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Sensor Kinase PA4398 Modulates Swarming Motility and Biofilm Formation in *Pseudomonas aeruginosa* PA14

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Pseudomonas aeruginosa **is an opportunistic human pathogen that is able to sense and adapt to numerous environmental stimuli by the use of transcriptional regulators, including two-component regulatory systems. In this study, we demonstrate that the sensor kinase PA4398 is involved in the regulation of swarming motility and biofilm formation in *P. aeruginosa* PA14. A PA4398$^-$ mutant strain was considerably impaired in swarming motility, while biofilm formation was increased by approximately 2-fold. The PA4398$^-$ mutant showed no changes in growth rate, rhamnolipid synthesis, or the production of the Pel exopolysaccharide but exhibited levels of the intracellular second messenger cyclic dimeric GMP (c-di-GMP) 50% higher than those in wild-type cells. The role of PA4398 in gene regulation was investigated by comparing the PA4398$^-$ mutant to the wild-type strain by using microarray analysis, which demonstrated that 64 genes were up- or downregulated more than 1.5-fold ($P < 0.05$) under swarming conditions. In addition, more-sensitive real-time PCR studies were performed on genes known to be involved in c-di-GMP metabolism. Among the dysregulated genes were several involved in the synthesis and degradation of c-di-GMP or in the biosynthesis, transport, or function of the iron-scavenging siderophores pyoverdine and pyochelin, in agreement with the swarming phenotype observed. By analyzing additional mutants of selected pyoverdine- and pyochelin-related genes, we were able to show that not only *pvdQ* but also *pvdR*, *fptA*, *pchA*, *pchD*, and *pchH* are essential for the normal swarming behavior of *P. aeruginosa* PA14 and may also contribute to the swarming-deficient phenotype of the PA4398$^-$ mutant in addition to elevated c-di-GMP levels.**

Pseudomonas aeruginosa is a ubiquitous Gram-negative water and soil bacterium as well as an important opportunistic human pathogen, causing many serious acute and chronic infections, such as sepsis in immunocompromised individuals and burn wound patients, catheter-associated urinary tract infections, and nosocomial pneumonia (1). Moreover, this bacterium is the prevalent pathogen that colonizes and persists in the lungs of individuals suffering from the genetic disease cystic fibrosis (CF), leading to chronic and eventually fatal pulmonary infections (2, 3). Due to the high intrinsic resistance of this organism to a wide range of antibiotics (4), the secretion of numerous virulence factors, including siderophores, exotoxins, proteases, and type III secretion effectors (5, 6), and the ability to form robust biofilms, treatment of *P. aeruginosa* infections still remains difficult.

Motility in *P. aeruginosa* is an important process that enables the bacterium to colonize different environments, to adhere to a number of biotic and abiotic surfaces, and to form biofilms on them (7). Motility is strongly associated with the virulence of this human pathogen (8). *P. aeruginosa* is capable of exerting three major types of motility: swimming motility in aqueous environments, which is mediated by its polar flagellum; type IV pilus-mediated twitching motility on solid surfaces or interfaces (9); and swarming motility on viscous media (10). The latter represents a second surface-associated multicellular behavior in addition to biofilms and requires both a functional flagellum and type IV pili (11). Swarming is characterized by rapid, coordinated migration of bacterial cells across semisolid surfaces (12, 13) and is highly dependent on cell-to-cell signaling and the production and secretion of rhamnolipids, which are proposed to act as biosurfactants that lower the strong surface tension between bacterial cells and the surrounding environment (11, 14, 15). Previously, studies by us and others were able to show that swarming in *P. aeruginosa* is more than just a form of locomotion: it is a complex adaptation process resulting in cellular changes, such as cell elongation or hyperflagellation, as well as increased virulence gene expression and antibiotic resistance (11, 13, 16, 17).

The ability of *P. aeruginosa* to rapidly colonize different ecological niches is mediated, at least in part, by its large and complex genome and a remarkable proportion of transcriptional regulatory units (9.4% of the genome) (1). In addition to sigma factors and other one-component regulators, two-component systems (TCSs) play an important part in sensing diverse stimuli and enable an appropriate and rapid adaptive physiological response (18). Two-component systems classically comprise a membrane-bound histidine kinase and a cytoplasmic response regulator. The genome of *P. aeruginosa* encodes 64 sensor kinases and 73 response regulators (19, 20), whose genes are most commonly organized in operons (18). However, 13 sensor kinases and 15 response regulators are not linked to any other genes and are termed

Received 28 August 2014 **Accepted** 5 December 2014

Accepted manuscript posted online 12 December 2014

Citation Strehmel J, Neidig A, Nusser M, Geffers R, Brenner-Weiss G, Overhage J. 2015. Sensor kinase PA4398 modulates swarming motility and biofilm formation in *Pseudomonas aeruginosa* PA14. Appl Environ Microbiol 81:1274–1285. doi:10.1128/AEM.02832-14.

Editor: H. Nojiri

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Supplemental material for this article may be found at http://dx.doi.org/10.1128/AEM.02832-14.

doi:10.1128/AEM.02832-14

TABLE 1 Strains and plasmids used in this study

Strain or plasmid	Characteristic(s)[a]	Source or reference
Strains		
P. aeruginosa		
PA14 WT	Wild-type *P. aeruginosa* PA14	22
PA_57140 mutant	PA14 transposon insertion mutant; ID 30693	22
fpvA mutant	PA14 transposon insertion mutant; ID 43776	22
pvdA mutant	PA14 transposon insertion mutant; ID 30448	22
pvdR mutant	PA14 transposon insertion mutant; ID 53759	22
pvdL mutant	PA14 transposon insertion mutant; ID 24971	22
pvdS mutant	PA14 transposon insertion mutant; ID 34241	22
fptA mutant	PA14 transposon insertion mutant; ID 41286	22
pchF mutant	PA14 transposon insertion mutant; ID 24493	22
pchD mutant	PA14 transposon insertion mutant; ID 42509	22
pchA mutant	PA14 transposon insertion mutant; ID 35443	22
PA4398$^-$ mutant	PA4398 chromosomal mutation in PA14; Gm^r	This study
PA4398c strain	PA4398 mutant with pUCP20::PA4398; Cb^r	This study
pUCP20 strain	PA14 WT with pUCP20; Cb^r	This study
E. coli		
DH5α	F$^-$ ϕ80*lacZ*ΔM15 Δ(*lacZYA-argF*)*U169 deoR recA1 endA1 hsdR17*(r_K^- m_K^+) *supE44* λ$^-$ *thi-1 gyrA96 relA*	Invitrogen
S17-1	*galU galK rpsL*(Str^r) *endA1 nupG thi pro hsdR hsdM*$^+$ *recA* (RP4-2Tc::Mu Km::Tn*7*) λ*pir*	68
Plasmids		
pUCP20	*E. coli-Pseudomonas* shuttle vector; Ap^r Cb^r	69
pUCP20::PA4398	pUCP20 with PA4398 gene; Ap^r Cb^r	This study
pEX18Ap	Suicide plasmid carrying *oriT*$^+$ *sacB*$^+$; Ap^r	25
pPS856	Carrying Gm^r cassette of pUCGM; Ap^r Gm^r	25

[a] Ap, ampicillin; Cb, carbenicillin; Gm, gentamicin; Km, kanamycin. PA14 transposon mutants harbor a Gm^r cassette.

"orphans" (18). The large number of TCSs, as well as the diversity of sensor kinase input and response regulator output domains, enables the regulation of numerous processes in *P. aeruginosa*, including motility, biofilm formation, antibiotic resistance, and virulence factor production and secretion, among many others (18, 20, 21).

