5 and 8 days. The experiments were repeated at least three times on independent days. Bacterial growth and viability were tested as follows. After 0, 5, and 8 days of incubation at 37°C, three inoculated leaves were weighed and disrupted with a scalpel for each strain. The mixture was covered with 5 ml of LB broth and vigorously mixed. After a short pulse centrifugation to pellet the chicory waste, the supernatant was diluted and plated out onto agar-solidified LB plates. Plates were incubated at 37°C overnight, and bacteria were numerated. Results are the means of three independent experiments and are expressed as the number of CFU per 1 g of leaf.

Cellular culture. Primary culture of glial cells was achieved as previously described (53). The Caco-2/TC7 clonal cell line, derived from parental Caco-2 cells, was kindly provided by A. Servin (INSERM, UMR-S756, Châtenay-Malabry, France) at passage 23 and was used between passages 25 and 35. Cells were routinely grown at 37°C in a controlled atmosphere containing 5% CO_2 in Dulbecco's modified essential medium (DMEM) supplemented with 15% heat-inactivated fetal calf serum (FCS) and 1% nonessential amino acids. The medium was changed three times a week. The growth and the viability of strains H103, H636, and H636O were tested by plate numeration after incubation of the bacteria for 3 and 14 h (corresponding to the adhesion and cytotoxicity assays, respectively) in the eukaryote medium and under the eukaryote culture conditions.

Cell adherence assays. The adhesive behavior (binding index) of *P. aeruginosa* strains onto glial cells and intestine Caco-2/TC7 cells was investigated using the procedure described by Picot et al. (54) adapted to *P. aeruginosa*. Cells were allowed to adhere onto eukaryotic cells for 3 h.

Cytotoxicity assays. The cytotoxic potential of *P. aeruginosa* strains was determined by measurement of lactate dehydrogenase (LDH) release by eukaryotic cells upon cytoplasmic membrane destabilization. LDH is a stable cytosolic enzyme, the release of which is considered an indicator of necrosis (53). Confluent Caco-2/TC7 and glial cells were infected with 10^8 CFU/ml and 10^6 CFU/ml of the three strains, respectively, for 14 h. The amount of LDH released by eukaryotic cells was determined using the Cytotox 96 enzymatic assay (Promega, Charbonnières, France), as previously described (53).

T3SS assays. We used the pEXSAind plasmid, which contains *exsA* under the control of an isopropyl-β-D-thiogalactopyranoside (IPTG)-inducible promoter and a gene encoding the fusion protein ExoS-GP100, used as a marker of secretion (21). Overnight cultures of the different *P. aeruginosa* strains were resuspended at an OD_{600} of 0.2 in LB medium containing 5 mM EGTA and 20 mM $MgCl_2$ supplemented or not with 0.5 mM IPTG and grown to an OD_{600} of 1.5. Growth and viability of strains H103, H636, and H636O were assayed by cell numeration in LB medium containing EGTA and $MgCl_2$, and those of strains H103(pEXSAind) and H636(pEXSAind) were monitored under T3SS-inducing conditions supplemented with 0.5 mM IPTG. Bacterial cultures were centrifuged

at 17,000 × *g* for 15 min, and 1 ml of the supernatant was recovered. Proteins were precipitated from the supernatant with 15% trichloroacetic acid (TCA) at 4°C overnight. The precipitated protein fraction was pelleted at 17,000 × *g*, washed twice with acetone, dried at room temperature, and resuspended in 60 μl denaturation buffer (Tris-HCl, 0.2 M, pH 6.8; dithiothreitol, 2%; SDS, 10%; glycerol, 10%; bromophenol blue, 0.02%), prior to analysis by SDS-PAGE. Bacterial lysates were obtained after resuspension into denaturation buffer. For immunoanalyses of ExoS/ExoT proteins, normalized supernatants or bacterial lysates were separated on 15% SDS-polyacrylamide gels, transferred onto nitrocellulose membranes, and revealed with an anti-ExoS/ExoT serum (a gift from I. Attree, Grenoble, France) at a 1:1,000 dilution.

Virulence factor production. (i) Pyocyanin production assays. Bacteria were grown overnight in 5 ml of LB medium at 37°C with orbital shaking at 180 rpm. After centrifugation at 10,000 × *g* for 10 min, the phenazine pigment was extracted from the supernatant with 3 ml of chloroform and 0.2 N HCl. The amount of pyocyanin was estimated by the ratio of the absorbance measured at 520 and the absorbance measured at 600 nm.

(ii) Secreted elastase activity assay. The secreted elastase activity of *P. aeruginosa* strains was determined on LB medium culture supernatants using as a probe elastin-Congo red (ECR; Sigma, St. Louis, MO). Briefly, 100 μl of bacterial supernatant was sterilized by filtration and added to 900 μl of ECR buffer (100 mM Tris-HCl, pH 7.5, 1 mM $CaCl_2$, 20 mg ECR). This mixture was incubated overnight at 37°C with agitation. Insoluble ECR was removed by centrifugation, and the absorption of the supernatant was measured at 495 nm. Fresh sterile LB medium was used as a negative control. Secreted elastase activity was expressed as the ratio of the OD_{495} and OD_{600} absorbance values.

(iii) Secreted exotoxin A production assay. Secreted exotoxin A production was evaluated as previously described by Gaines et al. (30).

(iv) Assay of *lecA* mRNA encoding lectin PA-1L. Extraction of RNAs, synthesis of cDNAs, and real-time PCR were achieved as previously described (4, 32) using primers lecA 1 (TGGAAAGGTGAGGTTCTGAC) and lecA 2 (AATCG ACGTTACCTGCCCTG). Each RNA extraction was tested by PCR for the absence of contaminating DNA prior to reverse transcription. The PCRs were performed in triplicate, and the standard deviations (SDs) were lower than 0.15 threshold cycle (C_T) unit. The relative quantification of the mRNAs was obtained by the comparative C_T ($2^{-\Delta\Delta CT}$) method (39), using 16S rRNA as an endogenous control (sense primer, CAGGATTAGATACCCTGGTAGTC CAC; reverse primer, GACTTAACCCAACATCTCACGACAC [15]). ΔC_T values were calculated by subtracting the 16S rRNA C_T value from the C_T value of the *lecA* mRNA from the same sample. $\Delta\Delta C_T$ values were then obtained by calculating the difference between (i) the ΔC_T value of *lecA* mRNA resulting from *P. aeruginosa* H636 cells grown to a specific stage and (ii) the ΔC_T value of the *lecA* mRNA from *P. aeruginosa* H103 (wild-type) cells grown to the same stage. Relative mRNA level values are equal to $2^{-\Delta\Delta CT}$.

