Journal of Bacteriology

# Transcription of the *oprF* Gene of Pseudomonas aeruginosa Is Dependent Mainly on the SigX Sigma Factor and Is Sucrose Induced

**Emeline Bouffartigues, Gwendoline Gicquel, Alexis Bazire, Manjeet Bains, Olivier Maillot, Julien Vieillard, Marc G. J. Feuilloley, Nicole Orange, R. E. W. Hancock, Alain Dufour and Sylvie Chevalier**

***J. Bacteriol.*** **2012, 194(16):4301. DOI: 10.1128/JB.00509-12. Published Ahead of Print 8 June 2012.**

JB Journals.ASM.org

# Transcription of the *oprF* Gene of *Pseudomonas aeruginosa* Is Dependent Mainly on the SigX Sigma Factor and Is Sucrose Induced

**Emeline Bouffartigues,[a] Gwendoline Gicquel,[a] Alexis Bazire,[b] Manjeet Bains,[c] Olivier Maillot,[a] Julien Vieillard,[d] Marc G. J. Feuilloley,[a] Nicole Orange,[a] R. E. W. Hancock,[c] Alain Dufour,[b] and Sylvie Chevalier[a]**

Laboratoire de Microbiologie-Signaux et Micro-Environnement (LMSM), Université de Rouen, Rouen, France[a]; Laboratoire de Biotechnologie et Chimie Marines (LBCM), Université de Bretagne Sud (UEB), IUEM, Lorient, France[b]; R. E. W. Hancock Laboratory, Department of Microbiology and Immunology, University of British Columbia, Vancouver, British Columbia, Canada[c]; and UMR CNRS 6014 COBRA, Université de Rouen, Rouen, France[d]

**The OprF porin is the major outer membrane protein of *Pseudomonas aeruginosa*. OprF is involved in several crucial functions, including cell structure, outer membrane permeability, environmental sensing, and virulence. The *oprF* gene is preceded by the *sigX* gene, which encodes the poorly studied extracytoplasmic function (ECF) sigma factor SigX. Three *oprF* promoters were previously identified. Two intertwined promoters dependent on $\sigma^{70}$ and SigX are located in the *sigX-oprF* intergenic region, whereas a promoter dependent on the ECF AlgU lies within the *sigX* gene. An additional promoter was found in the *cmpX-sigX* intergenic region. In this study, we dissected the contribution of each promoter region and of each sigma factor to *oprF* transcription using transcriptional fusions. In Luria-Bertani (LB) medium, the *oprF*-proximal region (*sigX-oprF* intergenic region) accounted for about 80% of the *oprF* transcription, whereas the AlgU-dependent promoter had marginal activity. Using the *sigX* mutant PAOSX, we observed that the SigX-dependent promoter was largely predominant over the $\sigma^{70}$-dependent promoter. *oprF* transcription was increased in response to low NaCl or high sucrose concentrations, and this induced transcription was strongly impaired in the absence of SigX. The lack of OprF itself increased *oprF* transcription. Since these conditions led to cell wall alterations, *oprF* transcription could be activated by signals triggered by perturbation of the cell envelope.**

*Pseudomonas aeruginosa* is a ubiquitous Gram-negative bacterium capable of surviving in a broad range of natural environments, although it is best known as an human pathogen associated with hospital-acquired infections in immunocompromised patients and as a leading cause of death in cystic fibrosis patients (8, 56).

The outer membrane (OM) of *P. aeruginosa* is directly exposed to the environment, and OM components are involved in the adaptation process of the bacterium. OprF is the most abundant OM protein of bacteria belonging to the *Pseudomonas* genus (20). First described as a structural protein involved in bacterial morphology by anchoring the outer membrane to the peptidoglycan layer (44), OprF is also a hydrophilic channel-forming protein contributing to OM permeability. Since no specific substrate has been discovered to date, OprF was proposed to allow the nonspecific diffusion of ions and low-molecular-mass sugars (5, 37), or even antibiotics (31), with a cutoff size of 1,519 Da (37). Its functions in host-pathogen interactions started to be deciphered within the last decade. OprF is involved in adhesion to eukaryotic cells (3) and in biofilm formation under anaerobic conditions, possibly through nitrate or nitrite diffusion (47, 56). OprF binds gamma interferon, which leads to the stimulation of the quorum sensing (QS) network and consequently to the production of two bacterial virulence factors, the lectin PA1 and the phenazine pyocyanin (55). OprF has therefore been suggested to be a bacterial sensor, involved in the perception of the host immune state (55) and, more generally, of environmental modifications (7, 29, 48). The importance of OprF in *P. aeruginosa* pathogenicity was further highlighted by our recent data, which showed that the lack of OprF resulted in impaired production of a number of virulence factors (22).

Although OprF is involved in the important functions mentioned above, the understanding of its gene expression regulation remains fragmentary. A first transcriptional study suggested that *oprF* was constitutively transcribed as a monocistronic unit from a $\sigma^{70}$-type promoter (20). However, a more complex view emerged from subsequent work by Brinkman et al. (9), who confirmed that the predominant *oprF* mRNA is monocistronic but observed other, larger *oprF* transcripts, including the upstream *sigX* and *oprF* genes. These authors mapped two intertwined promoters just upstream of *oprF* (within the *sigX-oprF* intergenic region): the $\sigma^{70}$-type promoter previously identified by Duchêne et al. (20) and a new promoter depending on the extracytoplasmic function (ECF) sigma factor SigX encoded by the *sigX* gene lying just upstream of *oprF* (Fig. 1A). This led to the notion that *oprF* is not simply constitutively expressed. In addition, a search for promoters dependent on the ECF factor AlgU led to the identification of a third *oprF* promoter lying within the *sigX* coding sequence (21) (Fig. 1A). The fact that ECF sigma factors are activated in response to specific extracellular signals (28, 38) is consistent with the involvement of OprF in adaptation to environments. AlgU (also known as AlgT, RpoE, $\sigma^{E}$, and $\sigma^{22}$) was widely studied because its hyperactivity leads to overproduction of the exopolysaccharide alginate, inducing the mucoid phenotype responsible for chronic infection of the lungs of cystic fibrosis (CF) patients (24, 43). Mucoid strains result in most cases from spontaneous mutations in the *mucA* gene, which encodes the anti-sigma factor specific for

**Received** 31 March 2012 **Accepted** 2 June 2012
**Published ahead of print** 8 June 2012
Address correspondence to Sylvie Chevalier, sylvie.chevalier@univ-rouen.fr.
Supplemental material for this article may be found at http://jb.asm.org/.

doi:10.1128/JB.00509-12

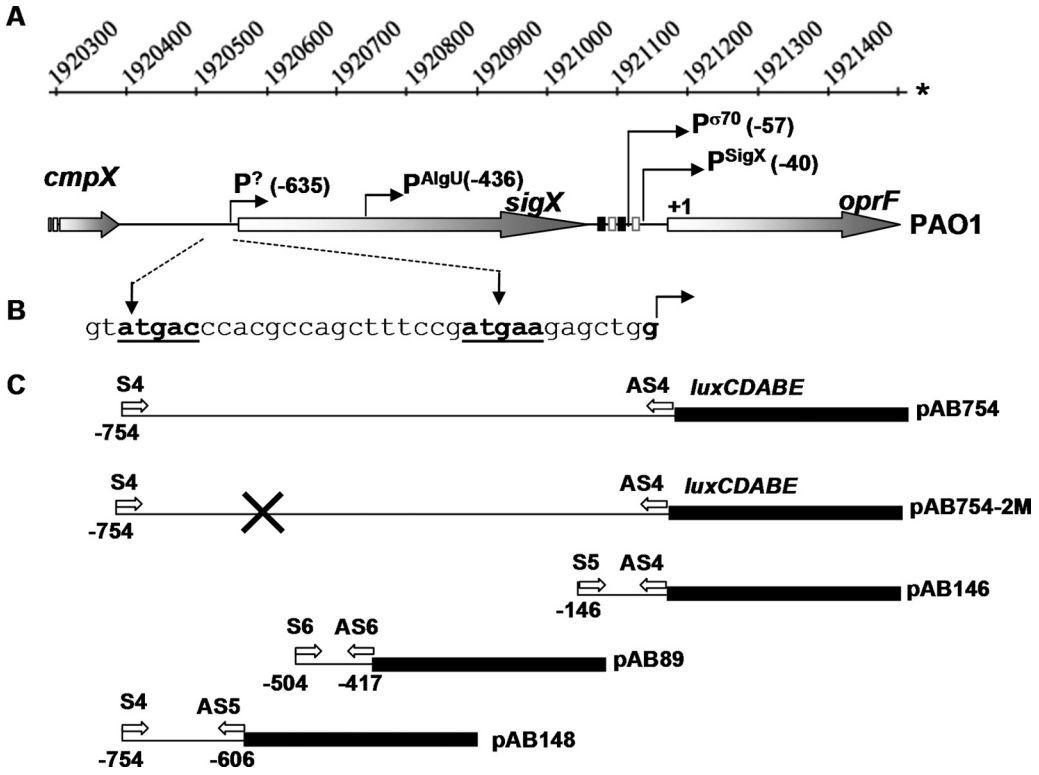


**FIG 1** Schematic representation of the promoter regions of *oprF* (A and B) and of transcriptional fusions (C). (A) The genomic positions are indicated on the top of the genetic map (*) (see reference 49 and http://www.pseudomonas.com). The transcriptional initiation sites of the four promoters lying upstream of the *oprF* gene ($P^{SigX}$, $P^{\sigma 70}$, $P^{AlgU}$, and $P^{?}$, corresponding to SigX-, σ70-, and AlgU-dependent promoters and *sigX*'s own promoter, respectively) are indicated by arrows. Their positions are indicated relative to the translational initiation start of *oprF* in brackets. Squares indicate the −10 and −35 sequences of the two overlapping promoters $P^{SigX}$ and $P^{\sigma 70}$. (B) Identification of a putative transcriptional start site (in bold, black arrow) for *sigX* in the *cmpX-sigX* intergenic sequence. The deduced −10 and −35 boxes are underlined and in bold. (C) Black bars represent the beginning of the *luxCDABE* reporter cassette of the promoterless pAB133 vector. The position of the promoter region studied is indicated relative to the translational initiation start of *oprF*. The primers used for PCR amplifications are represented by short white arrows. The black cross indicates the *sigX* mutation in pAB754-2 M. The names of the resulting plasmids are indicated on the right.

