RESEARCH ARTICLE

Specific Control of *Pseudomonas aeruginosa* Surface-Associated Behaviors by Two c-di-GMP Diguanylate Cyclases

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ABSTRACT The signaling nucleotide cyclic diguanylate (c-di-GMP) regulates the transition between motile and sessile growth in a wide range of bacteria. Understanding how microbes control c-di-GMP metabolism to activate specific pathways is complicated by the apparent multifold redundancy of enzymes that synthesize and degrade this dinucleotide, and several models have been proposed to explain how bacteria coordinate the actions of these many enzymes. Here we report the identification of a diguanylate cyclase (DGC), RoeA, of *Pseudomonas aeruginosa* that promotes the production of extracellular polysaccharide (EPS) and contributes to biofilm formation, that is, the transition from planktonic to surface-dwelling cells. Our studies reveal that RoeA and the previously described DGC SadC make distinct contributions to biofilm formation, controlling polysaccharide production and flagellar motility, respectively. Measurement of total cellular levels of c-di-GMP in Δ*roeA* and Δ*sadC* mutants in two different genetic backgrounds revealed no correlation between levels of c-di-GMP and the observed phenotypic output with regard to swarming motility and EPS production. Our data strongly argue against a model wherein changes in total levels of c-di-GMP can account for the specific surface-related phenotypes of *P. aeruginosa*.

IMPORTANCE A critical question in the study of cyclic diguanylate (c-di-GMP) signaling is how the bacterial cell integrates contributions of multiple c-di-GMP-metabolizing enzymes to mediate its cognate functional outputs. One leading model suggests that the effects of c-di-GMP must, in part, be localized subcellularly. The data presented here show that the phenotypes controlled by two different diguanylate cyclase (DGC) enzymes have discrete outputs despite the same total level of c-di-GMP. These data support and extend the model in which localized c-di-GMP signaling likely contributes to coordination of the action of the multiple proteins involved in the synthesis, degradation, and/or binding of this critical signal.

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The molecule cyclic di-GMP (c-di-GMP) has earned a reputation as an important signal for biofilm formation (1) and, in particular, regulating bacterial phenotypes associated with a sessile lifestyle, including localization of cell surface adhesins (3, 40), production of the exopolysaccharide (EPS) matrix (4–6), and suppression of various forms of motility (7–9). The amount of c-di-GMP within the cell is mediated by the opposing activities of two classes of proteins. Diguanylate cyclases (DGCs) are responsible for the synthesis of c-di-GMP from GTP, while phosphodiesterases (PDEs) degrade c-di-GMP to an inactive, linear form. DGCs contain a well-conserved GGDEF domain (10), and PDEs specific for c-di-GMP are characterized by the presence of an EAL or HD-GYP domain (11, 12).

One perplexing aspect of c-di-GMP-mediated signaling is the observed multitude of GGDEF and EAL domain-containing proteins encoded in individual bacterial genomes. For example, *Pseudomonas aeruginosa* PA14 expresses up to 16 GGDEF, 5 EAL, and 16 GGDEF/EAL domain proteins (13). Despite this apparent redundancy, mutating a single DGC, for example, the enzyme encoded by the *sadC* gene, has been shown to impact both swarming motility and the transition from reversible to irreversible attachment during early biofilm formation by *P. aeruginosa* (14). While the DGC SadC is important for biofilm formation, it has only a small effect on EPS production, despite the fact that production of the Pel EPS in *P. aeruginosa* is regulated by c-di-GMP (14). These findings evoke an important open question: how are such precise phenotypic outputs specified in the context of almost 40 enzymes with the predicted capacity to synthesize and/or degrade c-di-GMP?

In this report, we describe the roles of two c-di-GMP DGCs in the regulation of biofilm formation via their respective control of flagellar motility and EPS production. Our data show conclusively that the phenotypic outputs controlled by these DGCs are independent of the total level of c-di-GMP and suggest that alternative mechanisms such as localized production or sensing of c-di-GMP must be considered with regard to the control of swarming motil-

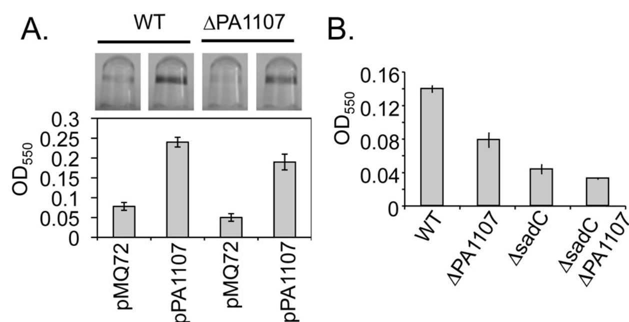

FIG 1 Identification of a DGC involved in biofilm formation. (A) Biofilms at 24 h comparing the effects of expressing a His-tagged variant of PA1107 in multicopy plasmid pPA1107 to its vector control (pMQ72) in either the WT or the ΔPA1107 mutant background. The static biofilm assay was performed with M63 G/CAA medium. The bar graph represents quantification of the biofilm assays using OD_{550} readings of solubilized (CV) solution averaged from four wells of each strain, and the error bars indicate standard deviation. Shown are CV-stained wells (top) and quantification of biofilm formation (bottom). (B) Biofilms at 24 h comparing the WT, the ΔPA1107 and Δ*sadC* single mutants, and the Δ*sadC* ΔPA1107 double mutant. Assays were performed as described for panel A.

ity and EPS production by *P. aeruginosa*, as is likely the case in other c-di-GMP-regulated processes.