AHL extraction and quantification. AHLs were extracted, identified, and quantified as previously described (4, 47), using reverse-phase liquid chromatography coupled to positive-ion electrospray ionization and ion trap mass spectrometry (Brucker Esquire-LC ESI-MS/MS; Bruker Daltonic, Germany).

Quantification of HAQs and of *pqsH* expression. HHQ and PQS were extracted and quantified as described by Lépine et al. (37). Expression of *pqsH* was evaluated by assaying the β-galactosidase activity produced from the *pqsH-lacZ* transcriptional fusion, according to a protocol adapted from Miller (46).

OM physicochemical properties. (i) MICs of hydrophobic antibiotics. MICs were obtained by the agar dilution method as previously described (1). Briefly, antibiotics were incorporated at various concentrations into LB agar plates. Bacteria were grown overnight and diluted 100-fold, and then 0.01 ml bacterial suspension was spotted onto the plates. The plates were incubated for 18 h at 37°C. MIC values were defined as the lowest concentration of antibiotic which produced inhibition of growth. Antibiotics were gifts from the manufacturers.

Gentamicin-assisted lysozyme lysis assay. The gentamicin-assisted lysozyme lysis assay was conducted as previously described (1, 40), except that the aminoglycoside used was gentamicin (10 $\mu g \cdot ml^{-1}$) instead of streptomycin and that the buffer used was sodium HEPES (5 mM, pH 7) instead of sodium phosphate buffer (pH 7).

RESULTS

Growth characteristics. 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. Under our conditions, the three growth curves obtained in LB medium at 37°C with shaking were closely similar, with a doubling time of 45 min (Fig. 1). Since it has previously been shown that the *oprF* mutant had a growth defect under certain growth conditions (12, 57, 69), its growth and viability were systematically compared to those of the two other strains in the subsequent experiments.

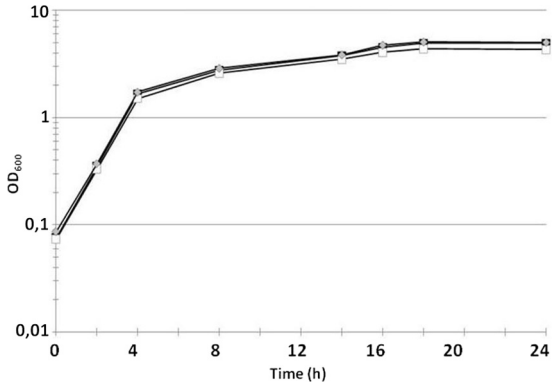

FIG. 1. Growth of the *oprF* mutant is not drastically altered in LB medium. The growth kinetics of H103 (wild-type strain; black squares), H636 (*oprF* mutant; white squares), and H636O (*oprF*-complemented mutant strain; gray diamonds) in LB medium are shown. Experiments have been performed at least in triplicate. Values are expressed as means. In each case, the standard error of the mean was lower than 10%.

OprF is involved in *P. aeruginosa* virulence. The virulence of each strain was assessed using two complete eukaryote model organisms, the Belgian endive (*Cichorium intylus*) and the nematode *C. elegans*, and two different mammalian cell lines, primary cultures of rat glial cells and cells of the immortalized human cell line Caco-2/TC7 (Fig. 2). Plants have been used as a fast and inexpensive tool to identify putative virulence determinants (56). The wild-type *P. aeruginosa* H103 strain inoculated in the middle vein was able to cause significant necrosis on the leaves of Belgian endives 5 and 8 days after inoculation, while the *oprF* mutant caused limited necrosis, even 8 days after inoculation. The control, consisting of bacterium-free buffer, did not cause significant necrosis symptoms even at 8 days postinoculation. Complementation with *oprF* in *trans* fully restored the virulence of the *oprF* mutant toward Belgian endive (Fig. 2A). Growth and viability of the *oprF* mutant H636 were altered, since 5 days after inoculation of a young chicory leaf, 10^5 H636 cells/g were numerated, whereas about 10^8 of both H103 and H636O cells/g were numerated, explaining at least partially the lower necrosis symptoms observed in the case of the *oprF* mutant (Fig. 2A). However, after 8 days the *oprF* mutant reached a density similar to that of H103 and H636O after 5 and 8 days (about 10^8 cells/g of tissue). At this density, the necrosis observed for H636 (8 days postinoculation) was lower than that observed for the other strains at 5 and 8 days postinoculation (Fig. 2A). *P. aeruginosa* is able to kill the worm *C. elegans* in an infection-like process which requires the ingestion of bacteria, followed by proliferation in the worm gut (64). As shown in Fig. 2B, the *P. aeruginosa* H103 wild-type strain and strain H636O (*oprF* complemented) were toxic for the worms, since about 50% and 87% of the population died within 6 and 8 days, respectively. In contrast, the