AlgU (15, 24, 43). Alternatively, AlgU activity can be induced by a regulated proteolysis pathway (12, 17, 40, 41, 50, 51). AlgU was shown to be involved in response to various stresses (such as oxidative, heat, and hyperosmotic stresses) and to cell wall-inhibitory antibiotics (2, 38, 51). We recently reported that AlgU is necessary for the formation of robust biofilms by the nonmucoid *P. aeruginosa* PAO1 strain (5). In contrast, the ECF factor SigX has not yet been the focus of detailed studies in *Pseudomonas* (38). SigX shares 49% similarity with the ECF factors SigX, SigV, and SigW of *Bacillus subtilis*, which are induced by alkaline and salt shock and by antibiotics that affect cell wall biosynthesis (10, 11, 30, 45). In *B. subtilis*, SigX has a major role in transcribing the gene cluster for sublancin (32). In *P. aeruginosa*, disruption of *sigX* not only reduced the OprF amount in the bacterial outer membrane but also led to OprF-independent phenotypes, including reduced growth rates, secretion of an unidentified pigment, and increased sensitivity to the antibiotic imipenem (9).

In this study, we dissected the relative roles of the three previously reported promoters and of the newly identified *sigX* promoter in *oprF* transcription. Since OprF has been shown to be necessary for *P. aeruginosa* growth in low-osmolarity media (9), and since osmolarity variations often occur in many environments (27), we examined the effects of NaCl and sucrose on the promoter activities.

## MATERIALS AND METHODS

**Bacterial strains and culture conditions.** The strains used are listed in Table 1. Bacteria were grown at 37°C on a rotary shaker (180 rpm) in Luria-Bertani (LB) broth containing 171 mM (10 g $l^{-1}$) NaCl (normal LB). We also used the following LB-derived media: LB0, NaCl-free LB; LB342S, LB0 supplemented with 342 mM sucrose; and LBPEG, LB0 supplemented with 1.4% polyethylene glycol 8000 (PEG 8000). Cultures were inoculated at an initial optical density at 580 nm ($OD_{580}$) of 0.08. When required (for plasmid maintenance), *Escherichia coli* strains were grown in the presence of ampicillin (Ap; 100 μg $ml^{-1}$), gentamicin (Gm; 15 μg $ml^{-1}$) or tetracycline (Tc; 10 μg $ml^{-1}$). *P. aeruginosa* strains were grown in LB liquid cultures in the presence of carbenicillin (Cb; 300 μg $ml^{-1}$), Gm (30 μg $ml^{-1}$), or Tc (250 μg $ml^{-1}$) or on LB agar (1.5%) plates containing Cb (600 μg $ml^{-1}$), Gm (100 μg $ml^{-1}$), or Tc (250 μg $ml^{-1}$).

**General DNA procedures.** Restriction enzymes, T4 DNA ligase, and alkaline phosphatase were purchased from New England BioLabs (Ipswich, MA) and used according to the manufacturer's instructions. PCR assays were carried out with 1 μg of *P. aeruginosa* strain H103 chromosomal DNA, 20 pmol of each primer, and *Taq* DNA polymerase (Roche Molecular Biochemicals). When necessary, PCR products and plasmids were purified with the QIAquick and QIAprep Spin Miniprep kits (Qiagen), respectively. *E. coli* and *P. aeruginosa* were transformed by electroporation (Gene Transformer GTF100; Savant) as described by Dower et al. (19) and Choi et al. (14), respectively. When indicated, *P. aeruginosa* was transformed by conjugation (13). Briefly, a drop of an exponential-phase culture of the donor *E. coli* S17.1 (carrying the plasmid to transfer)

**TABLE 1** Bacterial strains and plasmids used in this study

| Strain or plasmid | Characteristics | Source or reference |
|---|---|---|
| *P. aeruginosa* PAO1 strains | | |
| H103 | Wild-type strain H103; prototroph derivative of PAO1 | 26 |
| H636 | PAO1 H103 *oprF*::Ω | 52 |
| PAOSX | PAO1 H103 *sigX*::*lox* | This study |
| PAOSX+ | PAOSX containing pAB754 | This study |
| PAOU | PAO1 Δ*algU*::*lox* | 5 |
| MUC-N1 | Mucoid clinical isolate, *mucA* mutant | J. Caillon, Nantes |
| *E. coli* strains | | |
| JM109 | Competent cells used as cloning host | Promega |
| S17.1 | *recA pro* (RP4-2Tet::Mu Kan::Tn*7*), donor and helper strain for conjugation | 46 |
| Plasmids | | |
| pGEM-T Easy | PCR cloning vector | Promega |
| pEX100Tlink | Ap$^r$ *sacB*; pUC19-based gene replacement vector with MCS[a] | 42 |
| pUCGm*lox* | Ap$^r$ Gm$^r$ ; pUC18-based gene replacement vector with MCS | 42 |
| pCM157 | Tc$^r$; *cre* expression vector | 35 |
| pEXΔSGm | pEX100Tlink containing 5′ and 3′ flanking sequences of *sigX*::Gm*lox* | This study |
| pAB133 | Gm$^r$; pBBR1MCS-5-based cloning vector containing the promoterless *luxCDABE* operon | 4 |
| pAB754 | Gm$^r$; pAB133-based plasmid with the 754-bp *oprF* upstream fragment at the SacI and SpeI sites | This study |
| pAB7542 M | Gm$^r$; pAB754-based plasmid containing an inactive copy of *sigX* | This study |
| pAB146 | Gm$^r$; pAB133-based plasmid containing the proximal promoter region of *oprF* at the SacI and SpeI sites | This study |
| pAB148 | Gm$^r$; pAB133-based plasmid containing the promoter region of *sigX* at the SacI and SpeI sites | This study |
| pAB89 | Gm$^r$; pAB133 derivative plasmid containing the *oprF* promoter; AlgU dependent | This study |

[a] MCS, multiple cloning site.

and a drop of the *P. aeruginosa* recipient strain taken from the stationary growth phase were mixed on a solidified LB0 agar plate and incubated overnight at 37°C. The next day, the bacteria were harvested and resuspended into 1 ml of liquid LB medium. To select for *Pseudomonas* transconjugants, 100 μl of this suspension was plated onto *Pseudomonas* isolation agar (Difco, France) containing the appropriate antibiotic selection.

**Extraction of total RNA from *P. aeruginosa*.** Total RNA was prepared by the hot acid-phenol method. Briefly, cells were lysed and RNA was extracted three times with an equal volume of acidic hot phenol and once with chloroform. RNA was ethanol precipitated, air dried, and dissolved in water. Contaminating DNA was removed from total RNA by using 10 U of RNase-free DnaseI (New England BioLabs) in a 50-μl mixture containing 6.25 mM $MgCl_2$ and approximately 3 μg/μl of total RNA. The reaction mixture was incubated 30 min at 37°C, and the DNase I was then inactivated by adding 1 μl of 0.5 M EDTA to the mixture and incubating the mixture for 10 min at 65°C. The concentration was determined by measuring the absorbance at 260 nm. The quality of the RNA was then checked on a 2% agarose gel prior to use.

**Mapping of *sigX* transcriptional start site.** The 5′ end of *sigX* mRNA was mapped using the 5′ RACE (rapid amplification of cDNA ends) procedure (3′/5′ RACE 2nd Generation kit; Roche Molecular Biochemicals) according to the manufacturer's instructions. Briefly, cDNA was produced from 1 μg of total RNA using primer AS1 (Table 2) and further purified using the High Pure PCR product purification kit (Roche). Nested PCR was further achieved using the *sigX*-specific AS2 and AS3 primers (Table 2) and oligo(dT) primer. Finally, the PCR product was cloned into pGEM-T Easy vector according to the manufacturer's instructions (Promega) and sequenced to identify the 5′ end of the *sigX* mRNA (Beckman Coulter Genomics, Villepinte, France).

**Construction of transcriptional fusions.** To monitor *oprF* transcription, *oprF* promoters were fused to the promoterless *luxCDABE* cassette in the replicative low-copy-number pAB133 vector (Gm$^r$) (3). The promoter regions of interest and the positions of the primers, relative to the *oprF* and *sigX* genes, which were used for PCR amplification are presented in Fig. 1. The larger *oprF* promoter region was PCR amplified with the S4 and AS4 primers (Table 2). The SacI-SpeI-digested PCR product was inserted into pAB133, yielding pAB754. The insert was verified by DNA sequencing (Beckman Coulter Genomics). Since this construct contained a functional *sigX* gene, the two putative translational start codons, located at positions −606 and −582 relative to the *oprF* translational starting site (see reference 49 and http://www.pseudomonas.com), were modified by site-directed mutagenesis (Quick Change II site-directed mutagenesis kit; Stratagene), using primers S7 and AS7 (Table 2). This construct, pAB754-2 M, was sequenced on both strands to confirm the two mutations (Beckman Coulter Genomics). The *oprF*-proximal, *sigX*-proximal, and AlgU-dependent promoter regions were PCR amplified using the primer pairs S5-AS4, S4-AS5, and S6-AS6 (Table 2), respectively. The SacI-SpeI-digested PCR products were then inserted into pAB133, yielding pAB146, pAB148, and pAB89, respectively. The inserts were verified by DNA sequencing (Beckman Coulter Genomics).