Recently, we screened the publicly available *P. aeruginosa* PA14 transposon insertion mutant library (22), which covers approximately 75% of the predicted 5,962 PA14 genes, and identified 233 transposon mutants with altered swarming phenotypes (23). *P. aeruginosa* strain PA14, in contrast to PAO1, is a primary clinical isolate that has not been passaged in the laboratory (22). In addition, PA14 is capable of infecting a variety of nonvertebrate and mammalian hosts and is therefore more suitable for virulence analysis, using different model systems, than strain PAO1 (22, 24). Besides genes involved in flagellum and type IV pilus biosynthesis and in quorum sensing (QS), 35 transcriptional regulators, including two-component regulatory proteins, were found to be involved in swarming motility during this screening (23). Among the TCSs, a mutant with a transposon insertion in the *P. aeruginosa* PA14_57170 gene (PA14 transposon insertion mutant library identification number [ID] 54440), coding for a sensor kinase, exhibited significant impairment in the ability to swarm on viscous surfaces.

In the present study, we demonstrate that this sensor kinase, PA14_57170 (termed PA14 PA4398, according to the corresponding locus in *P. aeruginosa* PAO1), is involved in the regulation of bacterial community behavior in *P. aeruginosa* PA14. We constructed a PA14 PA4398 insertion mutant and observed a strong swarming-deficient phenotype for this mutant, while the level of biofilm formation was significantly increased. Furthermore, gene expression analyses of the PA14 PA4398$^-$ mutant revealed strong downregulation of pyoverdine and pyochelin biosynthesis genes, in addition to dysregulation of genes coding for diguanylate cyclases and phosphodiesterases, which are involved in the synthesis and degradation, respectively, of the second messenger intracellular bis-(3′,5′)-cyclic dimeric GMP (c-di-GMP).

MATERIALS AND METHODS

Bacterial strains and growth conditions. The bacterial strains and plasmids used in this study are listed in Table 1. Bacteria were routinely grown in Luria-Bertani (LB) broth or BM2 minimal medium [62 mM potassium phosphate buffer (pH 7), 7 mM $(NH_4)_2SO_4$, 2 mM $MgSO_4$, 10 μM $FeSO_4$, 0.4% (wt/vol) glucose] at 37°C with shaking at 170 rpm. For plasmid selection or maintenance, antibiotics were added to the growth media at the following concentrations: for *Escherichia coli*, 100 μg/ml ampicillin or 10 μg/ml gentamicin, and for *P. aeruginosa*, 300 μg/ml carbenicillin or 30 μg/ml gentamicin, respectively.

PA14 PA4398 mutant and plasmid generation. The DNA primers used for genetic manipulation or gene expression analyses were obtained from Eurofins MWG Operon (Ebersberg, Germany) and are listed in Table 2. A PA4398 insertion mutant of *P. aeruginosa* PA14 was constructed according to the method described previously by using the suicide vector pEX18Ap in combination with a gentamicin resistance cassette (Gm) (25). Briefly, two terminal 500-bp and 600-bp fragments of the 2.3-kbp PA14 PA4398 gene were amplified by PCR, and the products were subsequently cloned into the broad-host-range vector pUCP20. Thus, a >1-kb fragment of PA4398 was deleted and was replaced with an Ω gentamicin resistance cassette as a selection marker via a BamHI restriction site. The disrupted PA4398ΩGm gene was amplified by blunt-end PCR, and the product was cloned into the SmaI-linearized suicide vector pEX18Ap (25). The gene replacement vector obtained was transformed into *E. coli* S17-1 λ*pir* and was conjugated into wild-type (WT) *P. aerugi-*

TABLE 2 Oligonucleotide sequences used in this study

Oligonucleotide	Sequence (5′ → 3′)[a]
Cloning primers	
PA4398_U1	AAAAAGAATTCCCTGCGCCA GCGCCTCGACAACCT
PA4398_D2	AAAAAAAGCTTCTTCCTCTTC GTCGCCCCGCCGCAG
PA4398_D1	AAAAAGGATCCCCTCGCGGGT GACCTGCCGCGACAG
PA4398_U2	AAAAAGGATCCCTTCGACTTC AAGTCGTTGCAGATC
PA4398-EcoRI_for	AAAAAGAATTCGTTTGCCCGA CCGCTAAAG
PA4398_*HindIII*_rev	AAAAAAAGCTTCGGGTGTAG ATCTTCGACAA
Primers for qRT-PCR	
qRT-*exoY*_for	AACTTCCCGGCCACCTTCTAC
qRT-*exoY*_rev	AAAGCTCTTCCGATCCGCC
qRT-*hasAp*_for	CGATTTCCTACAGCACCACC
qRT-*hasAp*_rev	GGTTGACGTCGCCGAAGTA
qRT-*pchF*_for	AAGTCGTCGAACATGGCTTC
qRT-*pchF*_rev	GGTGTTCACCAGTGGCATC
qRT-*pscJ*_for	ATTTCCCATACCCTTTCCGAGA
qRT-*pscJ*_rev	TGCGGTCGTAGCTGAGTCCT
qRT-*pvdA*_for	AGCCAGTACCTGGAACACATGG
qRT-*pvdA*_rev	TAGCTGTCGTTGAGGTCGATGA
qRT-*pvdD*_for	CAACCTGGCCTACGTGATCT
qRT-*pvdD*_rev	GAAGGCGTAGGAATGGAACA
qRT-*pvdQ*_for	CGACCAGACCGAGCTGTTTTC
qRT-*pvdQ*_rev	TCGCGAATGCTTAGCCGTT
qRT-*rpoD*_for	CAGTTCCACGGTACCCATTT
qRT-*rpoD*_rev	GGGGATCAACGTATTCGAGA
qRT-PA2200_for	ATCCTGCCGATACTGCTCACC
qRT-PA2200_rev	TGATCACGAAGATCCCGCA
qRT-PA2072_for	AGCCCATGCGAAGATCCAG
qRT-PA2072_rev	CTGGAACAGCTTGTTGCGGT
qRT-PA2870_for	CACAACCGCCACTCGTTCCT
qRT-PA2870_rev	GCCGTGGCTGTCGTTGATGT
qRT-PA4396_for	CTGGACGAAAGCATCAACCA
qRT-PA4396_rev	AGTTGCTCGTTCATCGCGG

[a] EcoRI (GAATTC), HindIII (AAGCTT), and BamHI (GGATCC) restriction sites are underlined.

nosa PA14 by allelic exchange. The resulting PA14 PA4398$^-$ insertion mutant was confirmed by PCR and subsequent restriction site mapping.

The PA14 PA4398$^-$ mutant was complemented by amplifying the PA4398 gene from PA14 genomic DNA using EcoRI- and HindIII-flanked oligonucleotides and subsequently cloning the fragment into pUCP20, resulting in pUCP20::PA4398. For complementation, this plasmid was transferred into the *P. aeruginosa* PA4398$^-$ mutant by electroporation (26), and the resulting strain was named the PA4398c strain.

Bacterial motility. The swimming, swarming, and twitching motilities of *P. aeruginosa* PA14 were tested on agar (Difco, Becton Dickinson GmbH, Heidelberg, Germany) plates as described previously (16, 23, 27). Swarming behavior on BM2 swarm plates (62 mM potassium phosphate buffer [pH 7], 2 mM $MgSO_4$, 10 μM $FeSO_4$, 0.4% [wt/vol] glucose, 0.1% [wt/vol] Casamino Acids [CAA], 0.5% [wt/vol] agar) was analyzed by measuring the agar plate surface coverage of the dendritic cell colonies by use of the open-source image-processing program ImageJ. The diameters of swim and twitch zones (determined by using LB medium containing 0.3% [wt/vol] and 1% [wt/vol] agar, respectively) were measured after 20 h at 30°C and 48 h at 37°C. All motility types were assayed in at least three independent experiments with five replicates for each mutant. Statistical significance was determined by the nonparametric Mann-Whitney test.