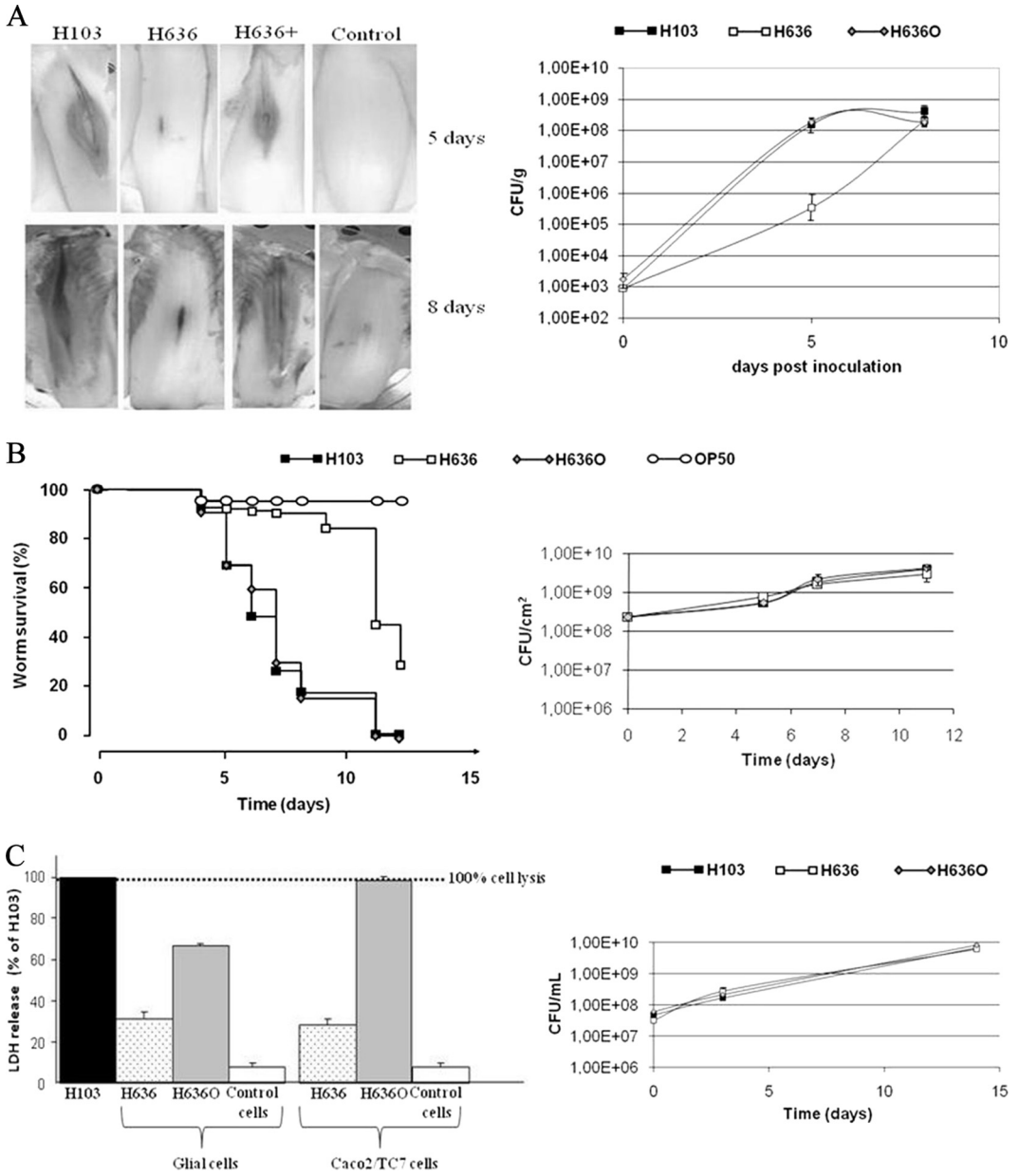

FIG. 2. The absence of OprF reduces *P. aeruginosa* virulence. (A) Hypersensitive response of *Cichorium intybus* 5 and 8 days after inoculation of 10 μl of bacterial suspensions containing 10^6 CFU in 10 mM $MgSO_4$ and bacterial numeration in chicory leaves 0, 5, and 8 days after inoculation, expressed as the number of CFU per 1 g of chicory leaf. The control was 10 μl of 10 mM $MgSO_4$. Experiments have been performed at least in triplicate. (B) Kaplan-Meier survival plots of *C. elegans* nematodes fed wild-type strain *P. aeruginosa* H103 ($n = 136$), *oprF* mutant H636 ($n = 136$), OprF-complemented mutant strain H636O ($n = 132$), and the nontoxic strain *E. coli* OP50 (negative control; $n = 118$). Each value reported for the assay is the mean of measurements of eight samples from three independent experiments. Pairwise strain comparisons (log-rank test) were as follows: H103 versus H636, $P < 0.0001$; H636 versus H636O, $P < 0.0001$; H103 versus H636O, $P = 0.5889$. The three bacterial strains were numerated (expressed as the number of CFU/cm^2) on NGM plates after 0, 5, 7, and 11 days under the growing conditions used to test *C. elegans* viability. Experiments were repeated three times. (C) Cytotoxic effect (through LDH release measurement) of the strains on rat glial cells and human Caco-2/TC7 cells and bacterial numeration after 3 and 14 h of growth in DMEM. The amount of LDH released upon infection by H103 corresponds to 100% LDH release. The control was natural cell death without infection with bacteria and with the same treatment used in the assays.

virulence of the *oprF* mutant H636 was drastically reduced since 80% of the worms were still alive after 9 days of contact. At 11 days postinfection, all worms exposed to H103 and H636O were dead, whereas about 50% of the initial worm population was surviving on plates seeded with H636. A control with the reference strain *E. coli* OP50 (a harmless food supply for *C. elegans*) showed that worm viability was stable until 12 days of contact with these nonvirulent bacteria (Fig. 2B). The numbers of viable H636 cells were similar to the numbers of viable H103 and H636O cells throughout the in-

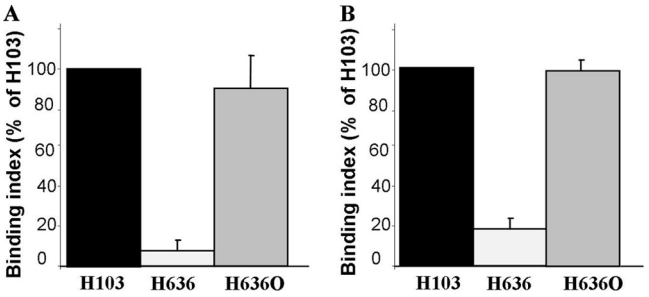

FIG. 3. OprF is required for bacterial adhesion onto eukaryotic cells. The binding indexes of bacteria onto glial (A) and Caco-2/TC7 (B) cells are shown. Each bar represents the mean number of adherent bacteria per cell (±SD) calculated over 100 cells and expressed as a percentage compared to the binding of the wild-type H103 strain. Experiments were performed at least three times.

cubation on nematode medium (Fig. 2B), showing that the virulence defect of H636 strain on *C. elegans* cannot be attributed to a growth or viability defect of this strain. We next tested the involvement of OprF in induction of cytotoxicity and cell lysis through the LDH release assay on rat glial cells (53) and on human Caco-2/TC7 cells (34). The amount of LDH release is related to eukaryotic cell death. Clear and comparable reductions in the cytotoxicity of the *oprF* mutant H636 were observed on these cultivated eukaryotic cells. Whereas wild-type strain H103 induced 100% LDH release on primary cultures of rat glial cells, mutant H636 provoked the release of only 34% ± 2.9% LDH (Fig. 2C). *trans* complementation with *oprF* partially restored the phenotype, with strain H636O inducing 68% ± 1.5% LDH release. The fact that the complementation was only partial, compared to the expression of the native *oprF* in the wild-type strain, could result from the overexpression of *oprF* from the complementing plasmid (see the Materials and Methods). Very similar results were obtained using the human-derived Caco-2/TC7 cell line (Fig. 2C): the H103 wild-type strain of *P. aeruginosa* provoked 100% LDH release, and the *oprF* mutant provoked only 29% ± 0.9%. In these cells, the virulence of the *oprF* mutant strain was almost completely restored in the complemented mutant H636O, resulting in 98% ± 1.2% LDH release. A control of growth and viability of H103, H636, and H636O under the culture conditions of the eukaryotic cells (DMEM) showed that the numbers of viable H636 cells were similar to those of H103 and H636O (Fig. 2C), showing that the virulence defect of the *oprF* mutant strain cannot be attributed to a growth or viability defect.