**Bioluminescence assays.** *P. aeruginosa* strains containing pAB133-derived plasmids were grown in covered, white 96-well OptiPlates with a flat transparent bottom (BD Falcon, San Jose, CA). Bioluminescence and absorbance were simultaneously measured throughout bacterial growth using a multimode plate reader (Flex-Xenius XM; SAFAS). The bioluminescence values (in relative light units [RLU] · 0.5 $s^{-1}$) were divided by the absorbance values at 580 nm, yielding to relative bioluminescence values (in RLU · 0.5 $s^{-1}$ · $A_{580}{}^{-1}$). The relative luminescence values of the negative-control strain *P. aeruginosa* harboring the empty vector pAB133 were subtracted from those of the studied *oprF* promoter region containing *P. aeruginosa*, as previously described (3). Each set of experiments was performed at least three times.

**Construction of the *sigX* mutant.** The *sigX* mutant was constructed by following the procedure described by Quénée et al. (42). Briefly, the *sigX*-flanking regions were PCR amplified using the primer pairs Sig1-Sig2 and Sig3-Sig4 (Table 2). After XbaI digestions, the two fragments were coligated, generating a 57-bp deletion within the sequence containing *sigX*. This fragment was cloned into the SacI-HindIII sites of the suicide vector pEX100Tlink, resulting in plasmid pEXΔS. The *lox-aacC1-lox*

**TABLE 2** Primers used in this study

| Primer | Sequence (5′–3′)[a] | Reference |
|---|---|---|
| Mapping of *sigX* transcriptional start site | | |
| AS1 | CGAAGCGAAGAACCAGAATC | This study |
| AS2 | TATCGGATTGACATGGACGA | This study |
| AS3 | ATCACCTCCTGGCACACG | This study |
| Construction of the transcriptional fusions | | |
| S4 | ttaatcgagctcGACGTGGCTGCTCTGCAGG | This study |
| AS4 | taataaactagtCCGTTAAATCCCCATCTTGATGGT | This study |
| S5 | ttaatcgagctcGCCTGCGCGAAAAGTTTTCA | This study |
| AS5 | taataaactagtGTGGAACAGCTCCGAGTGCG | This study |
| S6 | ttaatcgagctcCAGGAGGTGATGCTGAAAGTTTTGT | This study |
| AS6 | taataaactagtTTGTACGTGATGCTATATAGCCACG | This study |
| Site-directed mutagenesis | | |
| S7 | GCTGTTCCACtaGACGCGCGCCTATGAAGAATTGtaGCGGCGTTA | This study |
| AS7 | TAACGCCGCtaCAATTCTTCATAGGCGCGCGTCtaGTGGAACAGC | This study |
| Construction of the *sigX* mutant | | |
| Sig1 | taataagagctcCAGAGCTTGCTGACCGCGATG | This study |
| Sig2 | taataatctagaAGGTAGCGGGCACAGACGTTGAA | This study |
| Sig3 | taataatctagaGGGTTGAAGAACTTTGAGGGCAAGT | This study |
| Sig4 | taataaaagcttCGCCCAGGCCAGCCATC | This study |
| Quantification of mRNA levels by qRT-PCR | | |
| OprF1 | aggtctgcgtccgtacgtgt | This study |
| OprF2 | tggtgatgttctggtgagcc | This study |
| AlgDF | ACGTGATCTGCCAGGACCA | This study |
| AlgDR | GACGCATGTAGTAGCGCCAC | This study |
| 16SF | CAGGATTAGATACCCTGGTAGTCCAC | 16 |
| 16SR | GACTTAACCCAACATCTCACGACAC | 16 |

[a] All primers used in this study were synthesized by Eurogentec and are based on PAO1 genome sequence (see reference 49 and http://www.pseudomonas.com). Nucleotides in lowercase are not complementary to the target sequence. Underlined nucleotides indicate restriction endonuclease sites inserted within primer sequences.

cassette encoding Gm resistance was excised from pUCGm*lox* (42) using XbaI and was subcloned into the unique XbaI site of pEXΔS. The resulting pEXΔSGm was then introduced into the *E. coli* donor/helper strain S17.1 and transferred by conjugation into *P. aeruginosa* H103. Gm-resistant colonies grown on *Pseudomonas* isolation agar (PIA) plates were counterselected on 5% sucrose LB agar plates, and the double recombinants were selected for their Gm resistance and Cb sensitivity. Finally, the *aacC1* gene conferring Gm resistance was excised by the Cre recombinase encoded by pCM157 (35). The resulting PAOSX strain was checked by PCR using primers Sig1 and Sig4 (Table 2), and the resulting fragment was sequenced (Beckman Coulter Genomics). To complement the mutation, PAOSX was transformed with plasmid pAB754 containing a functional copy of the *sigX* gene under the control of its own promoter region.

**qRT-PCR experiments.** Quantitative reverse transcription-PCR (qRT-PCR) experiments were conducted as previously described by Guyard-Nicodème et al. (25).

**Viscosity measurement.** The viscosity of the growth media was measured using a classical glass capillary viscometer (diameter, 0.59 mm; Prolabo, France). Briefly, media were loaded into the viscosimeter and the time flow was measured. The viscosity ($\eta$) was deduced according to the equation $\eta = k \times t \times \rho$, where $k = 0.000095$, $t$ represents the time (s) and $\rho$ represents the density (g/cm$^3$).

**Gentamicin-assisted lysozyme lysis assay.** The gentamicin-assisted lysozyme lysis assay was conducted as previously described (1, 33), except that the aminoglycoside used was gentamicin (10 $\mu g \cdot ml^{-1}$) instead of streptomycin and the buffer utilized was sodium HEPES (5 mM, pH 7) instead of sodium phosphate buffer (pH 7) (19).

## RESULTS

**Identification of a *sigX* transcriptional start site.** Three promoters lying in two distinct regions have been described: the $\sigma^{70}$ and SigX-dependent promoters in the *sigX-oprF* intergenic region and the AlgU-dependent promoter within the *sigX* gene (Fig. 1A) (9, 21). Previously, Brinkman et al. (9) showed that the monocistronic *oprF* mRNAs were predominant, but they also detected the minor bicistronic *sigX-oprF* transcript, which indicates the presence of a promoter upstream of *sigX*. Since no promoter of the *sigX* gene was previously identified, we first mapped the *sigX* transcriptional start site. RACE-PCR experiments detected one transcriptional start site, located 30 bp upstream of the first of the two putative *sigX* translational start codons. This start site is located in the intergenic region between *cmpX* and *sigX* (Fig. 1B). From this site, the −35 and −10 putative sequences were identified, and these consisted of ATGAC-N16-ATGAA sequences, which shared approximately 40 and 80% identity with the −10 and −35 $\sigma^{70}$ consensus sequences, respectively (18). To investigate the activities of the promoters responsible for the *sigX-oprF* and *oprF* mRNA production, we constructed 5 transcriptional fusions, namely, pAB754, pAB754-2 M, pAB146, pAB89, and pAB148 (Fig. 1C), as described in Materials and Methods.

**Pattern of *oprF* transcription in the course of growth.** The activity of *oprF* promoters was monitored during growth of *P. aeruginosa* H103 in LB medium. The largest DNA fragment fused

to the *luxCDABE* reporter operon included the promoter region of the *sigX* gene, the AlgU-dependent promoter lying in *sigX*, and the *oprF*-proximal region (*sigX-oprF* intergenic region) with its two promoters (Fig. 1C). This DNA fragment is hereafter referred to as the "large promoter region." To avoid SigX overproduction, the *sigX* gene was inactivated by site-directed mutagenesis of its two putative translational start codons, leading to the pAB754-2 M plasmid (Fig. 1C). The relative luminescence activity resulting from pAB754-2 M increased during the exponential growth phase, reaching a peak toward the end of this phase, and decreased at the entry into and during the stationary phase (Fig. 2A). Since the promoter activity pattern could be biased by the *lux* reporter system (4), we assayed, using qRT-PCR, the *oprF* mRNAs during bacterial growth (Fig. 2B). The pattern obtained by qRT-PCR was similar to that observed by measuring reporter activity. We thus focused on the maximal promoter activity reached in the subsequent experiments. Since *P. aeruginosa* grows more slowly and to lower yields in microtiter wells (maximal $OD_{580}$ below 1), and since the *lux* reporter system requires large amounts of energy and oxygen, we questioned if these growth circumstances might be responsible for the large decrease in promoter activity during the stationary phase. We thus further tested the promoter activity in cells grown in Erlenmeyer flasks (10 ml of culture in 100-ml flasks) under vigorous shaking conditions (180 rpm). The growth was similar under each tested condition (see Fig. S1A and S1B in the supplemental material). As shown in Fig. 2C, results were similar in Erlenmeyer flasks and in microtiter plates, showing that the promoter activity decrease was not due to the specific growth condition imposed by using microtiter plates but, rather, reflected decreased transcription of *oprF* upon entry into stationary phase.