Rhamnolipid measurement by LC–MS-MS. Cultures of *P. aeruginosa* PA14 grown for 20 h in BM2 swarm medium supplemented with 0.1% (wt/vol) CAA at 37°C under shaking conditions were harvested by centrifugation (20 min, 9,000 × *g*). Mono- and dirhamnolipids were extracted from 5 ml of cell-free culture supernatants by adding 3 ml chloroform, shaking vigorously for 10 min, and incubating overnight at −20°C for phase separation. The lower organic layer containing the rhamnolipids was transferred to a new reaction tube, followed by evaporation under nitrogen gas at room temperature. The remaining pellet was resuspended in a 1:1 (vol/vol) acetonitrile–ammonium acetate (10 mM) mixture, followed by liquid chromatography–tandem mass spectrometry (LC–MS-MS) quantification using a PerkinElmer (Überlingen, Germany) series 200 quaternary high-performance liquid chromatography (HPLC) pump with an autosampler. Chromatographic separation was performed on a LiChrospher 100 RP-18 ec HPLC column (length, 150 mm; inside diameter, 4.0 mm; particle size, 5 μm; Ziemer Chromatographie, Germany) by the use of an isocratic mobile phase consisting of 10 mM acetonitrile–ammonium acetate (65:35 [vol/vol]) at a flow rate of 0.3 ml/min and a sample volume of 25 μl. Electrospray ionization (ESI)-MS was performed on an API 365 triple quadrupole mass spectrometer (PE Sciex, Toronto, Canada) with a turbo ion spray interface used in negative mode. Particular instrument adjustments of the mass spectrometer (voltage, temperature, gas flows, etc.) were performed by infusion experiments using nitrogen as the curtain gas, nebulizer gas, and collision gas. Rhamnolipids were identified and quantified by multiple-reaction monitoring (MRM) by use of the following specific mass transitions from molecule ion to product ion: *m*/*z* 503.3/169.2 (MRM1) and *m*/*z* 503.3/333.3 (MRM2) for monorhamnolipids; *m*/*z* 649.5/479.4 (MRM1) and *m*/*z* 649.5/169.2 (MRM2) for dirhamnolipids. Quantification was performed through external calibration using rhamnolipid standard solutions with concentrations ranging from 0.2 μg/ml to 6.0 μg/ml.

Rhamnolipid concentrations were normalized to the cell density (expressed as the optical density at 600 nm [OD_{600}]) of each sample. Analysis was performed with three independent bacterial cultures, and statistical significance was analyzed by the Mann-Whitney test.

Biofilm formation and rapid adhesion. Biofilm formation was analyzed in 96-well polystyrene microtiter plates (Nunc, Thermo Fisher Scientific, St. Leon-Rot, Germany) by using an abiotic solid-surface assay (28). Overnight PA14 cultures, grown in BM2 minimal medium, were diluted 1:100 in a BM2 medium supplemented with 0.5% (wt/vol) CAA, which is named BM2 biofilm medium. After incubation for 24 h at 37°C, biofilms were stained with crystal violet (29), and the absorbance at 595 nm was measured using a microtiter plate reader (Tecan Group Ltd.).

Rapid adhesion was assayed as described by O'Toole and Kolter (28). BM2 medium-grown *P. aeruginosa* cultures were adjusted to a cell density (OD_{600}) of 1 in BM2 biofilm medium, and 100-μl aliquots of each culture were added to the wells of a 96-well plate. Following attachment for 1 h at 37°C, bacterial adhesion was determined by crystal violet staining as described above. Both assays were performed at least three times with six technical repeats for each strain, and statistical significance was analyzed by the Mann-Whitney test.

CR plate assay. The Congo red (CR) binding assay was performed as described previously (30). Briefly, overnight cultures of *P. aeruginosa* PA14, grown in tryptone broth (10 g/liter tryptone, 25 g/liter LB medium) were diluted to an OD_{600} of 0.025. Aliquots of 10 μl were spotted onto CR plates (10 g/liter tryptone and 1% [wt/vol] Difco agar with 40 μg/ml Congo red and 20 μg/ml Coomassie brilliant blue G, both solubilized in 70% [vol/vol] ethanol) and were incubated at room temperature for 10 days to assess the colony morphology.

Extraction and quantification of c-di-GMP. Intracellular bis-(3′,5′)-cyclic dimeric GMP (c-di-GMP) was extracted and quantified as described previously (31) with some modifications. *P. aeruginosa* PA14 cultures were grown for 24 h in BM2 biofilm medium at 37°C under shaking

conditions. Subsequently, 10 ml of each culture, adjusted to a cell density (OD_{600}) of 0.5, was harvested by centrifugation for 2 min at 8,000 × *g* and 4°C. The cell pellets obtained were resuspended in 590 μl ice-cold extraction buffer (40% [vol/vol] acetonitrile, 40% [vol/vol] methanol, and 20% [vol/vol] double-distilled water [ddH_2O]) and 10 μl (10 ppm) 3′,5′-cyclic XMP (cXMP) (Biolog Life Science Institute, Bremen, Germany) as an internal standard by vigorous vortexing. After 15 min of incubation on ice, cells were lysed at 95°C for 10 min, followed by centrifugation at 15,000 × *g* and 4°C for 5 min. Supernatants were collected in a new 1.5-ml reaction tube and were stored on ice. The remaining pellets were again resuspended in 400 μl extraction buffer, incubated on ice for 15 min, and subjected to centrifugation (15,000 × *g*, 4°C, 5 min). This procedure was repeated, and supernatants from all three extraction steps were pooled and were subsequently evaporated under nitrogen gas at 40°C. The remaining pellets were then resuspended in 400 μl ddH_2O, and c-di-GMP levels were quantified by LC–MS-MS as described above for rhamnolipid quantification, with the following modifications. Chromatographic separation was performed on a Multospher AQ RP 18 HPLC column (length, 250 mm; inside diameter, 4.0 mm; particle size, 5 μm; CS Chromatography Service GmbH, Langerwehe, Germany) by use of a binary mobile phase consisting of methanol and ammonium acetate (10 mM)–0.1% acetic acid (55:45 [vol/vol]) at a flow rate of 0.3 ml/min. The injection volume of each sample was set to 20 μl. ESI-MS was performed on an API 365 triple quadrupole mass spectrometer (PE Sciex, Toronto, Canada) with a turbo ion spray interface used in positive mode at an ionization potential of 5,000 V and a temperature of 400°C. Particular instrument adjustments for the mass spectrometer were performed by infusion experiments using nitrogen as the curtain gas, nebulizer gas, heater gas, and collision gas at a purity rate of 5.0. c-di-GMP was identified and quantified by MRM by use of the following specific mass transitions from molecule ion to product ion: *m/z* 691/152 (MRM1) and *m/z* 691/540 (MRM2). Quantification was performed through external calibration using a cXMP standard solution at concentrations ranging from 50 ng to 600 ng in 400 μl methanol.

c-di-GMP concentrations were normalized to the total-protein concentration of each culture, determined by the bicinchoninic acid assay (32), and c-di-GMP was quantified in three independent cultures, with two technical repeats for each strain. Statistical significance was analyzed by the Mann-Whitney test.

Growth curves. *P. aeruginosa* PA14 cells were grown overnight in BM2 minimal medium. Cultures were diluted to an OD_{600} of 0.1 in the medium to obtain equal optical densities. One hundred microliters of these dilutions was added to 96-well microtiter plates (Nunc, Thermo Fisher Scientific, St. Leon-Rot, Germany), and growth was monitored with a Tecan Infinite 200 Pro system (Tecan Group Ltd.) under shaking conditions at 37°C by determining the absorbance at 595 nm every 30 min for 24 h. For each medium condition, three independent experiments were performed with at least four replicates for each mutant.