OprF is involved in *P. aeruginosa* adhesion onto eukaryotic cells. Since OprF is an OM porin, we first confirmed that the involvement of OprF in virulence and in related phenotypes is at least partly linked to the attachment of bacteria to cells, as previously described on pulmonary epithelial cells (2). As shown in Fig. 3A and B, the adhesion potential (binding index) of the *oprF* mutant was drastically reduced on both rat glial cells and human Caco-2/TC7 cells (representing only 7.8% ± 2.6% and 20% ± 1.6%, respectively) compared to the binding index of wild-type strain *P. aeruginosa* H103. Complementation in *trans* restored the adhesion property onto both rat glial cells (with the binding index reaching 87.9% ± 16% that of the wild type) and Caco-2/TC7 cells (94.3% ± 0.3% binding compared to that of H103). As indicated above, growth and viability were similar for the three strains in DMEM (Fig. 2C).

Alterations of type III secretion system in *oprF* mutant. Since the *oprF* mutant displays reduced cytotoxicity toward eukaryotic cells, we examined the functionality of its T3SS, which is used by *P. aeruginosa* to translocate toxins into eukaryotic cells (18, 74, 75), using the secretion assay achieved under calcium-chelating conditions (28). The secretion of several proteins was induced when H103 and H636O were cultivated in calcium-depleted medium, which activates T3SS (Fig. 4A, EGTA+). Two of these proteins were previously identified to be type III secreted effectors (ExoS and ExoT) in the control *P. aeruginosa* CHA strain isolated from a patient with cystic fibrosis (17). The *oprF* mutant failed to secrete ExoS and ExoT, even under calcium-depleted conditions (Fig. 4A), showing that T3SS activity is impaired in the absence of OprF. Under the conditions used, the growth and the viability of the three bacterial strains were not deeply modified, even if the cell number of H636 was lower than that of H103 (Fig. 4A), indicating that the T3SS alteration cannot be attributed to a growth or viability defect of the *oprF* mutant. This defect could result from an alteration of OM properties due to the absence of OprF, which could prevent T3SS functioning. Alternatively, the expression of genes encoding T3SS and its effectors could be impaired. Since ExsA is the transcription activator which positively regulates the expression of the T3SS and effector genes (74), we tested if increasing the level of *exsA* transcription would restore T3SS activity in strain H636. The strains were grown under T3SS-inducing (with EGTA [$EGTA^+$]) or noninducing (without EGTA [$EGTA^-$]) conditions and *exsA*-inducing (with IPTG [$IPTG^+$]) or noninducing (without IPTG [$IPTG^-$]) conditions. The supernatant protein pattern was analyzed on SDS-polyacrylamide gels. As shown in Fig. 4B, the fusion protein ExoS-GP100 was observed in H103(pEXSAind) supernatants under calcium-depleted conditions without IPTG, which confirms that the expression level of the native chromosomal copy of *exsA* was sufficient in the wild-type strain. Accordingly, adding IPTG to H103(pEXSAind) under calcium-depleted conditions led to larger amounts of secreted ExoS-GP100. In the opposite situation, ExoS-GP100 secretion by the H636(pEXSAind) strain required IPTG induction of the plasmid-borne *exsA* gene, showing that the T3SS defect of the *oprF* mutant can be bypassed by overexpressing *exsA*. Since the plasmidic ExsA was functional in H636(pEXsAind) and both the natural and plasmidic ones were functional in H103(pEXSAind), the amount of ExoS-GP100 secretion in H636 under *exsA*-inducing conditions remained lower than that in the parental H103 strain. Cell numeration did not reveal a deep viability defect of the *oprF* mutant, even if the number of *oprF* mutant H636 cells remained lower than that of H103 cells (Fig. 4B). If the T3SS defect observed in H636 was solely due to alterations of the OM properties, one would expect that the T3SS effectors ExoT, ExoS, and ExoS-GP100 would accumulate in the bacterial cytoplasm. Figure 4C and D shows that this is not the case, since the effectors were found in larger amounts in H636 supernatants than in H636 cell lysates. It is therefore likely that the genes encoding T3SS and its effectors are at least partially underexpressed in strain H636.