**Most of *oprF* transcription in LB medium arose from the *oprF*-proximal region.** The luminescence activities were then assayed from constructions separately carrying each of the three promoter regions described above, using pAB146, pAB89, and pAB148 (Fig. 1). The activities resulting from these three regions followed time courses similar to that of pAB754-2 M but differed from each other in their levels of expression (Fig. 2A and 3A; Table 3). The activities of pAB146 (*oprF*-proximal region), pAB148 (*cmpX-sigX* intergenic region), and pAB89 (*algU* promoter region) in strain H103 were 79, 11, and 3%, respectively, of the H103 (pAB754-2 M) activity, indicating a major involvement of the *oprF*-proximal region (promoter region in pAB146) in *oprF* transcription under our culture conditions. Since similar results were obtained when bacteria were grown in Erlenmeyer flasks with vigorous shaking (Fig. 3B), we conducted the subsequent experiments in microtiter wells. Bicistronic mRNAs, including the *sigX* and *oprF* genes, have been previously described (9), and it has been concluded that the *sigX* promoter region contributes to *oprF* transcription. This was indeed the case in our experiments, but the activity of this promoter region (pAB148) remained weak compared to that of the proximal promoter region (Fig. 3A and Table 3). The pAB89 activity is expected to result from the AlgU-dependent promoter (Fig. 1) previously identified by Firoved et al. (21). We therefore measured this activity in the *algU* mutant strain PAOU (4). The maximal activity of PAOU(pAB754-2 M) was detected during the exponential growth phase and was only 15% lower than the activity of the equivalent H103(pAB754-2 M) wild-type construct (data not shown). This confirmed that AlgU was only weakly involved in *oprF* transcription in *P. aeruginosa* strain

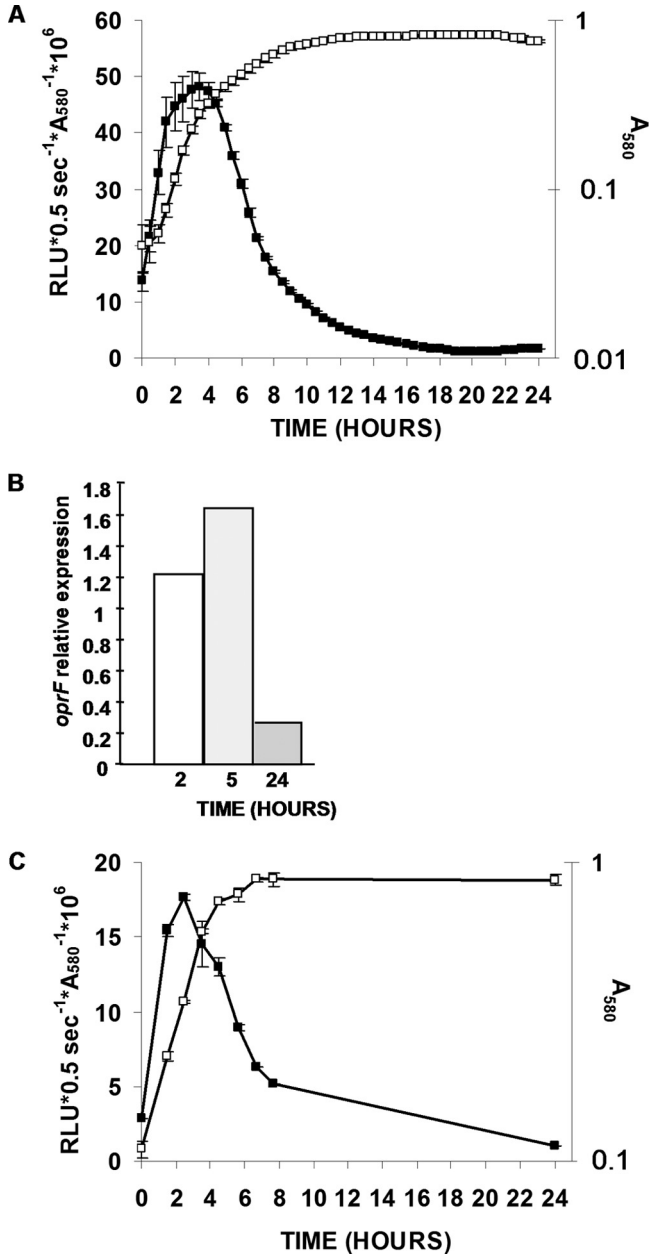


**FIG 2** Relative activity of the large promoter region of *oprF* in *P. aeruginosa* H103 during growth in LB medium. Shown are relative bioluminescence levels generated by pAB754-2 M (filled squares) when *P. aeruginosa* H103 was grown in LB medium in microtiter wells (A) or Erlenmeyer flasks (C). The growth of H103 containing the different plasmids is shown (corresponding open symbols) (A and C). (B) The level of *oprF* mRNA in *P. aeruginosa* relative to the 16sRNA transcript was evaluated in the course of bacterial growth by quantitative reverse transcription-PCR. Reactions were performed in triplicate with independent bacterial cultures, and the standard deviations were lower than 0.15 threshold cycle ($C_T$) unit. PCR was performed on total RNA extracts prior to reverse transcription-PCR to check for the absence of contaminating DNA (data not shown).

H103, consistent with the low contribution of the pAB89 promoter to *oprF* transcription. To clarify the AlgU activity level in *P. aeruginosa* H103, which is a prototroph derivative of PAO1, we compared four strains: the nonmucoid strains H103 and PAO1, PAOU (which is an *algU* mutant of PAO1), and the *P. aeruginosa*

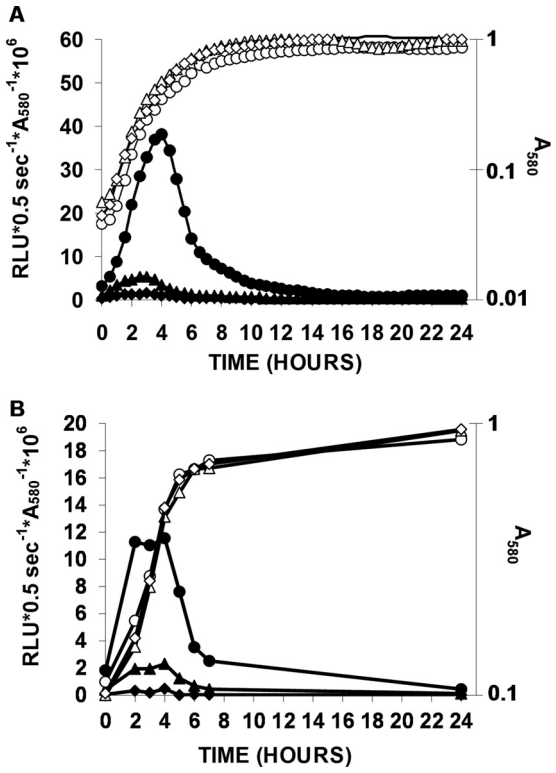


**FIG 3** Relative activity of the promoter regions of *oprF* in *P. aeruginosa* H103 during growth in LB medium. Shown are relative bioluminescence levels generated by pAB146 (SigX- and σ70-dependent proximal promoter region; filled circles), pAB148 (*sigX*'s own promoter region; filled triangles), and pAB89 (AlgU-dependent promoter region; filled diamonds) when H103 was grown in microtiter wells (A) or Erlenmeyer flasks (B). The growth of H103 containing the different plasmids is shown (corresponding open symbols) (A and B). Standard deviations were lower than 10% and 4% for the bioluminescence and growth assays, respectively, in microplates and 17% and 10% in Erlenmeyer flasks. The experiments were repeated at least three times.

clinical mucoid isolate MUC-N1 (which carries a mutation in the *mucA* gene encoding an anti-sigma-factor protein for AlgU). We assayed by qRT-PCR the *algD* mRNA levels, since the transcription of *algD* is AlgU dependent (54). The results are shown in Fig. 4A and led to the conclusion that AlgU activities were similar in H103 and PAO1, strongly increased in the MUC-N1 strain (about 7-fold), and reduced (about 4-fold) in the PAOU strain when grown in LB medium. The *oprF* mRNA levels were, however, similar in all four strains (Fig. 4B), suggesting that the AlgU-dependent promoter upstream of *oprF* was poorly active even in the MUC-N1 strain, which displayed a high AlgU activity level. Alternatively, the AlgU-dependent promoter might be more active, while other promoter regions might be less active. Unfortunately, we could not further examine these hypotheses, since we were unable to transform the MUC-N1 strain with our reporter plasmids despite repeated attempts.