DNA microarray and qPCR experiments. For global gene expression analyses, microarray experiments were performed on three independent *P. aeruginosa* PA14 wild-type and PA4398$^-$ mutant cultures. Cells were grown on BM2 swarm plates containing 0.1% (wt/vol) CAA and 0.5% (wt/vol) agar (Difco; Becton Dickinson GmbH, Heidelberg, Germany) for 20 h at 37°C. As described previously (8), bacteria were harvested from the leading edges of the dendritic swarm colonies and were resuspended in RNAprotect reagent (Qiagen, Hilden, Germany). Total RNA isolation, DNase digestion, synthesis of first-strand cDNA, cDNA fragmentation, biotin labeling, and finally hybridization to Affymetrix GeneChip Pae_G1a DNA microarrays (Affymetrix UK Ltd., Freiburg, Germany) were carried out as described by Strempel et al. (33). As a technical repeat, each sample was hybridized to two microarray chips. Only genes that exhibited significant ($P \leq 0.05$) ≥1.5-fold changes in expression in the PA4398$^-$ mutant from that in the respective wild-type control were included in further analyses. For the validation of microarray results, quantitative real-time PCR (qPCR) was performed as described previously (24) using the KAPA SYBR Fast PCR master mix (Peqlab Biotechnologie GmbH, Erlangen, Germany) in an ABI 7300 real-time PCR system (Applied Biosystems Deutschland, Darmstadt, Germany). Analysis of melting curves of PCR products ensured the specificity of the PCRs. Relative gene expression was calculated using the $\Delta\Delta C_T$ method (34), whereby the threshold cycle (C_T) values obtained were normalized to those for the housekeeping sigma factor gene *rpoD*, which was not differentially expressed in the PA4398$^-$ sensor kinase mutant, as shown by the microarray analysis. All reactions were assayed three times in duplicate ($n = 6$).

Microarray data accession number. The complete microarray data obtained in this study have been deposited in ArrayExpress under accession number E-MTAB-2540.

RESULTS

The swarming motility of the PA14 PA4398$^-$ mutant is impaired. A previous screening of the Harvard Medical School *P. aeruginosa* PA14 transposon mutant library (22) indicated a defect in the ability of the PA14 PA4398 transposon mutant (PA14 transposon insertion mutant library ID 54440) to swarm (23). For further characterization, we constructed a PA14 PA4398 insertion mutant (the PA4398$^-$ mutant) in *P. aeruginosa* PA14 in which a ~1-kb region of the sensor kinase gene was deleted and replaced by the insertion of a gentamicin resistance gene cassette. The resultant mutant strain was verified by PCR and restriction site mapping.

The abilities of the PA4398$^-$ mutant to swim, twitch, and swarm were examined on 0.3%, 1%, and 0.5% (wt/vol) agar plates, respectively. The PA14 PA4398$^-$ mutant exhibited only very minor differences from wild-type PA14 cells in flagellum-mediated swimming and type IV pilus-mediated twitching motility (data not shown). In contrast, the ability to swarm on viscous surfaces was strongly affected in this mutant. The swarming-defective phenotype of the PA4398$^-$ mutant could be restored to wild-type levels by heterologous expression of the wild-type PA4398 gene in the insertion mutant (Fig. 1). To investigate whether the swarming-deficient phenotype observed for the PA4398$^-$ mutant could be related to differences in growth behavior, the OD_{600} values of the sensor kinase mutant and the wild-type strain were monitored over 24 h of incubation at 37°C. As shown in Fig. 2A, the PA4398$^-$ mutant exhibited no growth defect relative to WT PA14 in liquid BM2 swarm medium. In addition, we also analyzed the growth behavior of swarmer cells grown on BM2 swarm plates according to the method described by Butler et al. (35). After 20 h at 37°C, all strains exhibited 1×10^7 to 2×10^7 cells/ml, and no differences in CFU counts per milliliter could be observed among the PA4398$^-$ mutant, the PA4398c complemented strain, WT PA14, and the pUCP20 empty-vector control strain (data not shown).

In addition to flagella, rhamnolipids are very important for the swarming of *P. aeruginosa* due to their ability to lower surface tension. Using LC–MS-MS to quantify synthesized mono- and dirhamnolipids, we analyzed whether the swarming deficiency of the PA4398$^-$ mutant was caused by differences in the production of rhamnolipids. When cells were grown in BM2 swarm medium, no differences in rhamnolipid concentrations were observed between WT PA14 and the PA4398$^-$ mutant strain (Table 3).

PA4398$^-$ exhibits increased biofilm formation and c-di-GMP levels. Since previous studies have shown inverse regulation of swarming motility and biofilm formation (23, 36, 37), we were interested in whether PA4398 is involved in biofilm development in *P. aeruginosa*. In order to analyze the ability of the PA4398$^-$

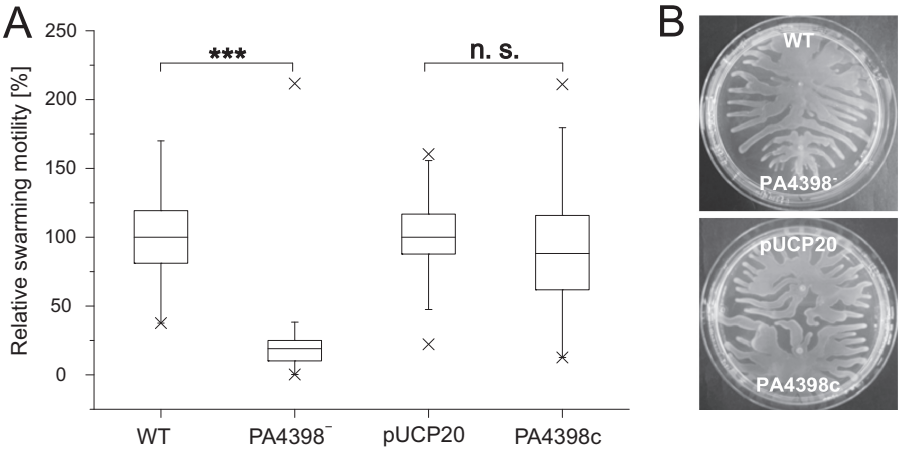

FIG 1 Swarming motilities of the *P. aeruginosa* PA14 WT, PA4398$^-$, pUCP20, and PA4398c strains. Swarming motility was assayed on BM2 swarm plates after incubation for 20 h, and agar plate surface coverage was determined in at least three independent experiments with five replicates for each mutant. (A) The swarming-defective phenotype of the PA4398$^-$ mutant was restored to the level of the empty-vector control by introducing the wild-type PA4398 gene into the mutant (PA4398c). Statistical significance was calculated by the nonparametric Mann-Whitney test (***, $P \leq 0.001$; n. s., not significant). The highest and lowest outliers are indicated by "×." (B) Representative swarm plates after 20 h of incubation. WT, wild-type PA14; PA4398$^-$, PA4398$^-$ mutant; pUCP20, empty-vector control; PA4398c, complemented PA4398 mutant.

mutant to form biofilms, static microtiter-based biofilm assays were performed; they demonstrated a significant 1.8-fold increase in biofilm biomass for the PA4398$^-$ mutant after 24 h (Fig. 3A). This hyperbiofilm phenotype could be complemented by introducing the wild-type gene PA4398 into the PA4398$^-$ strain. To determine whether this phenotype is initiated in the early stages of the biofilm-forming process, a rapid adhesion assay was performed. After 1 h, no significant differences in early attachment between WT PA14 and the PA4398$^-$ mutant strain could be observed (Fig. 3B). As confirmed by growth analysis in BM2 biofilm medium, this hyperbiofilm formation is also not caused by enhanced growth behavior of the mutant strain (Fig. 2B).

Motility and biofilm formation are in large part controlled by the intracellular level of the second messenger c-di-GMP in *P. aeruginosa* and various other Gram-negative bacteria (38, 39). To determine whether this small signaling molecule is implicated in the swarming deficiency and hyperbiofilm phenotype of the PA4398$^-$ mutant, cyclic nucleotides were extracted from liquid-grown cultures, and c-di-GMP levels were quantified by LC–MS-MS. The PA4398$^-$ mutant strain exhibited a statistically significant (P, ≤0.01 by the Mann-Whitney test) 50% increase in the intracellular c-di-GMP concentration over that in the PA14 wild-type control (Fig. 4). No statistically significant difference was observed between the vector control strain (pUCP20) or the complemented strain (PA4398c).