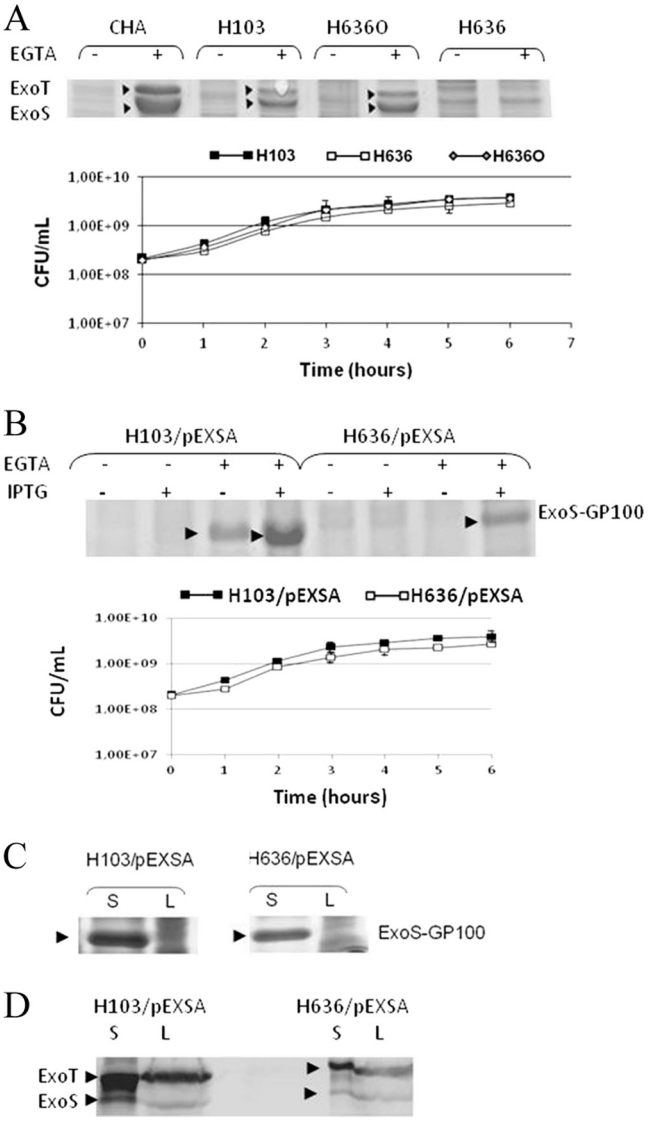

FIG. 4. OprF is involved in T3SS expression. (A) *oprF* mutation reduces protein secretion through T3SS. Strains H103, H636, and H636O and wild-type strain CHA were grown under T3SS-inducing ($EGTA^+$) or noninducing ($EGTA^-$) conditions until the OD_{600} reached 1.5. Supernatants were normalized and subjected to TCA precipitation. The extracellular proteins were separated by 15% SDS-PAGE and stained with Coomassie blue. Bands corresponding to previously identified SSTT toxins (ExoS and ExoT) are indicated by black arrowheads. Experiments were repeated three times. Numeration of the H103, H636, and H636O bacteria (expressed in numbers of CFU per ml) was performed under T3SS-inducing conditions to test the viability of the strains. Experiments were repeated in triplicate. (B) *exsA* overexpression restores T3SS activity in the absence of OprF. Wild-type strain H103 and mutant H636 express ExsA from an IPTG-inducible promoter on plasmid pEXSAind, which also contains a gene encoding the fusion protein ExoS-GP100. The strains were grown under T3SS-inducing ($EGTA^+$) or noninducing ($EGTA^-$) conditions, with or without 0.5 mM IPTG, until the OD_{600} reached 1.5. The extracellular proteins were separated by 15% SDS-PAGE and stained with Coomassie blue. The arrowheads indicate secreted ExoS-GP100. Experiments were repeated three times. Cell numeration of H103/pEXSAind and H636/pEXSAind was performed under T3SS- and *exsA*-inducing conditions. Experiments were repeated in triplicate. (C) In the absence of OprF, ExoS-GP100 is not sequestered inside the cells. H103 and H636 containing plasmid pEXSAind were cultured under inducing conditions with IPTG and EGTA. Normalized TCA-precipitated supernatants (lanes S) or total cell lysates (lanes L) were separated by 15% SDS-PAGE and stained with Coomassie blue. Arrowheads point to ExoS-GP100. The experiment was repeated twice. (D) ExoS/ExoT do not accumulate inside the *oprF* mutant cells. Western blots of normalized supernatants (lanes S) or bacterial lysates (lanes L) obtained under inducing conditions after separation by 15% SDS-PAGE, transfer onto nitrocellulose membranes, and revelation with an anti-ExoS/ExoT serum at a 1:1,000 dilution are shown.

Altered pyocyanin, exotoxin A, lectin PA-1L, and elastase production by *oprF* mutant. The bacterial culture supernatants were then investigated for the presence of major virulence factors, namely, pyocyanin, exotoxin A, lectin PA-1L, and elastase. In agreement with the reduced virulence phenotype of the *oprF* mutant, all of these factors are strongly affected by the inactivation of *oprF*. As described above, the growth of the *oprF* mutant was not deeply altered under our culture conditions (Fig. 1). Measurement of the relative pyocyanin production by the different strains revealed that the production of this phenazine is not detected in the *oprF* mutant supernatant grown to the entry of the stationary phase and is strongly reduced when it is grown to the stationary phase (9% ± 1% that of the wild type) (Fig. 5A). In complemented mutant H636O, the relative production of pyocyanin was restored to 126% ± 5% and 63% ± 4% of that of the wild-type strain in the early stationary and stationary phases, respectively. The peak of exotoxin A production was obtained during the entry into the stationary growth phase in the wild-type strain but was strongly decreased in the *oprF* mutant, representing only 21% ± 1% of the wild-type strain production. The *oprF* complementation partially restored this phenotype (Fig. 5B). Likewise, the expression of *lecA*, which encodes the lectin PA-1L, was strongly reduced in the *oprF* mutant and was fully restored in the *oprF*-complemented strain (Fig. 5C). Finally, when the cells were grown to the entry into the stationary phase, we observed a significant reduction of the elastase amount secreted by *oprF* mutant H636 (18% ± 0.1% that of the wild type) and good restoration of the phenotype in complemented strain H636O (77% ± 0.1% that of the wild type) (Fig. 5D). However, when it was grown to stationary phase, the elastolytic activity of the *oprF* mutant strain was found to be similar to the activities of the two other strains, indicating that the production of elastase is delayed but not abrogated.

Altered patterns of quorum-sensing molecule accumulation in culture supernatants of *oprF* mutant. Since the syntheses of pyocyanin, elastase, lectin PA-1L, and exotoxin A are under QS control (61) and since OprF was proposed to transmit signals to the QS network (67), we investigated whether the *oprF* mutation has an effect on the accumulation of the major intercellular communication molecules in the culture supernatants. Production of 3O-C_{12}-HSL by wild-type strain H103 increased during the exponential growth phase and reached a peak during the entry into the stationary phase (Fig. 6A). 3O-C_{12}-HSL almost disappeared from culture supernatants after 14 h of culture (Fig. 6A). In the *oprF* mutant strain, the maximal level of 3O-C_{12}-HSL was reduced, representing 46.9% ± 0.5% of the wild-type strain production (Fig. 6A). This phenotype was fully restored in *oprF*-complemented strain H636O. Production of C_4-HSL by H103 increased