**The transcription of *oprF* in LB medium relied mainly on SigX.** Since the above-described experiments indicated that the largest proportion of *oprF* transcription originates from the *oprF*-proximal region under the growth conditions tested here, our subsequent experiments focused on this region. The proximal region contains two intertwined promoters dependent on $\sigma^{70}$ and SigX (9, 20); therefore, we examined the contribution of SigX to *oprF* transcription. We constructed a stable *sigX* deletion mutant (PAOSX) of *P. aeruginosa* H103, using the *cre-lox* procedure (42). As shown in Fig. 5A, the growth of PAOSX was impaired in LB medium, with a doubling time of 149 min, compared to 58 min for the wild-type H103 strain. The transcriptional fusion pAB754-2 M containing the larger promoter region was then introduced into the mutant PAOSX. The resulting luminescence activity reached a peak during the exponential growth phase (Fig. 5). The maximal relative bioluminescence detected in PAOSX represented 35% of that of H103(pAB754-2 M) (Fig. 5B and Table 3), indicating that SigX is responsible for about two-thirds of *oprF* transcription. Since pAB754 contains the wild-type *sigX* gene in addition to the three promoter regions (Fig. 1A), we used this plasmid both to complement the *sigX* mutant and to assay the effect of this complementation on the promoter activity. The complementation was effective, as judged by the restoration of growth in LB medium (Fig. 5A), with a doubling time of 62 min for PAOSX(pAB754), which was similar to that of the wild-type H103 strain (Fig. 2A). This complementation also restored the activity of the larger promoter region, since the bioluminescence activity of PAOSX-

**TABLE 3** Maximal relative transcriptional activities generated by the *lux* transcriptional fusions in *P. aeruginosa* H103, PAOSX (*sigX* mutant), and H636 (*oprF* mutant) strains cultured in various LB media[a]

| Strain | Plasmid | Maximal relative transcriptional activity ($10^7$ RLU · 0.5 $s^{-1}$ · $A_{580}{}^{-1}$) in medium | | | |
|---|---|---|---|---|---|
| | | LB | LB0 | LB342S | LBPEG |
| H103 | pAB754-2 M | 4.8 ± 0.2 | 5.9 ± 0.4 | 11.1 ± 1 | 4.8 ± 0.3 |
| | pAB146 | 3.8 ± 0.2 | 3.8 ± 0.3 | 7.7 ± 0.2 | 2.95 ± 0.5 |
| | pAB148 | 0.54 ± 0.07 | 0.95 ± 0.05 | 1.58 ± 0.19 | ND |
| | pAB89 | 0.13 ± 0.02 | 0.15 ± 0.05 | 0.2 ± 0.05 | ND |
| PAOSX | pAB754-2 M | 1.7 ± 0.1 | NG | 2.5 ± 0.6 | ND |
| | pAB754 | 4.6 ± 0.1 | NG | 11.2 ± 0.5 | ND |
| | pAB146 | 0.39 ± 0.08 | NG | 0.37 ± 0.05 | ND |
| | pAB148 | 0.26 ± 0.03 | NG | 0.39 ± 0.05 | ND |
| H636 | pAB754-2 M | 10.5 ± 0.5 | NG | 17.7 ± 0.9 | ND |
| | pAB146 | 3.5 ± 0.2 | NG | 7.1 ± 0.13 | ND |
| | pAB148 | 0.9 ± 0.02 | NG | 1.34 ± 0.3 | ND |

[a] Strains were cultured in normal LB medium and LB media with low salt (LB0), sucrose (LB342S), and polyethylene glycol (LBPEG) in microtiter plates. Results are the means of at least three independent experiments. NG, no growth; ND, not determined.

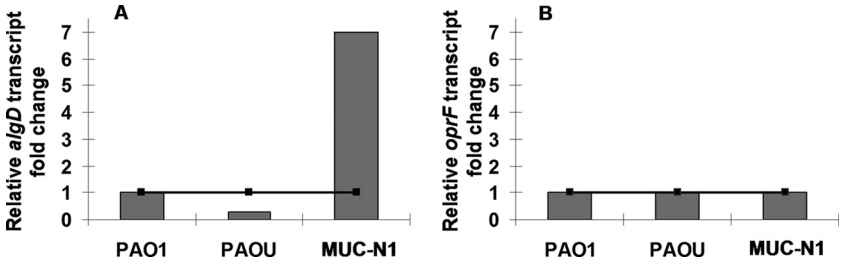


**FIG 4** Relative amounts of *algD* (A) and *oprF* (B) transcripts in *P. aeruginosa* PAO1, PAOU (*algU* mutant), and MUC-N1 (*mucA* mutant) strains compared to those of H103 in LB medium. The levels of *algD* (A) and *oprF* (B) mRNA in *P. aeruginosa* PAO1, PAOU, and MUC-N1 were evaluated by quantitative reverse transcription-PCR and compared to those in H103 (black bar). 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). PAOU is an AlgU mutant strain; MUC-N1 is a clinical mucoid strain mutated in the *mucA* gene.

(pAB754) was nearly identical to that of H103(pAB754-2 M) in terms of time course (Fig. 2A and 5B) and maximal level (Table 3). This confirmed the involvement of SigX in *oprF* transcription.

Since the *oprF*-proximal region contains the two overlapping SigX- and $\sigma^{70}$-dependent promoters, we introduced pAB146 into PAOSX. The maximal luminescence activity of PAOSX(pAB146) represented only 10% of the H103(pAB146) activity (Table 3), indicating that the SigX-dependent promoter was predominant over the $\sigma^{70}$-dependent promoter in the *oprF*-proximal region. Consistently, the latter region provided a lower contribution to the transcriptional activity in the absence of SigX (PAOSX strain) than in the wild-type strain: the maximal relative activity of PAOSX(pAB146) was only 23% of the PAOSX(pAB754-2 M) activity (Fig. 5; Table 3), whereas H103(pAB146) reached 79% of the H103(pAB754-2 M) activity (Fig. 2; Table 3). The promoter activity of the region located upstream of *sigX* (pAB148) was furthermore about 2-fold lower in a *sigX* mutant, suggesting that SigX, at least partially, positively controlled the transcription of its own gene (Table 3), even though the sequences in this promoter region were quite different from the *sigX*-dependent promoter sequences located upstream of *oprF* (Fig. 1B).

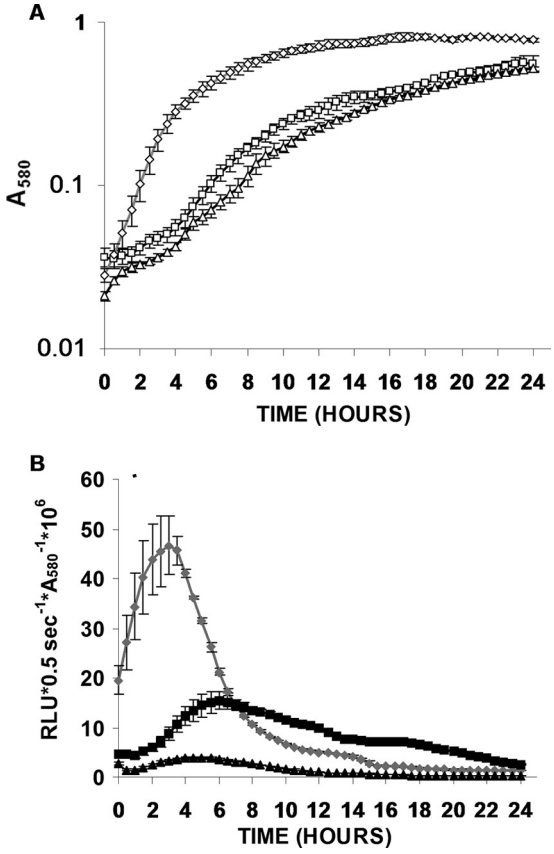


**FIG 5** SigX is the major ECF sigma factor involved in *oprF* transcription in LB medium. (A) Kinetics of growth of the PAOSX harboring the pAB754 plasmid (large promoter region containing a functional copy of the *sigX* gene; open diamonds), the pAB754-2 M (large promoter region including the four described promoters and containing a nonfunctional *sigX* copy; open squares) or the pAB146 (proximal promoter region containing the SigX- and σ70-dependent promoters; open triangles). (B) Relative bioluminescence levels generated by the *luxCDABE* cassette under the control of the *oprF* large promoter region (pAB754-2 M; filled squares) or the proximal promoter region (pAB146, filled triangles) in PAOSX. As a control, the relative bioluminescence generated by pAB754 (containing a functional *sigX* copy; filled gray diamonds) in PAOSX is shown. Experiments were repeated at least three times.

**The absence of NaCl increased *oprF* transcription.** OprF was previously shown to be necessary for *P. aeruginosa* growth in low-osmolarity LB medium (9). We therefore studied the effects of the absence of NaCl in LB medium (LB0 medium) on *P. aeruginosa* growth and on *oprF* transcription. As previously described (9), the degrees of growth of H103 were closely similar in LB and LB0 media (see Fig. S1A and S2A in the supplemental material). Regardless of the plasmid studied, the bioluminescence activity patterns were similar, reaching a peak during the exponential growth phase (data not shown). The relative activity of the large promoter region (pAB754-2 M) in the wild-type H103 strain was slightly but significantly increased in low-salt LB0 medium, since it reached 122% ± 2% of the activity in LB medium (Table 3). This increased activity resulted mainly from the *oprF*-proximal region, since the activity of H103(pAB146) represented 64% of the H103(pAB754-2 M) activity in LB0 medium (Table 3), whereas the activity of the AlgU-dependent promoter remained weak (see data for pAB89 in Table 3). The bioluminescence activities of H103(pAB146) and H103(pAB89) were not higher in LB0 than in LB medium (Table 3), suggesting that the increase of H103(pAB754-2 M) activity in LB0 medium resulted from an elevated activity of the promoter of the *sigX* gene. This was indeed the case, since the bioluminescence activity of H103(pAB148) in low-salt LB0 medium was 176% that in normal-salt LB medium (Table 3). Furthermore, PAOSX growth was nearly abolished in LB0 medium, and this phenotype was restored by in *trans* complementation of the PAOSX mutant with the *sigX* gene (see Fig. S2A in the supplemental material), suggesting that SigX is absolutely required

for *P. aeruginosa* growth in low-salt LB0 medium. Thus, the bioluminescence assays could not be conducted in LB0 medium.