To investigate whether this increased-biofilm-forming phenotype of the PA4398$^-$ mutant was also due to enhanced production of the glucose-rich exopolysaccharide Pel, which is, in addition to extracellular DNA, lipids, proteins, and ions (30, 40), an important component of the *P. aeruginosa* PA14 biofilm matrix and has been shown to be regulated by c-di-GMP (37, 41, 42), we evaluated exopolysaccharide production in a Congo red plate assay. As shown in Fig. 5, wild-type and PA4398$^-$ cells formed similar red, wrinkled colonies on agar plate surfaces due to the ability to bind CR, indicating that increased production of the biofilm matrix is most likely not responsible for the enhanced biofilm-forming phenotype of the sensor kinase mutant. This could be confirmed by further analysis of the expression of the polysaccharide biosyn-

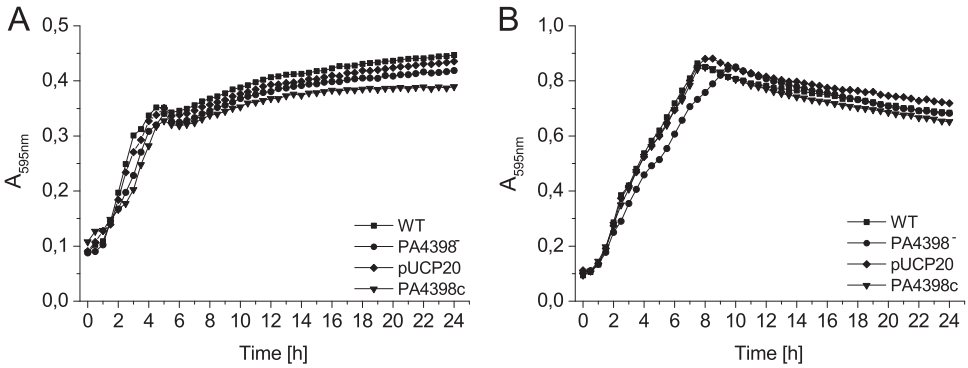

FIG 2 Growth curves of the *P. aeruginosa* PA14 WT, PA4398$^-$, pUCP20, and PA4398c strains. Cells were cultivated overnight in BM2 minimal medium, diluted to an OD_{595} of 0.1, and grown in BM2 swarm medium (A) or BM2 biofilm medium (B). Growth was measured every 30 min for 24 h at 37°C under shaking by the use of a microplate reader. The curves are representative of the results of three independent experiments with five technical replicates for each strain. Symbols: ■, WT PA14; ●, PA4398$^-$ mutant; ◆, pUCP20 strain; ▼, PA4398c strain.

TABLE 3 Rhamnolipid concentrations quantified by LC–MS-MS

P. aeruginosa PA14 strain	Concn (μg/ml)[a]	
	Monorhamnolipids	Dirhamnolipids
WT	24.8 ± 3.4	7.5 ± 1.5
PA4398⁻ mutant	24.3 ± 4.6	6.6 ± 0.5
pUCP20 strain	20.6 ± 7.2	6.1 ± 2.3
PA4398c strain	20.9 ± 9.3	5.9 ± 2.2

[a] Concentrations are normalized to the OD_{600} for each sample.

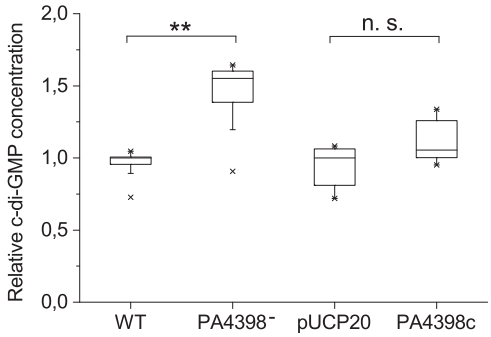

FIG 4 Relative intracellular c-di-GMP levels of PA14 wild-type and PA4398⁻ mutant cells. Intracellular c-di-GMP concentrations were measured by LC–MS-MS after the growth of three independent PA14 WT, PA4398 mutant, pUCP20, and PA4398c cultures in BM2 biofilm medium for 24 h at 37°C under shaking conditions. The c-di-GMP levels of the PA4398⁻ mutant were 1.5-fold higher than those of WT PA14, while the complemented PA4398c mutant did not exhibit significant differences in c-di-GMP levels from the pUCP20 control. The highest and lowest outliers are indicated by "×." Statistical significance was confirmed by the Mann-Whitney test (**, $P = 0.0086$; n. s., not significant).

thesis gene *pelA* under biofilm-forming conditions, which revealed no differences in transcriptional levels between the PA14 WT and PA4398⁻ mutant strains (data not shown).

Analyses of gene expression by the PA4398⁻ mutant. Initial semiquantitative real-time PCR analyses revealed that the histidine kinase-encoding gene PA4398 is constitutively expressed during the exponential phase and, to a lesser extent, during the stationary growth phase and that PA4398 is not differentially regulated in *P. aeruginosa* PA14 planktonic and swarmer cells (data not shown). In order to obtain detailed insight into the regulatory network of the histidine kinase PA4398, microarray studies comparing PA14 PA4398⁻ to wild-type cells were performed. For this purpose, RNA was isolated from the leading edges of PA14 wild-type and PA4398⁻ swarm zones after growth for 20 h at 37°C on BM2 swarm plates. Subsequently, microarray analysis identified 64 genes that were significantly ($P \leq 0.05$) dysregulated by more than 1.5-fold; of these, 25 genes were upregulated and 39 were downregulated. Of note, for transcriptional studies of the PA14 PA4398⁻ and wild-type strains, PAO1 DNA microarray slides were used, since no PA14-specific microarray slides are available. The PAO1 and PA14 genomes differ slightly in size (6.3 Mbp for PAO1 and 6.5 Mbp for PA14), and more than 92% of all genes present in PA14 are also present in PAO1 (8). Table 4 summarizes the functions of selected genes that are dysregulated in the PA4398⁻ mutant under swarming conditions relative to expression in wild-type swarmer cells. Additionally qPCR experiments confirmed the microarray data obtained for the *exoY*, $hasA_p$, *pchF*, *pscJ*, *pvdA*, *pvdD*, and *pvdQ* genes (Table 4).

In addition to an upregulation of genes associated with the type III secretion system (TTSS) (see Table S1 in the supplemental material), we observed a significant downregulation (approximately 2- to 44-fold) of genes involved in the biosynthesis and transport of the two major siderophores pyoverdine and pyochelin in PA4398⁻ cells relative to expression in WT PA14 cells (Table 4). In particular, microarray data analysis revealed downregulation by 44-fold and 28-fold, respectively, of genes coding for PvdA, one of the initial enzymes in the biosynthetic pathway of pyoverdine in the cytoplasm, and PvdQ, which is involved in pyoverdine maturation in the periplasm (43). Additionally, PvdQ, which exhibits an *N*-acyl homoserine lactone (HSL) acylase activity and has been shown to degrade the quorum-sensing signal 3-oxododecanoyl-HSL ($3OC_{12}$-HSL), is known for its role in biofilm formation, swarming motility, and virulence (16, 44). Moreover, the pyochelin synthesis genes coding for PchB (involved in the first pyochelin biosynthesis step, the conversion of chorismate to salicylate) and for PchD, PchF, and PchG (which play important roles in salicylate adenylation as well as in ring formation and pyochelin maturation) are downregulated 2- to 3-fold. In contrast, the ABC transporter PchH may have a redundant function in pyochelin transport (45).