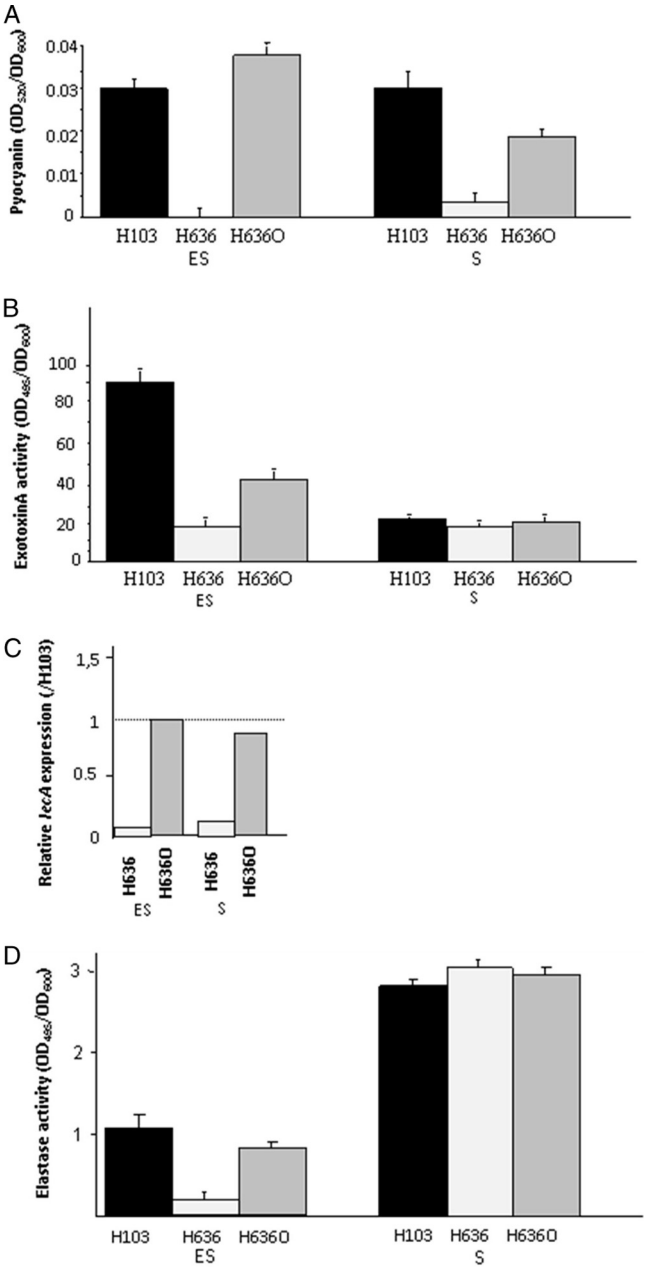

FIG. 5. Altered pyocyanin, exotoxin A, lectin PA-1L, and elastase production in the *oprF* mutant. Relative amounts of pyocyanin (A), exotoxin A (B), and elastase (D) extracted from strain H103, H636, and H636O supernatants after the bacteria were grown in LB medium until they reached entry into stationary phase (ES) and stationary phase (S) are shown. Experiments were repeated three times. (C) The level of *lecA* mRNA encoding the lectin PA-1L in H636 and H636O strains relative to that in H103 was evaluated by quantitative reverse transcription-PCR experiments. Reactions were performed in triplicate with independent bacterial cultures, and the standard deviations were lower than 0.15 C_T unit. PCR was performed on total RNA extracts prior to reverse transcription-PCR to check for the absence of contaminating DNA (data not shown).

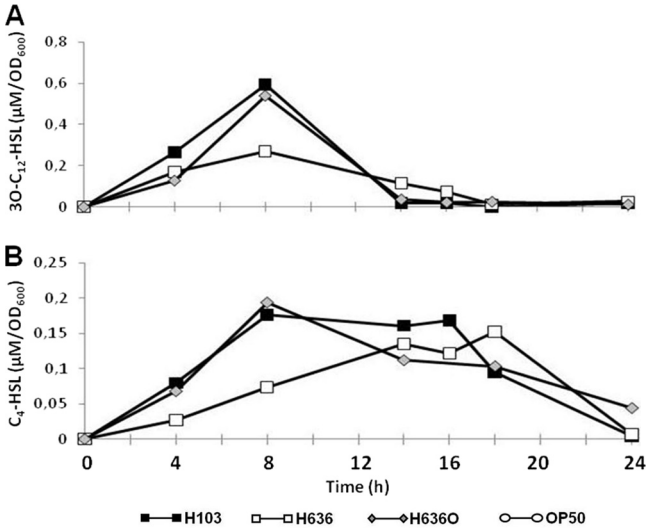

FIG. 6. Altered AHL production by the *oprF* mutant. Time courses of 3O-C_{12}-HSL (A) and C_4-HSL (B) production by the three strains are normalized to the OD_{600}. Standard deviations were lower than 10%. Experiments were repeated at least three times. Growth kinetics are indicated in Fig. 1.

throughout the exponential phase, reaching its maximal level at between 8 and 16 h of growth (Fig. 6B). The production of C_4-HSL increased more slowly for H636 than for H103 and H636O, reaching its maximum level after 18 h of growth (Fig. 6B). However, the maximal levels detected were similar for the tested strains (Fig. 6B). Production of HHQ and PQS was then followed in the supernatants of the three strains. The bacterial growth was monitored by the Bradford protein assay to verify that the insertion of a *pqsH-lacZ* fusion does not deeply affect growth: the *oprF* mutant growth curve was significantly shifted compared to that of H103 during the exponential growth phase; however, this difference disappeared during the stationary growth phase (Fig. 7A). Interestingly, we found that HHQ accumulated to very high concentrations in H636 cultures compared to the concentrations in H103 and H636O cultures (Fig. 7B), while production of PQS was only slightly delayed in the *oprF* mutant (Fig. 7C). Since HHQ is converted into PQS by the action of the PqsH mono-oxygenase and is therefore known to accumulate in a *pqsH* mutant background (24), we analyzed the expression of *pqsH* in the same cultures. A chromosomal *pqsH-lacZ* reporter showed that the transcription of this gene is not deeply altered in H636 with regard to the bacterial growth (Fig. 7D).

Alteration of OM hydrophobic properties due to lack of OprF. Since the loss of OprF has been described to cause changes in the overall OM structure, especially its hydrophobic properties (31, 50, 57, 68), we analyzed the sensitivity of the *oprF* mutant to three hydrophobic antibiotics. MIC determination revealed a higher susceptibility of H636 to novobiocin (128 versus 512 $\mu g \cdot ml^{-1}$ for H103), but the susceptibilities to erythromycin and fusidic acid were similar. Lysozyme targets the peptidoglycan layer but is normally unable to cross the OM barrier. Addition of aminoglycoside antibiotics disrupts the OM sufficiently to allow the lysozyme molecules access to the cell. As can be seen from Fig. 8, in the presence of 10 μg gentamicin ml^{-1}, *oprF* mutant H636 was more susceptible to lysozyme lysis than both wild-type strain H103 and *oprF*-complemented mutant strain H636O, suggesting that the OM of *oprF* mutant strain H636 was altered. Treatment of cultures