**Transcription of *oprF* was induced by sucrose.** Since the presence of NaCl in LB medium led to slightly lower *oprF* transcription, we tested if adding osmolytes other than NaCl to LB0 medium would have a similar effect on *oprF* expression. We first increased the osmolarity of the LB0 medium by adding sucrose at the same osmolarity as NaCl. LB contains 171 mM NaCl, i.e., 342 mM osmolytes, since it can dissociate into two ionic species. We therefore added 342 mM sucrose to LB0 medium, generating LB342S medium. In LB342S medium, the doubling times were 150 min for H103 and 294 min for PAOSX, indicating that the presence of sucrose impaired the growth of H103 and PAOSX strains (doubling times 2.6- and 2-fold longer than in LB medium, respectively) (see Fig. S2B in the supplemental material). Regardless of the strain or plasmid studied, the bioluminescence patterns in LB342S medium reached a peak during the exponential growth phase (data not shown). The maximal activities of H103(pAB754-2 M), H103(pAB146), H103(pAB148), and H103(pAB89) were 1.7- to 2.3-fold higher in LB342S medium than in LB0 and LB media (Table 3). As in the previously used media, the activity of the *oprF*-proximal region in LB342S medium accounted for the largest part (69%) of the total activity (Table 3; compare data for pAB146 and pAB754-2 M in H103), whereas the activity of the *sigX* promoter region represented 14% of the total activity (see data for pAB148 in Table 3) and that of the AlgU-dependent promoter only 2.3% of the total activity (see data for pAB89 in Table 3). The effects of 171 mM NaCl and 342 mM sucrose on the *oprF* expression were therefore opposite.

**SigX was involved in the sucrose-dependent increase of *oprF* transcription.** In the PAOSX mutant, the large promoter region (pAB754-2 M) displayed a slightly higher activity (about 1.4-fold) in LB342S than in LB medium (Table 3). Complementation of the *sigX* mutation with pAB754 restored the high increase of the large promoter activity in LB342S medium compared to LB medium (Table 3). The activity of the *oprF*-proximal region (pAB146), which contains the SigX-dependent promoter, was strongly reduced in the PAOSX mutant compared to H103 in LB and LB342S media. However, its activities were similar in LB342S and LB media in the absence of SigX, while it was increased in H103 (Table 3). These data suggest that SigX is involved in the sucrose-dependent activity of the proximal promoter region. The activity of the *sigX* promoter region (pAB148) was lowered in the PAOSX mutant compared to the wild-type strain (between 2-fold in LB medium and 4-fold in LB342S medium). In PAOSX, this promoter region was, however, more active in LB342S than in LB medium, and this could at least partially explain the higher *oprF* transcription observed in LB342S medium than in LB medium [see data for PAOSX(pAB754-2 M) in LB and LB342S media in Table 3]. Similar results were obtained when the concentration of sucrose was increased to 500 mM (data not shown). These data showed that the higher *oprF* transcription level induced by sucrose relied essentially on SigX and on the SigX-dependent promoter in the *oprF*-proximal region.

***oprF* transcription was not increased due to the sucrose-induced viscosity.** LB342S and LB media were similar in terms of osmolarity. However, we suspected that addition of sucrose might have an effect on the medium viscosity. To investigate if the effects observed on the transcription of *oprF* were due to the medium viscosity rather than sucrose itself, we increased the LB0 medium viscosity by adding 1.4% of polyethylene glycol 8000 (PEG 8000) (LBPEG medium), leading to a viscosity similar to that in LB342S medium (see Fig. S3 in the supplemental material). Under this condition, the growth of H103 in LBPEG medium was not altered (data not shown), and the activities of pAB146 and pAB754-2 M were not increased in this medium compared to those in LB medium (Table 3), suggesting that sucrose promoted *oprF* transcription through a means other than sucrose-induced viscosity increase.

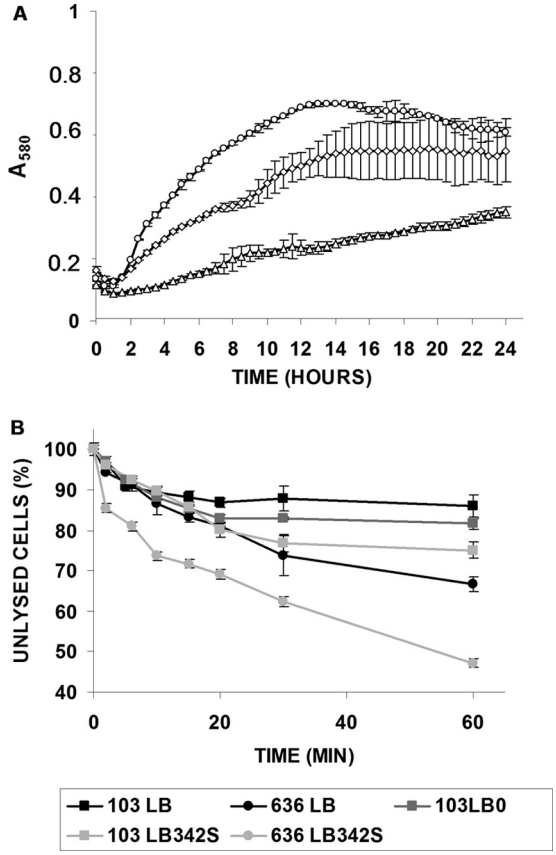


FIG 6 The lack of OprF results in growth and outer membrane permeability alterations in LB (open circles), LB0 (open triangles), and LB342S (open diamonds) media. (A) Kinetics of growth of H636 (*oprF* mutant) in LB, LB0, and LB342S media. (B) Gentamicin-assisted lysozyme lysis assay performed on H103 grown in LB, LB0, and LB342S media and H636 grown in LB and LB342S media. Experiments were repeated at least three times.

**Transcription of *oprF* was increased in an *oprF* mutant.** Since the growth of the *oprF* mutant H636 was impaired strongly in LB0 medium and to a lesser extent in LB342S medium (Fig. 6A), we studied the transcription of *oprF* in LB and LB342S media. As shown in Table 3, *oprF* transcription was increased 2.2-fold in LB medium, as determined by comparing the *oprF* mutant H636 to the wild-type H103 strain. The same held true in sucrose medium, in which there was a 1.6-fold increase observed when comparing H636 to H103 (see data for pAB754-2 M in Table 3). The activity of pAB148 followed a pattern similar to that of pAB754-2 M, suggesting that the absence of OprF led to increased transcription of *sigX*. The activities of pAB146 were similar in H103 and H636 in LB342S, indicating that OprF was largely not involved in the in-

crease of the sucrose-dependent activity of the proximal promoter region.

**LB342S and LB0 media provoke an alteration of the outer membrane permeability.** Since OprF was previously described as a structural protein involved in cell wall maintenance (52, 53), we further tested the effects of the addition of sucrose or NaCl to LB0 medium on outer membrane integrity, using the previously described gentamicin-assisted lysozyme lysis assay (19). As shown in Fig. 6B, addition of sucrose, and to a much lesser extent the absence of NaCl, led to an altered functioning of the H103 outer membrane. The lack of OprF had an effect on the bacterial gentamicin-promoted lysozyme lysis, the phenotype of which was exacerbated in LB342S medium. These data showed that the lack of OprF and the growth in LB342S medium resulted in higher sensitivity to lysozyme, suggesting that these two conditions led to cell envelope perturbations.

## DISCUSSION

We dissected in this study the transcription of the *oprF* gene, which encodes the multifunction and major outer membrane protein. The *oprF* gene was previously described as the last gene of a 7-gene-containing putative operonic structure. However, recent annotations of the *oprF* genomic environment, based on improved operon prediction algorithms and unpublished 5′ mapping of transcriptional start sites by RNA-Seq, have indicated that neither *oprF* nor *sigX* belongs to this operonic structure (see reference 49 and http://www.pseudomonas.com). Previously, monocistronic *oprF* and the bicistronic *sigX-oprF* mRNAs were detected (9). We thus focused our study on the *sigX* and *oprF* genes and promoter regions. We observed that the *oprF* transcription is growth phase dependent in LB medium, increasing throughout the exponential phase and decreasing from the entry into stationary phase. All three promoter regions displayed similar patterns of activity, but their contributions to *oprF* transcription were quite different, with the *oprF*-proximal region including the intertwined $\sigma^{70}$- and SigX-dependent promoters, which accounted for the largest part of *oprF* transcription (~80%). In contrast, the AlgU-dependent promoter lying within *sigX* made a marginal contribution to *oprF* expression under the conditions tested. This result was not surprising since AlgU is poorly active in H103 and PAO1 nonmucoid strains (4). Nevertheless, it was also observed that the global transcription of *oprF* remained similar in *mucA* and *algU* mutant backgrounds, in which AlgU was strongly active and absent, respectively. This is consistent with a previous study, in which a transcriptional *oprF-lacZ* fusion was not observed to increase significantly as a result of the *mucA* background (34). In contrast, Firoved et al. (21) showed by primer extension analysis that this AlgU promoter region was active in a *mucA* mutant strain, leading to a 2.5-fold increase in *oprF* transcription. Taken together, these results suggest that net *oprF* transcription might remain similar in mucoid and in nonmucoid strains, with the AlgU-dependent promoter being activated while other promoter region activity might be reduced. However, our study was conducted in LB liquid medium, which is a quite different condition from what occurs in the case of cystic fibrosis. The promoter region of the *sigX* gene contributed modestly to *oprF* transcription, compared to the *oprF*-proximal one under our conditions. Its activity was about 2-fold lowered in the *sigX* mutant, showing that SigX positively but partially controls the transcription of its own gene, which is a quite common feature of ECF sigma factors (36, 38). The promoter sequences we mapped showed some degrees of similarities to $\sigma^{70}$-dependent regions. Furthermore, Brinkman et al. (9) mapped the SigX-dependent promoter upstream of *oprF* and proposed −35 and −10 sequences that could be recognized by SigX. Using *in silico* analyses, we failed to identify such sequences upstream of *sigX*. Taken together, these data suggest that the identified promoter in the *cmpX-sigX* intergenic region might be indirectly activated by SigX, through *cis*- or *trans*-acting regulatory elements, for example. It is furthermore conceivable that other promoter regions initiating *sigX* transcription are present upstream of this intergenic region.