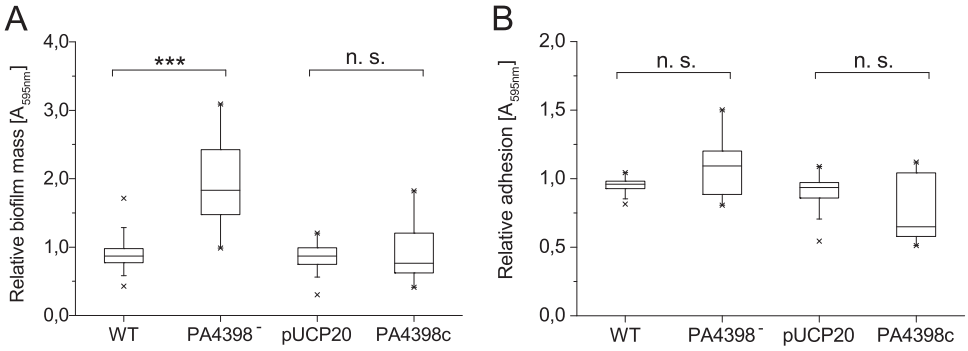

FIG 3 Biofilm formation and rapid attachment. Bacteria were grown for 24 h (A) or for 1 h (B) at 37°C in 96-well microtiter plates containing BM2 biofilm medium. Biofilm formation and adhesion ability were analyzed by staining of the adherent biomass with crystal violet, followed by quantification (A_{595}) for the stained wells. Shown are the results of at least three independent biological experiments, each with six technical repeats. The statistical significance of differences between the PA4398⁻ mutant and WT PA14, as well as between the PA4398c and pUCP20 strains, was determined by the Mann-Whitney test (***, $P \leq 0.001$; n. s., not significant). The highest and lowest outliers are indicated by "×."

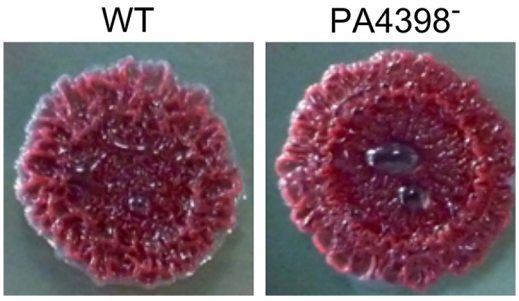

FIG 5 Congo red binding assay. Extracellular matrix production by *P. aeruginosa* strains was evaluated on tryptone agar plates containing CR and Coomassie brilliant blue G after incubation at room temperature for 10 days. Representative images of the colony morphologies of WT PA14 and the PA4398$^-$ mutant are shown.

Determination of intracellular nucleotides showed a strong increase in c-di-GMP levels in the PA4398$^-$ mutant over those in wild-type PA14 cells. To investigate the potential molecular basis for this observation, we performed more-sensitive qPCR of all known diguanylate cyclase (DGC) and phosphodiesterase (PDE) genes (shown to be involved in the synthesis and degradation of c-di-GMP, respectively) in *P. aeruginosa* PA14. These studies revealed that the GGDEF domain-containing DGCs PA2870 (fold change, +1.4 ± 0.1) and PA4396 (fold change, +3.4 ± 0.5), as well as the DGC/PDE hybrid PA2072 (fold change, +1.4 ± 0.1), were upregulated, whereas the EAL domain-containing PDE PA2200 was downregulated 1.7-fold (±0.4-fold), in the PA4398$^-$ sensor kinase mutant relative to expression in WT PA14. This dysregulation of c-di-GMP-related genes is consistent with the increase observed in intracellular c-di-GMP levels in the PA4398$^-$ mutant.

Knockout of PvdR, FptA, PchD, or PchA results in swarming defects. It was shown previously that siderophores play an important role in surface motility in *P. aeruginosa* (17). Since our microarray experiments revealed the downregulation of several genes involved in the synthesis and function of the two siderophores pyoverdine and pyochelin in the PA4398$^-$ mutant under swarming conditions, we were interested in whether siderophore-associated genes might contribute to swarming motility in *P. aeruginosa*. With this aim, we used the PA14 transposon mutant library (22) to screen mutants with transposon insertions in pyoverdine- and pyochelin-related genes for swarming behavior. This screen included genes with and without changes in expression as observed in our microarray experiments. No swarming defects were observed for mutants with mutations in *fpvA* (ID 43776), *pvdA* (ID 30448), *pvdL* (ID 24971), *pvdS* (ID 34241), or *pchF* (ID 24493) relative to the wild-type strain (Fig. 6). However, transposon insertions in *pvdR* (ID 53759), encoding a pyoverdine efflux transporter, and in *fptA* (ID 41286), encoding a pyochelin outer membrane receptor precursor, as well as in the pyochelin biosynthesis genes *pchD* (ID 42509) and *pchA* (ID 35443), re-

TABLE 4 Comparative analyses of gene expression by the PA4398$^-$ mutant and WT PA14

Locus tag	Gene name	Gene product	Fold change in gene expression[a]
Microarray analysis			
Pyoverdine biosynthesis, transport, and secretion			
PA2385	*pvdQ*	3-Oxo-C_{12}-homoserine lactone acylase (PvdQ)	−28.9
PA2386	*pvdA*	L-Ornithine N^5-oxygenase	−44.1
PA2390	*pvdT*	PvdT	−3.8
PA2392	*pvdP*	PvdP	−7.5
PA2393		Dipeptidase	−13.2
PA2394	*pvdN*	PvdN	−21.6
PA2397	*pvdE*	Pyoverdine biosynthesis protein (PvdE)	−11.4
PA2398	*fpvA*	Ferripyoverdine receptor	−17.4
PA2399	*pvdD*	Pyoverdine synthetase D	−18.8
PA2413	*pvdH*	L-2,4-Diaminobutyrate:2-ketoglutarate 4-aminotransferase (PvdH)	−7.9
PA2426	*pvdS*	Sigma factor (PvdS)	−12.8
Pyochelin biosynthesis and transport			
PA4223	*pchH*	Probable ATP-binding component of ABC transporter	−2.4
PA4224	*pchG*	Pyochelin biosynthetic protein (PchG)	−2.7
PA4225	*pchF*	Pyochelin synthetase	−3.0
PA4228	*pchD*	Pyochelin biosynthesis protein (PchD)	−2.4
PA4230	*pchB*	Salicylate biosynthesis protein (PchB)	−1.9
qPCR analysis			
PA1723	*pscJ*	Type III export protein (PscJ)	+2.12 ± 0.2
PA2191	*exoY*	Adenylate cyclase (ExoY)	+1.98 ± 0.1
PA2385	*pvdQ*	3-Oxo-C_{12}-homoserine lactone acylase (PvdQ)	−15.9 ± 3.8
PA2386	*pvdA*	L-Ornithine N^5-oxygenase	−465.8 ± 20.7
PA2399	*pvdD*	Pyoverdine synthetase D	−143.9 ± 70.9
PA3407	$hasA_p$	Heme acquisition protein ($HasA_p$)	−9.2 ± 4.4
PA4225	*pchF*	Pyochelin synthetase	−10.4 ± 2.8

[a] Fold regulation of genes differentially expressed in the PA4398$^-$ mutant relative to expression in wild-type PA14. A positive number indicates upregulation, and a negative number indicates downregulation, of transcripts in the mutant.

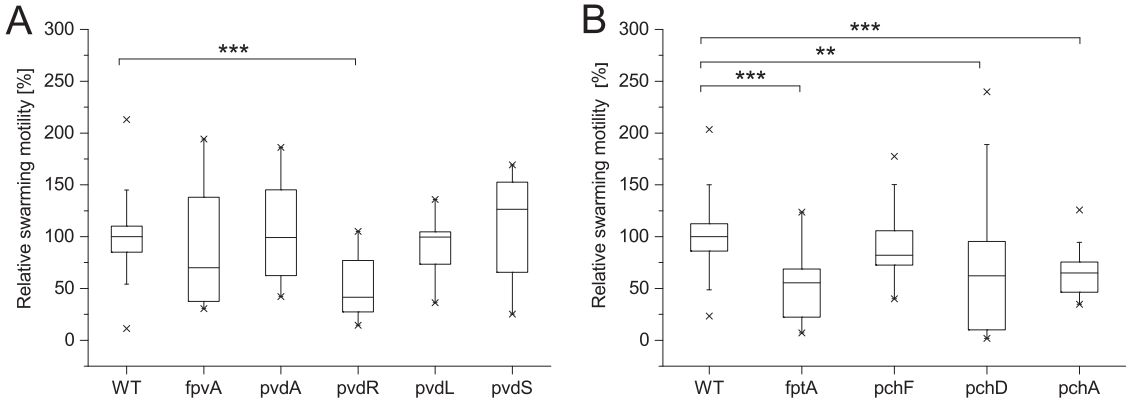

FIG 6 Swarming motilities of selected *P. aeruginosa* PA14 pyoverdine and pyochelin mutants. Swarming motility was assayed on BM2 swarm plates after a 20-h incubation, and agar plate surface coverage was determined by at least three independent experiments with five replicates for each mutant. (A) Among pyoverdine biosynthesis or transporter gene mutants, only the *pvdR* (ID 53759) mutant showed impaired swarming motility, while the *fpvA* (ID 43776), *pvdA* (ID 30448), *pvdL* (ID 24971), and *pvdS* (ID 34241) mutants exhibited swarming motilities comparable to that of the wild type. (B) Mutation of the pyochelin gene *fptA* (ID 41286), *pchD* (ID 42509), or *pchA* (ID 35443) resulted in impaired swarming motility relative to that of the parental wild-type strain. In contrast, a *pchF* (ID 24493) mutant showed no alteration in swarming motility. The highest and lowest outliers are indicated by "×." Statistical significance was calculated by the nonparametric Mann-Whitney test (***, $P \leq 0.001$; **, $P \leq 0.01$).