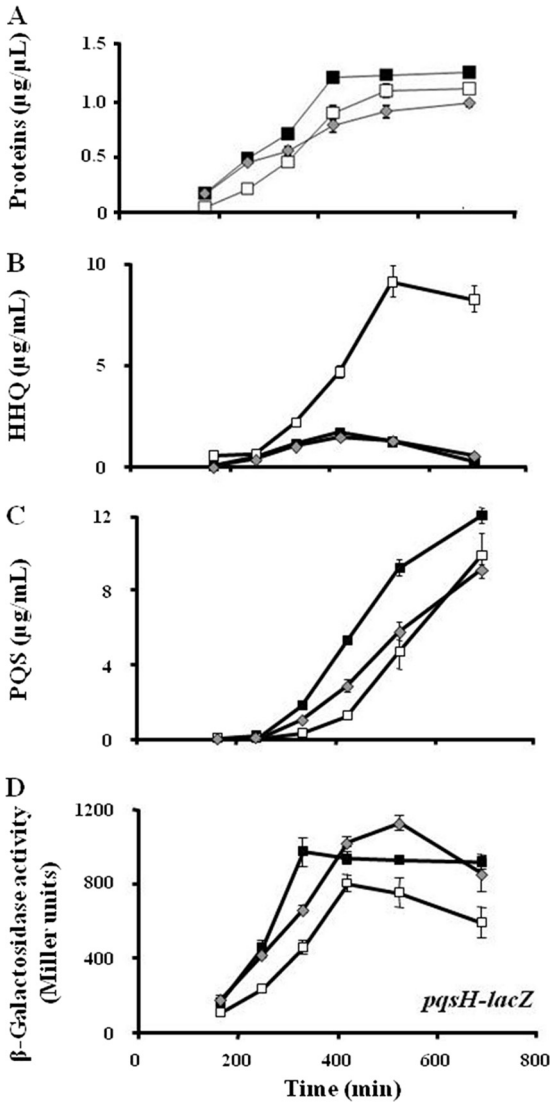

FIG. 7. Lack of OprF disturbs the PQS system. H103 (black squares), H636 (white squares), and H6360 (gray diamonds) were transformed by a chromosomal *pqsH-lacZ* fusion insertion. (A) Growth of the three strains containing this insertion; (B and C) kinetics of HHQ (B) and PQS (C) production in the three strains; (D) expression of *pqsH* through the β-galactosidase activity assay of the transcriptional fusion *pqsH-lacZ*. The values are expressed as the means ± standard errors of the means. Experiments have been repeated at least three times.

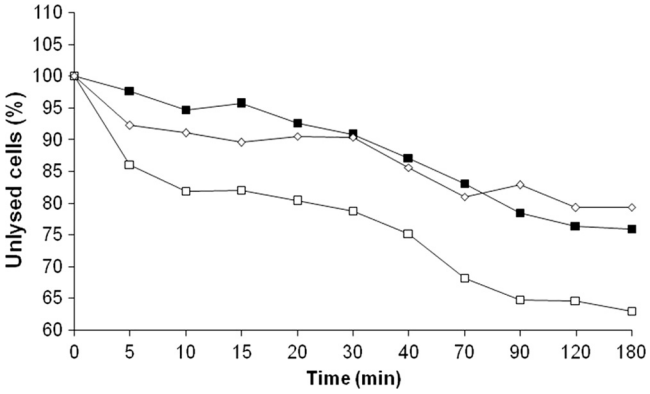

FIG. 8. Lack of OprF alters OM physicochemical properties. Gentamicin-assisted lysozyme lysis of strains H103 (wild type; black squares), H636 (*oprF* mutant; white squares), and H636O (OprF-complemented mutant; gray diamonds) is shown. Results are the means of three independent experiments using 10 μg gentamicin ml^{-1} and 50 μg lysozyme ml^{-1}, and the standard deviations were lower than 15%. Strains were grown in LB medium, and lysis was carried out in 5 mM HEPES.

with 10 μg gentamicin ml^{-1} in the absence of lysozyme caused minimal cell lysis, and no lysis was seen in cells treated with lysozyme alone (data not shown).

DISCUSSION

Since the mechanisms involved in *P. aeruginosa* virulence are numerous and complex, several infection models have been used to study host-pathogen interactions and the role of many factors in the bacterial virulence, contributing to a broader understanding of *P. aeruginosa* pathogenesis. In this study, we demonstrate the major contribution of the porin OprF to the bacterial pathogenicity, using four host models: two complete metazoans, namely, the plant *Cichorium intybus* and the nematode *C. elegans*, and two cellular models, namely, primary cultures of rat glial cells and human Caco-2/TC7 adenocarcinoma cells. To date, very few porins have been shown to be involved in bacterial virulence. This is the case for the OprF homologue OmpA, present in the *Enterobacteriaceae* family and some other *Gammaproteobacteria* class members, which contributes to interactions of *Acinetobacter baumannii* with eukaryotic cells (29), to astrocyte colonization by *E. coli* (71), and to invasion of human brain microvascular endothelial cells by *Cronobacter sakazakii* (48). In *P. aeruginosa*, a role of OM proteins in virulence recently emerged, since the OM protein OprG, an anaerobically induced porin, was shown to contribute to early-stage infections of human bronchial epithelial cells (45). To understand the mechanisms linking OprF and virulence, we hypothesized that OprF is involved in cellular adhesion and/or production of virulence-related factors. Using two cellular models, we show here that the decreased toxicity of the *oprF* mutant is at least partially due to its lowered ability to adhere to cells. This confirms the role of OprF as a cellular adhesin, as previously described using lung epithelial cells (2), fibronectin (58), and wheat root cells, in the case of *Pseudomonas fluorescens* OE28.3 (20). Furthermore, the reduction of the *oprF* mutant toxicity is unlikely ascribed only to a lessened affinity to target cells, since a strong decrease was also observed (i) in the production of the exoproducts, namely, pyocyanin, lectin PA-1L, exotoxin A, and elastase, which have cytotoxic properties contributing to tissue damage and to the pathogenesis of *P. aeruginosa* (36, 41, 61, 77), and (ii) in the activity of T3SS. We show that production, but likely not secretion, of toxins ExoT and ExoS by T3SS is impaired in the absence of OprF, suggesting that the native *exsA* gene is downregulated or that ExsA is kept inactive in the *oprF* mutant. It is, furthermore, well-known that a large part of T3SS regulation occurs downstream of ExsA transcriptional activation through complex posttranscriptional regulatory circuits involving small regulatory RNA (for a review, see references 11 and 76), suggest-

ing that at least a part of these regulatory circuits may remain disturbed in the *oprF* mutant even after ExsA induction.