A major result of our study concerned the *oprF* transcriptional increase in response to a high sucrose concentration and, to a lesser extent, to lowered NaCl concentrations. We demonstrated that *oprF* transcription was not modulated, *per se*, in response to osmolarity or viscosity variations. The *oprF* transcriptional increase in LB0 medium was mainly due to the activity of the *sigX* promoter region, leading to *sigX* transcription. Since the growth of the *sigX* mutant was nearly abolished in LB0 medium, this indicates that SigX was required for growth in low-NaCl media, as previously shown (9), thus contributing to initiation of the transcription of target genes other than *oprF*, leading *P. aeruginosa* to adapt to LB0 medium. The effect of sucrose on *oprF* transcription resulted from an increased activity of the *oprF*-proximal promoter and, to a lesser extent, of the *sigX* promoter region. While the absence of SigX resulted in a 4-fold-decreased activity of the latter region in high-sucrose LB342S medium, that of the *oprF*-proximal region was about 21-fold reduced, suggesting that SigX was activated by the high sucrose concentration. Furthermore, while the increase due to sucrose was abolished in the proximal promoter region, a residual increase in the activity of the *sigX* promoter region was observed, suggesting that in the latter case this increase was only partly due to SigX. It is noticeable that in the absence of SigX, the activity of the proximal promoter region remained low and similar in all tested media, suggesting that it might correspond to a basal level of *oprF* transcription, which could putatively be linked to $\sigma^{70}$ activity. Although the absence of SigX is likely to be directly responsible for these observations, it is possible that the altered growth of the *sigX* mutant might have had some effects on promoter activities.

Since OprF was previously proposed to allow the nonspecific diffusion of low-molecular-mass sugars (6), we suspected that OprF might contribute to the entrance of sucrose into the cell. However, the large, *oprF*-proximal and *sigX* promoter regions were activated in the presence of sucrose, indicating that OprF is not required for the sucrose-dependent increase of *oprF* transcription. Furthermore, *oprF* transcription was increased in an *oprF* mutant not only in response to a high sucrose concentration but also in LB medium. Interestingly, it has been recently shown that in *Pseudomonas putida*, utilization of glucose as a carbon source led to modulation of OprF levels in the outer membrane via the ColRS two-component regulatory system, which ensures the integrity of the cell membrane during the increased expression of certain outer membrane proteins (39). In *Pseudomonas reinekei*, OprF was upregulated under 4-chlorosalicylate carbon-limiting conditions (7). In *P. aeruginosa*, OprF was overproduced in response to amino acids in ASM medium, a rich medium that mimics the nutrient conditions in the lung during cystic fibrosis (CF)

(47), which is consistent with the fact that OprF is always overproduced in mucoid strains, leading OprF to be a marker of *P. aeruginosa* infection during CF (27, 34). Although these studies indicate that OprF can be dynamically regulated, under our conditions the carbon source was not limiting (LB rich medium) and sucrose, which induced OprF, cannot be metabolized by *P. aeruginosa* (6). Thus, the increased *oprF* transcription in response to sucrose is unlikely to be linked to nutrient signals *per se*. However, OprF seems to play an important role in nutrient uptake, although the mechanisms involved in this process appear to be numerous and complex, and closely linked to the specific growth conditions.

Since OprF is a structural protein, anchoring the outer membrane on the peptidoglycan layer (52, 53) and interacting with lipopolysaccharide (23), the H636 *oprF* mutant cells display an altered rounded morphology (53), indicating that the cell wall and/or outer membrane may be perturbed. Accordingly, through a gentamicin-induced lysozyme lysis assay, we demonstrated that the lack of OprF, as well as LB342S or LB0 medium, induced cell wall or envelope perturbations. The latter could provide the signals that lead to increased *oprF* transcription, thus producing OprF in larger amounts to contribute to maintenance of outer membrane integrity. It is noticeable that the *sigX* promoter region was activated under each of the three tested conditions, indicating that SigX may be important in the cell wall stress response. However, the *oprF*-proximal promoter region, containing the SigX-dependent promoter, was activated only in response to sucrose, even in the *oprF* mutant. This was quite surprising, particularly when considering the role of OprF in maintaining envelope integrity. While this issue remains to be more deeply investigated, our study provides guidance for future experiments addressing this point. It would be of interest to examine the effect of treatments that induce different envelope stress responses on *oprF* transcription and SigX activity. The aim of this study was to decipher the transcription of *oprF*. However, in some cases the abundance of OprF in the outer membrane is altered even though the mRNA level is not apparently modified (34), indicating a potential for posttranscriptional regulation. Further studies should address this important issue.

## ACKNOWLEDGMENTS

We are grateful to J. Caillon (CHU Nantes, France) for providing the MUC-N1 *P. aeruginosa* MucA strain. We also thank A. Kolb, F. Norel-Bozouklian, and A. Benachour for helpful discussions.

This work was supported by the French government and Région Haute Normandie, France (part of the doctoral fellowship to G. Gicquel), the Grand Evreux Agglomération (O. Maillot), and European FEDER funds no. 32789 for the LMSM (postdoctoral fellowship to E. Bouffartigues).

## REFERENCES

1. **Angus BL, Fyfe JA, Hancock REW.** 1987. Mapping and characterization of two mutations to antibiotic supersusceptibility in *Pseudomonas aeruginosa* 1. J. Gen. Microbiol. **133**:2905–2914.
2. **Aspedon A, Palmer K, Whiteley M.** 2006. Microarray analysis of the osmotic stress response in *Pseudomonas aeruginosa*. J. Bacteriol. **188**: 2721–2725.
3. **Azghani AO, Idell S, Bains M, Hancock REW.** 2002. *Pseudomonas aeruginosa* outer membrane protein F is an adhesin in bacterial binding to lung epithelial cells in culture. Microb. Pathog. **33**:109–114.
4. **Bazire A, et al.** 2005. Osmotic stress and phosphate limitation alter production of cell-to-cell signal molecules and rhamnolipid biosurfactant by *Pseudomonas aeruginosa*. FEMS Microbiol. Lett. **253**:125–131.
5. **Bazire A, et al.** 2010. The sigma factor AlgU plays a key role in formation of robust biofilms by nonmucoid *Pseudomonas aeruginosa*. J. Bacteriol. **192**:3001–3010.
6. **Bellido F, Martin NL, Siehnel RJ, Hancock REW.** 1992. Reevaluation, using intact cells, of the exclusion limit and role of porin OprF in *Pseudomonas aeruginosa* outer membrane permeability. J. Bacteriol. **174**: 5196–5203.
7. **Bobadilla Fazzini RA, Preto MJ, Quintas AC, Bielecka A, Dos Santos VA.** 2010. Consortia modulation of the stress response: proteomic analysis of single strain versus mixed culture. Environ. Microbiol. **12**:2436–2449.
8. **Bodey GP, Bolivar R, Fainstein V, Jadeja L.** 1983. Infections caused by *Pseudomonas aeruginosa*. Rev. Infect. Dis. **5**:279–313.
9. **Brinkman FS, Schoofs G, Hancock REW, De Mot R.** 1999. Influence of a putative ECF sigma factor on expression of the major outer membrane protein, OprF, in *Pseudomonas aeruginosa* and *Pseudomonas fluorescens*. J. Bacteriol. **181**:4746–4754.
10. **Cao M, Helmann JD.** 2004. The *Bacillus subtilis* extracytoplasmic-function sigmaX factor regulates modification of the cell envelope and resistance to cationic antimicrobial peptides. J. Bacteriol. **186**:1136–1146.
11. **Cao M, Wang T, Ye R, Helmann JD.** 2002. Antibiotics that inhibit cell wall biosynthesis induce expression of the *Bacillus subtilis* sigma (W) and sigma (M) regulons. Mol. Microbiol. **45**:1267–1276.
12. **Cezairliyan BO, Sauer RT.** 2009. Control of *Pseudomonas aeruginosa* AlgW protease cleavage of MucA by peptide signals and MucB. Mol. Microbiol. **72**:368–379.
13. **Chevalier S, et al.** 2000. Characterization of an OprF-deficient mutant suggests that OprF is an essential protein for *Pseudomonas fluorescens* strain MF0. Res. Microbiol. **151**:619–627.
14. **Choi KH, Kumar A, Schweizer HP.** 2006. A 10-min method for preparation of highly electrocompetent *Pseudomonas aeruginosa* cells: application for DNA fragment transfer between chromosomes and plasmid transformation. J. Microbiol. Methods **64**:391–397.
15. **Ciofu O, et al.** 2008. Investigation of the *algT* operon sequence in mucoid and non-mucoid *Pseudomonas aeruginosa* isolates from 115 Scandinavian patients with cystic fibrosis and in 88 *in vitro* non-mucoid revertants. Microbiology **154**:103–113.
16. **Corbella ME, Puyet A.** 2003. Real-time reverse transcription-PCR analysis of expression of halobenzoate and salicylate catabolism-associated operons in two strains of *Pseudomonas aeruginosa*. Appl. Environ. Microbiol. **69**:2269–2275.
17. **Damron FH, Qiu D, Yu HD.** 2009. The *Pseudomonas aeruginosa* sensor kinase KinB negatively controls alginate production through AlgW-dependent MucA proteolysis. J. Bacteriol. **191**:2285–2295.
18. **Dominguez-Cuevas P, Marques S.** 2004. Compiling sigma-70-dependent promoters, p 319–343. *In* Ramos J-L (ed), Pseudomonas: virulence and gene regulation, vol II. Kluwer Academic/Plenum Publishers, New York, NY.
19. **Dower JW, Miller JF, Ragsdale CW.** 1988. High efficiency transformation of *E. coli* by high voltage electroporation. Nucleic Acids Res. **16**:6127–6145.
20. **Duchêne M, et al.** 1988. Sequence and transcriptional start site of the *Pseudomonas aeruginosa* outer membrane porin protein F gene. J. Bacteriol. **170**:155–162.
21. **Firoved AM, Boucher JC, Deretic V.** 2002. Global genomic analysis of AlgU ($\sigma^E$)-dependent promoters (sigmulon) in *Pseudomonas aeruginosa* and implications for inflammatory processes in cystic fibrosis. J. Bacteriol. **184**:1057–1064.
22. **Fito-Boncompte L, et al.** 2011. Full virulence of *Pseudomonas aeruginosa* requires OprF. Infect. Immun. **79**:1176–1186.
23. **Freulet-Marrière MA, et al.** 2000. Evidence for association of lipopolysaccharide with *Pseudomonas fluorescens* strain MF0 porin OprF. Res. Microbiol. **151**:873–876.
24. **Govan JRW, Deretic V.** 1996. Microbial pathogenesis in cystic fibrosis: mucoid *Pseudomonas aeruginosa* and *Burkholderia cepacia*. Microbiol. Rev. **60**:539–574.
25. **Guyard-Nicodème M, et al.** 2008. Outer membrane modifications of *Pseudomonas fluorescens* MF37 in response to hyperosmolarity. J. Proteome Res. **7**:1218–1225.
26. **Hancock REW, Carey AM.** 1979. Outer membrane of *Pseudomonas aeruginosa*: heat- and 2-mercaptoethanol-modifiable proteins. J. Bacteriol. **140**:902–910.
27. **Hassett DJ, et al.** 2002. Anaerobic metabolism and quorum sensing by *Pseudomonas aeruginosa* biofilms in chronically infected cystic fibrosis