vealed statistically significant ($P \leq 0.01$) impairments in swarming, indicating that these genes are involved in the swarming motility of *P. aeruginosa*.

Mutation of the adjacent two-component response regulator PA14_57140 (PA4396) demonstrates phenotypes different from those of the PA4398⁻ mutant. The two-component response regulator PA14_57140 (corresponding to *P. aeruginosa* PAO1 locus PA4396) is located only two genes upstream of the histidine kinase PA4398. Since PA4396, which harbors a degenerate GGDEF domain (46), was also upregulated in the PA4398 mutant, we wondered if this adjacent regulator might be involved in swarming and biofilm formation in *P. aeruginosa* PA14. To test this, we phenotypically characterized the corresponding PA14_57140 transposon insertion mutant (ID 30693) (22) with regard to motility and the ability to form biofilms. In contrast to the PA4398⁻ mutant, no differences from wild-type PA14 in swarming motility, biofilm formation, or swimming and twitching motility could be observed for the PA14_57140 mutant (data not shown), indicating that regulation, at least for these phenotypes, is independent of this response regulator.

DISCUSSION

In this study, we investigated the role of the sensor kinase PA4398 in the swarming motility and biofilm formation of *P. aeruginosa* PA14. For this purpose, a PA4398 insertion mutant was constructed; it showed strong impairment in swarming motility on viscous surfaces but exhibited a 2-fold increase in biofilm formation.

The swarming motility of *P. aeruginosa* requires both a functional flagellum and type IV pili, as well as the production of rhamnolipids, which function as biosurfactants lowering the surface tension (11, 14, 15). The ability of the PA4398⁻ mutant to swim and twitch at wild-type levels indicated that the sensor kinase mutant is most likely not attenuated in the biosynthesis and functionality of flagella and type IV pili, respectively. However, changes in the frequency of flagellum reversals, which have been shown to impact swarming motility in a viscosity-dependent manner (36, 37, 47), cannot be completely excluded. Growth analyses in liquid cultures, as well as determination of the CFU counts of swarmer cells grown on BM2 swarm agar plates, also showed no growth defects for the PA4398⁻ mutant under planktonic and swarming growth conditions. Furthermore, determination of mono- and dirhamnolipid concentrations by LC–MS-MS revealed no significant differences between the wild-type PA14 strain and the PA4398⁻ mutant. These results are in accordance with those of the microarray studies, in which genes related to motility and rhamnolipid biosynthesis were not dysregulated in the mutant, indicating that PA4398 may affect other swarming-related genes in *P. aeruginosa*.

One important modulator governing swarming motility and biofilm formation in *P. aeruginosa* and other Gram-negative bacteria is the intracellular second messenger c-di-GMP, which is synthesized and hydrolyzed by diguanylate cyclases and phosphodiesterases, respectively. While lower concentrations of this molecule support motility, higher concentrations result in cell aggregation and subsequent biofilm formation (39, 48). In agreement with the phenotypes observed, we detected 50% higher intracellular c-di-GMP concentrations in PA4398⁻ cells than in wild-type or PA4398c complemented mutant cells. Since our microarray analyses failed to identify any significant (≥1.5-fold) dysregulation of genes coding for diguanylate cyclases or phosphodiesterases, we performed more-sensitive qPCR studies of all genes known so far that are involved in c-di-GMP synthesis or hydrolysis in *P. aeruginosa* PA14. These investigations revealed >3-fold upregulation of the PA4396 open reading frame (ORF) in the PA4398⁻ mutant. The PA4396 ORF exhibits homologies to two-component response regulators in addition to a degenerate GGDEF domain (DEQHF) (46) with an intact c-di-GMP binding I-site motif. However, while it was shown previously that PA4396 is able to bind c-di-GMP, no diguanylate cyclase activity was detected under the conditions tested (49), indicating that PA4396 most likely is not responsible for the increased intracellular c-di-GMP levels in the PA4398⁻ mutant. In addition to PA4396, we observed the dysregulation of three other genes encoding DGCs or PDEs. The two DGC-encoding genes, PA2072 and PA2870, were slightly upregulated, whereas the gene encoding the c-di-GMP-hydrolyzing PDE PA2200 was downregulated almost 2-fold in the

PA4398⁻ mutant relative to the WT, providing an explanation for the increased c-di-GMP levels. Recently, it was shown that changes in the expression of the PA2200 gene indeed affect the swarming behavior of *P. aeruginosa* PA14 strongly; however, only semiquantitative real-time PCR was performed, and therefore, no exact expression levels were determined (50). However, our analyses suggest that the sensor kinase PA4398 modulates intracellular c-di-GMP levels by affecting the expression of a set of DGC and PDE genes in *P. aeruginosa* PA14. In this context, it is also worth mentioning that total intracellular c-di-GMP levels are not the only important determinant of cellular behavior, since comparison of different diguanylate cyclases, e.g., RoeA and SadC, revealed distinct phenotypes for each individual DGC, despite similar levels of total c-di-GMP (47). This indicates that a mechanism independent of simple modified gene expression of diguanylate cyclases or phosphodiesterases—an interplay of multiple c-di-GMP-synthesizing or -hydrolyzing enzymes and effector proteins—is also important for c-di-GMP-dependent phenotypes. Since the Congo red binding assay and qPCR analysis of the Pel biosynthesis gene *pelA* showed no differences in polysaccharide synthesis between the PA4398⁻ mutant and WT PA14, the molecular mechanisms by which enhanced c-di-GMP levels influence biofilm formation in the PA4398⁻ mutant still remain to be investigated. In addition to the biofilm matrix, cellular appendages, including type IV pili, flagella, und Cup fimbriae, are important adhesins that facilitate biofilm formation by *P. aeruginosa* and are also regulated in part via the second messenger c-di-GMP (21, 51–53). Moreover, it has been shown recently that c-di-GMP controls flagellum reversal rates, thus impacting bacterial motility and biofilm formation (36, 47), which might also relate to the PA4398⁻ phenotypes observed.