Pyocyanin, lectin PA-1L, exotoxin A, and elastase are clearly under the control of the QS network. On the other hand, how QS affects T3SS is still unclear at the moment, as this system seems to be regulated by the *rhl* system (7, 35), by the major Gac/RsmA sensor system (62), by HAQs (60), and by other not yet characterized signals derived from tryptophan catabolism (59). Here, we show that 3O-C_{12}-HSL is present in the *oprF* mutant culture supernatants at lower concentrations than in the wild-type strain culture supernatants. Not surprisingly, since the *las* system upregulates the initial activation of the *rhl* system, the accumulation of C_4-HSL is slower in the *oprF* mutant H636 culture supernatant. Although a similar concentration of the latter signal is reached in the late stationary phase, out of all the QS-dependent virulence factors studied, only elastase is eventually produced at wild-type concentrations. Only the reduction in the level of 3O-C_{12}-HSL and the resulting delayed induction of the *rhl* system, seen from the delayed accumulation of C_4-HSL, cannot completely account for the impaired production of these factors. In a *lasR* mutant, which is severely defective in 3O-C_{12}-HSL production, the activity of the *rhl* system is only delayed, allowing the expression of *rhl*-dependent determinants, including pyocyanin and lectin PA-1L (19). An additional explanation for the phenotypes displayed by the *oprF* mutant might come from the interesting observation that, in this strain, production of HHQ and PQS signals, involved in a third level of QS in *P. aeruginosa*, is imbalanced. HHQ accumulates to high levels in the cultures of the *oprF* mutant, while PQS production is delayed. Such accumulation of HHQ has seldom been reported but has notably been observed in a *lasR* mutant (22). However, transcription of *pqsH* was not deeply modified in the *oprF* mutant, suggesting that posttranscriptional modifications might occur or that PqsH mono-oxygenase activity could be altered. This imbalance in HHQ and PQS in the *oprF* mutant cultures can have a number of effects on the bacteria. Hence, while PQS is associated with formation of microvesicles, which act as vehicles for QS signals (42), HHQ cannot perform this function (44). On the other hand, HHQ also acts as a signal and was proposed to perform an essential role in a two-way conversional cell-cell communication system in *P. aeruginosa* (22).

Whether OprF plays a direct or indirect role in the observed phenotypes is not a trivial question. A possibility is that the lack of OprF can change the cell surface and the OM composition, inducing a cell wall stress signal, which in turn could be responsible for the observed phenotypes. In favor of this hypothesis are the numerous reports indicating a modification of the *oprF* mutant against various classes of antibiotics. In *P. aeruginosa* PA14, loss of OprF induces susceptibility to a very broad spectrum of antimicrobials, such as carbapenem (ertapenem), cephalosporins (cefotaxime), aminoglycosides (levofloxacin), tetracyclines (tigecycline) (25), and also the fluoroquinolone ciprofloxacin (10). In their study, Woodruff and Hancock (68) showed that *oprF* mutant strain H636 was slightly more resistant to several β-lactam antibiotics, suggesting that the loss of OprF resulted in a modification of the OM structure, thus enhancing antibiotic uptake via nonporin pathways and counteracting the effects of the loss of OprF. Since the OM protein pattern of H636 was not strongly altered, the OM lipidic composition could be modified (68), in particular, with increased amounts of lipopolysaccharide (LPS) and phospholipids (31). Accordingly, the *oprF* mutant showed (i) increased permeability for the hydrophobic fluorophore *N*-phenylnaphthylamine (NPN), which is largely excluded by the wild-type cells (1, 68), and for the highly hydrophobic steroids (55) and (ii) showed increased susceptibility to the hydrophobic antibiotic novobiocin. Previous studies reported that an *oprF* mutant could not grow in low-salt medium and that these cells had substantially altered morphology (12, 57, 69), indicating the importance of OprF in stabilizing the OM of *P. aeruginosa.* Accordingly, we observed a higher susceptibility of the *oprF* mutant to lysozyme through the gentamicin-assisted lysozyme lysis, suggesting that the OM is more fragile in the absence of OprF. Likewise, we observed that H636 had difficulties growing under other laboratory conditions (minimal medium, poor oxygenation; our unpublished results) and in the chicory *in vivo* environment, suggesting that in this case, the reduced virulence of H636 was due, at least partially, to a growth defect. However, at a similar cellular density, the *oprF* mutant did not provoke the same necrosis that the wild-type strain did, suggesting that growth alteration was not the only cause for reduced virulence. Furthermore, we did not observe drastic growth or viability alterations of the *oprF* mutant under a majority of conditions tested in this study. Likewise, OprF deficiency did not affect the *in vivo* growth of an OprF-deficient strain in a mouse chamber model (57). This was also the case for the *P. aeruginosa* PA14 *oprF* mutant strain, the growth of which was not altered in LB medium (25).

Recently, examples of interplay between membrane properties and QS signaling have been described. Alterations in membrane fluidity are known to contribute to protection against antimicrobial peptides (51). Subinhibitory concentrations of colistin, a cationic antimicrobial interacting with the LPS, result in the upregulation of the HHQ and PQS biosynthesis genes (16). Accordingly, it has been reported that high inocula of *P. aeruginosa* attenuate colistin bactericidal activity, suggesting that bacteria were releasing signal molecules stimulating a phenotypic change that inhibits killing (13). PQS but not HHQ interacts strongly with the acyl chains and 4′-phosphate of bacterial LPS, whereby it maintains the LPS in a well-ordered state and potentially stimulates formation of OM vesicles (43). Since these vesicles have been associated with OM damage (65), it should be of interest to study the production of such vesicles in the *oprF* mutant. Furthermore, it has also been suggested that alterations in membrane fluidity can act as a trigger for an adaptative cellular response. An *lptA* mutant, altered in the fatty acid profile of the phospholipids, showed decreased membrane fluidity. This resulted in premature production of the QS signals C_4-HSL and C_6-HSL and a repression of PQS synthesis at later growth phases, supporting the concept that alterations in membrane properties can act as a trigger for stress-related gene expression (3).

The alternative or additional possibility that the absence of OprF impairs the observed phenotypes by means other than OM disorganization cannot be disregarded, considering the major role of OprF in binding gamma interferon and activation of the QS network (72). Wu and collaborators have indeed shown that OprF is the target of gamma interferon and that this interaction results in production of lectin PA-1L and pyo-

cyanin through activation of the QS system (72). These results suggested that OprF is a host immune system sensor, modulating QS to enhance virulence when the bacteria are in contact with the host. Very interestingly, sera from OprF/I fusion protein-vaccinated humans inhibited *P. aeruginosa* binding to gamma interferon, suggesting a novel mechanism in which blocking of gamma interferon binding abrogates the virulence enhancement of *P. aeruginosa*, thereby contributing to efficient host defense against *P. aeruginosa* infection (24). In our experiments, no gamma interferon was present and the absence of OprF still reduced the QS network activity, indicating that OprF might sense signals other than gamma interferon. In this hypothesis, OprF functions in *P. aeruginosa* virulence may be linked to its ability to transmit or to transduce unknown signals through the OM, which could be detected by a sensor(s) of the cytoplasmic membrane, leading to QS modulation, T3SS activation, and production of virulence factors.

Finally, the attenuation in virulence functions of the *oprF* mutant through a direct (signaling) and/or indirect (membrane alteration) effect leads to the major conclusion that OprF is a major therapeutic target. Since the absence of OprF leads to attenuated bacterial virulence, understanding the mechanisms through which *oprF* expression is regulated will now constitute an important challenge.

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