airways: rethinking antibiotic treatment strategies and drug targets. Adv. Drug Deliv. Rev. **54**:1425–1443.
28. **Helmann JD.** 2002. The extracytoplasmic function (ECF) sigma factors. Adv. Microb. Physiol. **46**:47–110.
29. **Hémery G, Chevalier S, Bellon-Fontaine MN, Haras D, Orange N.** 2007. Growth temperature and OprF porin affect cell surface physicochemical properties and adhesive capacities of *Pseudomonas fluorescens* MF37. J. Ind. Microbiol. Biotechnol. **34**:49–54.
30. **Ho TD, Hastie JL, Intile PJ, Ellermeier CD.** 2011. The *Bacillus subtilis* extracytoplasmic function σ factor σ(V) is induced by lysozyme and provides resistance to lysozyme. J. Bacteriol. **193**:6215–6222.
31. **Kadry AA.** 2003. Lack of efflux mechanism in a clinical isolate of *Pseudomonas aeruginosa* highly resistant to beta-lactams and imipenem. Folia Microbiol. **48**:529–533.
32. **Luo Y, Helmann JD.** 2009. Extracytoplasmic function sigma factors with overlapping promoter specificity regulate sublancin production in *Bacillus subtilis*. J. Bacteriol. **191**:4951–4958.
33. **Macfarlane EL, Kwasnicka A, Hancock REW.** 2000. Role of *Pseudomonas aeruginosa* PhoP-PhoQ in resistance to antimicrobial cationic peptides and aminoglycosides. Microbiology **146**:2543–2554.
34. **Malhotra S, Silo-Suh LA, Mathee K, Ohman DE.** 2000. Proteome analysis of the effect of mucoid conversion on global protein expression in *Pseudomonas aeruginosa* strain PAO1 shows induction of the disulfide bond isomerase, dsbA. J. Bacteriol. **182**:6999–7006.
35. **Marx CJ, Lidstrom ME.** 2002. Broad-host-range *cre-lox* system for antibiotic marker recycling in gram-negative bacteria. Biotechniques **33**: 1062–1067.
36. **Missiakas D, Raina S.** 1998. The extracytoplasmic function sigma factors: role and regulation. Mol. Microbiol. **28**:1059–1066.
37. **Nestorovich EM, Sugawara E, Nikaido H, Bezrukov SM.** 2006. *Pseudomonas aeruginosa* porin OprF: properties of the channel. J. Biol. Chem. **281**:16230–16237.
38. **Potvin E, Sanschagrin F, Levesque R.** 2008. Sigma factors in *Pseudomonas aeruginosa*. FEMS Microbiol. Rev. **32**:38–55.
39. **Putrinš M, Ainelo A, Ilves H, Hõrak R.** 2011. The ColRS system is essential for the hunger response of glucose-growing *Pseudomonas putida*. BMC Microbiol. **11**:170.
40. **Qiu D, Eisinger VM, Rowen DW, Yu HD.** 2007. Regulated proteolysis controls mucoid conversion in *Pseudomonas aeruginosa*. Proc. Natl. Acad. Sci. U. S. A. **104**:8107–8112.
41. **Qiu D, Eisinger VM, Head NE, Pier GB, Yu HD.** 2008. ClpXP proteases positively regulate alginate overexpression and mucoid conversion in *Pseudomonas aeruginosa*. Microbiology **154**:2119–2130.
42. **Quénée L, Lamotte D, Polack B.** 2005. Combined *sacB*-based negative selection and cre-lox antibiotic marker recycling for efficient gene deletion in *Pseudomonas aeruginosa*. Biotechniques **38**:63–67.
43. **Ramsey DM, Wozniak DJ.** 2005. Understanding the control of *Pseudomonas aeruginosa* alginate synthesis and the prospect for management of chronic infections in cystic fibrosis. Mol. Microbiol. **56**:309–322.
44. **Rawling EG, Brinkman FS, Hancock REW.** 1998. Roles of the carboxy-terminal half of *Pseudomonas aeruginosa* major outer membrane protein OprF in cell shape, growth in low-osmolarity medium, and peptidoglycan association. J. Bacteriol. **180**:3556–3562.
45. **Schöbel S, Zellmeier S, Schumann W, Wiegert T.** 2004. The Bacillus subtilis sigmaW anti-sigma factor RsiW is degraded by intramembrane proteolysis through YluC. Mol. Microbiol. **52**:1091–1105.
46. **Simon R, Priefer U, Pühler A.** 1983. A broad range mobilization system for *in vivo* genetic engineering: transposon mutagenesis in Gram-negative bacteria. Biotechnology (NY) **1**:784–791.
47. **Sriramulu DD, Lünsdorf H, Lam JS, Römling U.** 2005. Microcolony formation: a novel biofilm model of *Pseudomonas aeruginosa* for the cystic fibrosis lung. J. Med. Microbiol. **54**:667–676.
48. **Wagner VE, Frelinger JG, Barth RK, Iglewski BH.** 2006. Quorum sensing: dynamic response of *Pseudomonas aeruginosa* to external signals. Trends Microbiol. **14**:55–58.
49. **Winsor GL, et al.** 2011. *Pseudomonas* Genome Database: improved comparative analysis and population genomics capability for *Pseudomonas* genomes. Nucleic Acids Res. **39**(database issue):D596–D600.
50. **Wood LF, Ohman DE.** 2009. Use of cell wall stress to characterize $\sigma^{22}$ (AlgT/U) activation by regulated proteolysis and its regulon in *Pseudomonas aeruginosa*. Mol. Microbiol. **72**:183–201.
51. **Wood LF, Leech AJ, Ohman DE.** 2006. Cell wall-inhibitory antibiotics activate the alginate biosynthesis operon in *Pseudomonas aeruginosa*: roles of $\sigma^{22}$ (AlgT) and the AlgW and Prc proteases. Mol. Microbiol. **62**:412–426.
52. **Woodruff WA, Hancock REW.** 1989. *Pseudomonas aeruginosa* outer membrane protein F: structural role and relationship to the Escherichia coli OmpA protein. J. Bacteriol. **171**:3304–3309.
53. **Woodruff WA, Hancock REW.** 1988. Construction and characterization of *Pseudomonas aeruginosa* protein F-deficient mutants after *in vitro* and *in vivo* insertion mutagenesis of the cloned gene. J. Bacteriol. **170**:2592–2598.
54. **Wozniak DJ, Ohman DE.** 1994. Transcriptional analysis of the *Pseudomonas aeruginosa* genes *algR*, *algB*, and *algD* reveals a hierarchy of alginate gene expression which is modulated by *algT*. J. Bacteriol. **176**:6007–6014.
55. **Wu L, et al.** 2005. Recognition of host immune activation by *Pseudomonas aeruginosa*. Science **309**:774–777.
56. **Yoon SS, et al.** 2002. Pseudomonas aeruginosa anaerobic respiration in biofilms: relationships to cystic fibrosis pathogenesis. Dev. Cell **3**:593–603.