In addition to elevated c-di-GMP concentrations, our microarray analysis in combination with follow-up experiments demonstrated the downregulation of several genes required for normal swarming behavior in *P. aeruginosa* PA14. In particular, genes coding for proteins involved in the biosynthesis and transport of the iron-scavenging siderophores pyochelin and pyoverdine were downregulated approximately 2- to 44-fold in the PA4398⁻ mutant strain. PA14 mutants with insertions in *pvdQ*, *pvdD*, *pvdR*, *fptA*, *pchD*, *pchA*, or *pchH* exhibited significant swarming defects in this and previous studies (16, 23). Bacteria secrete siderophores to sequester iron from the environment, including the human body, and to transport it into the cell (44). Interestingly, it was shown previously that both synthesis pathways were highly upregulated in swarmer cells relative to planktonically grown bacteria (16), whereas downregulation of these genes in cells of the swarm colony tendril tips relative to the swarm center was demonstrated by Tremblay and Déziel (54), suggesting that swarmer cells, on the one hand, have a greater need for iron than free-swimming bacteria and that iron availability, on the other hand, is restricted in the swarm center (54). Since swarming motility is positively influenced by iron-depleted conditions and is inhibited under iron-rich conditions, iron seems to play an important role in the initiation of this multicellular behavior (55). The siderophore pyoverdine, a complex molecule used by *P. aeruginosa* as the primary iron source (44, 56), is an important virulence factor that is implicated in biofilm control and cell-to-cell communication and has also been demonstrated to influence the expression of virulence genes, including exotoxin A and PrpL protease (44, 56). Our data demonstrated approximately 4- to 44-fold downregulation of pyoverdine biosynthesis and transport genes in the PA4398⁻ mutant relative to WT PA14 under swarming conditions. Among others, *pvdQ* exhibited a strong, 28-fold downregulation. Besides its role in pyoverdine biosynthesis, PvdQ has been shown to hydrolyze the HSL 3-oxo-C_{12}-HSL, the major signaling molecule of the QS system, by its acylase activity (44, 57) and to control swarming motility, either through QS signal degradation or through involvement in pyoverdine production (16, 44). Jimenez et al. demonstrated that the swarming defect of a *pvdQ* mutant could be restored by exogenous iron (44). The addition of iron or pyoverdine also complemented the swarming-deficient phenotype of a *Pseudomonas putida* KT2440 pyoverdine mutant (17). Furthermore, this group stated that this pyoverdine-related mutant exhibited swarming motility at wild-type levels in the presence of the exogenous iron chelate complex ferric citrate, whose uptake occurs independently of pyoverdine, and proposed that the intracellular iron concentration or active transport of chelated iron is the important signal for swarming motility (17). Concerning these observations, we analyzed the swarming of the PA4398⁻ mutant in the presence of different iron sources. However, no complementation of swarming to wild-type levels could be achieved using $FeSO_4$ and $FeCl_3$ or ferric citrate in a range of 0 to 300 μM (data not shown), indicating that iron by itself is not responsible for the observed swarming-deficient phenotype of the PA4398⁻ mutant. In addition to *pvdQ*, we were able to show that *pvdR* is also connected to the swarming motility of *P. aeruginosa* PA14, since the *pvdR* mutant had a significant impairment in swarming. The PvdR protein is part of the *pvdRT-opmQ* operon, coding for an efflux system, which is supposed to be involved in the recycling of the apo-siderophores as well as in the secretion of newly synthesized pyoverdine (56, 58, 59). Although the precise roles of pyoverdine and the pyoverdine biosynthesis and transport proteins in swarming motility are still unknown, it is noteworthy that pyoverdine also plays an important role in the swarming of *Pseudomonas syringae*, where it probably functions as a signaling molecule that regulates not only swarming motility but also cellular processes, including biofilm formation, antibiotic tolerance, and virulence (60).

Pyochelin represents a second siderophore of *P. aeruginosa* that exhibits low iron affinity and is encoded by 3 divergent operons—*pchDCBA* and *pchEFGHI*, coding for biosynthesis genes, and *fptABCX*, coding for a membrane transporter—and its transcriptional regulator, *pchR* (45). The pyochelin biosynthesis genes *pchD*, *pchB*, *pchF*, and *pchG*, as well as *pchH*, a probable ABC transporter, were found to be downregulated in the PA4398⁻ mutant. Analyses of selected mutants with transposon insertions in pyochelin-related genes identified *fptA*, *pchA*, *pchD* (this study), and *pchH* (23) as essential for normal swarming motility in *P. aeruginosa*. Our results indicate that not only *pvdQ* but also *pvdR*, *fptA*, *pchA*, *pchD*, and *pchH* may contribute to the swarming deficiency of the PA4398⁻ mutant. Interestingly, *pchH* was found to play a role in the virulence of *P. aeruginosa*, as evidenced by the fact that a *pchH* transposon mutant displayed a strong decrease in virulence against the social amoeba *Dictyostelium discoideum*, probably through repression of the TTSS, while other genes of the same gene cluster, such as *pchE* or *pchF*, exhibited only very minor virulence attenuation (61), suggesting that different phenotypes are caused by individual mutations in the pyochelin biosynthesis pathway.

Most strikingly, the microarray data obtained revealed induc-

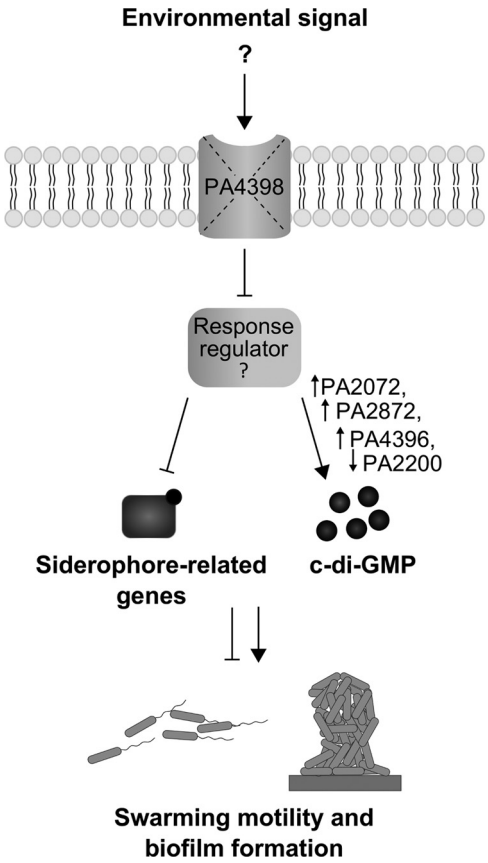

FIG 7 Effects of PA4398 inactivation in *P. aeruginosa* PA14. The *P. aeruginosa* PA14 sensor kinase PA4398 is activated by an unknown environmental signal, leading to signal transduction via an unidentified transcriptional regulator. Inactivation of the sensor kinase in the PA4398$^-$ mutant resulted in a swarming-deficient phenotype but a higher level of production of biofilm biomass than that of the PA14 wild-type control. In agreement with the observed swarming defect, gene expression analysis under swarming conditions revealed downregulation of genes involved in the biosynthesis, transport, and function of the siderophores pyoverdine and pyochelin. In addition, higher intracellular c-di-GMP levels could be quantified in the PA4398$^-$ mutant than in WT PA14.

tion of TTSS biosynthesis genes in the PA4398$^-$ mutant under swarming conditions relative to expression in the wild-type strain (see Table S1 in the supplemental material). This is in contrast to general knowledge about *P. aeruginosa* lifestyles in that motility is associated with TTSS-dependent acute infections, whereas the formation of biofilms contributes to chronic infections (62–64). However, a study by Matz et al. demonstrated that *P. aeruginosa* PAO1 biofilms respond to colonization by the environmental amoeba *Acanthamoeba castellanii* with the upregulation of TTSS genes (65). Moreover, small-colony variants isolated from chronically infected CF patients exhibited both enhanced production of biomass and increased TTSS expression and cytotoxicity, demonstrating that the inverse regulation of biofilm formation and the TTSS is not absolute (66, 67).

Overall, the data presented here demonstrate that the histidine kinase PA4398 plays a role in the regulation of swarming motility and biofilm formation in *P. aeruginosa* PA14 (Fig. 7). The PA4398$^-$ mutant of PA14 exhibited attenuated expression of genes associated with c-di-GMP metabolism and with the production of the siderophores pyoverdine and pyochelin. Future work will focus on identifying the cognate response regulator and unraveling the precise role of this histidine kinase in the regulatory network of *P. aeruginosa*.

ACKNOWLEDGMENTS

This work was supported by the BioInterfaces (BIF) program of Karlsruhe Institute of Technology (KIT) in the Helmholtz Association, the "Concept for the Future" of KIT within the German Excellence Initiative, and the Deutsche Forschungsgemeinschaft. The funders had no role in study design, data collection and analysis, the decision to publish, or the preparation of the manuscript.

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