RESULTS

A DGC important for biofilm formation. To better understand the impact of c-di-GMP on biofilm initiation, we tested strains from the nonredundant *P. aeruginosa* PA14 transposon library carrying mutations in genes coding for putative DGCs using a microtiter dish assay (15). We identified three candidate strains with biofilm defects under our standard biofilm assay conditions that harbored mutations in the PA1107 (PA14_50060), PA3177 (PA14_23130), and PA3343 (PA14_20820) genes. Single-crossover insertion mutations were introduced into these open reading frames (ORFs), as well as PA2870 (PA14_26970), because this ORF was not available from the nonredundant library. Only the single-crossover mutation in PA1107 resulted in a consistent observable biofilm defect (not shown). A deletion mutation of the PA1107 ORF was constructed (ΔPA1107), and a biofilm formation defect was observed at 24 h in the microtiter dish assay (Fig. 1A). We observed a biofilm defect in the PA1107 mutant as early as 8 h (not shown). The phenotype of this mutant was complemented by a plasmid containing a His-tagged variant of PA1107 (pPA1107; Fig. 1A). Furthermore, a plasmid expressing PA1107 was also capable of stimulating biofilm formation in a wild-type (WT) background (Fig. 1A).

To our knowledge, there has been only one other *P. aeruginosa* gene encoding a DGC that, when deleted, produces a biofilm formation defect under the conditions tested here. Like the ΔPA1107 mutant strain, the Δ*sadC* (PA4332, PA14_56280) mutant has reduced but not completely defective biofilm formation when assayed in a microtiter dish at 8 h (14) or 24 h (Fig. 1B). Thus, we constructed a strain in which the genes encoding both DGCs are deleted. We find that a Δ*sadC* ΔPA1107 double mutant has a more severe biofilm defect than a strain individually lacking either the PA1107 or the *sadC* gene (Fig. 1B).

RoeA (PA1107) is a DGC. The presence of a well-conserved GGDEF domain is a hallmark of DGCs. PA1107 has the alternative motif GGEEF found in other active DGC enzymes (13), but this protein does not have an EAL domain. Furthermore, a previous study showed that extracts made from a strain carrying a PA1107 expression plasmid had detectable DGC activity (13). To investigate whether PA1107 acts as a DGC, we assessed the effect of overexpressing this protein on the total levels of cellular c-di-GMP. Total c-di-GMP levels were visibly elevated in the presence of plasmid-expressed PA1107 (Fig. 2A, box), and the c-di-GMP level was found to be significantly higher in the presence of multicopy PA1107 than in the vector control (Fig. 2B). The increased c-di-GMP level supports the idea that PA1107 is a DGC and is in agreement with a previous finding that a plasmid-encoded copy of PA1107 increased c-di-GMP levels *in vivo* (13).

To confirm that the GGDEF domain of PA1107 is important for function, we tested the impact of mutating this domain on the production of the biofilm-associated Pel polysaccharide, which is know to respond to c-di-GMP (5, 14, 16, 17). His-tagged PA1107 expressed from a multicopy plasmid in either the WT or the ΔPA1107 mutant resulted in stimulation of Pel polysaccharide (Fig. 2C), and this stimulation was completely dependent on a functional *pelA* gene (Fig. 2D). Mutating the critical GGEEF motif to GGAAF resulted in loss of stimulation of Pel polysaccharide production, despite an apparent increased abundance of the mutant protein (Fig. 2C). Similarly, expressing the GGAAF variant of PA1107 from a plasmid could not stimulate biofilm formation or suppress swarming motility, as was observed when expressing WT PA1107 (see Fig. S1 in the supplemental material).

Using the mutant protein described above, we also assessed the ability of PA1107 expressed on a plasmid, versus the mutant variant PA1107-AA, to stimulate c-di-GMP levels *in vivo* as measured by liquid chromatography-mass spectrometry (LC-MS). Starting in a ΔPA1107 genetic background, we found that expressing PA1107 on a plasmid significantly stimulated c-di-GMP levels versus those of the ΔPA1107 strain carrying the vector control (22-fold, $P < 0.05$). In contrast, expressing the PA1107-AA mutant variant did not result in a significant change in c-di-GMP levels versus those of the vector control (0.6-fold change, $P = 0.088$).

We attempted to demonstrate DGC activity with purified PA1107 but were not successful despite the finding that PA1107 increased c-di-GMP levels *in vivo* (Fig. 2A and B) and the fact that DGC activity has been reported in a crude extract prepared from a strain carrying a PA1107 expression plasmid (13). To overcome the difficulties of working with this predicted membrane protein, chimeric proteins were created that replaced the GGDEF domain of PleD* with either the WT or a catalytic mutant version of the PA1107 GGDEF domain based on annotation generated using SMART (http://smart.embl-heidelberg.de/) (Fig. 2E). Thus, these hybrid proteins contain the N-terminal portion of PleD*, containing two REC domains, and the C terminus of PA1107, including its entire GGDEF domain or the corresponding catalytic mutant form. These proteins were stably expressed *in vivo* and tested for the ability to increase extracellular polysaccharide (EPS) production; a phenotype commonly observed upon the overexpression of a DGC. The chimera with the WT GGEEF domain of PA1107, but not the GGAAF-containing chimera, was capable of stimulating Pel polysaccharide production (Fig. 2F). We constructed similar chimeras with glutathione *S*-transferase (GST) and observed consistent results, with expression of the WT GST-GGEEF chi-

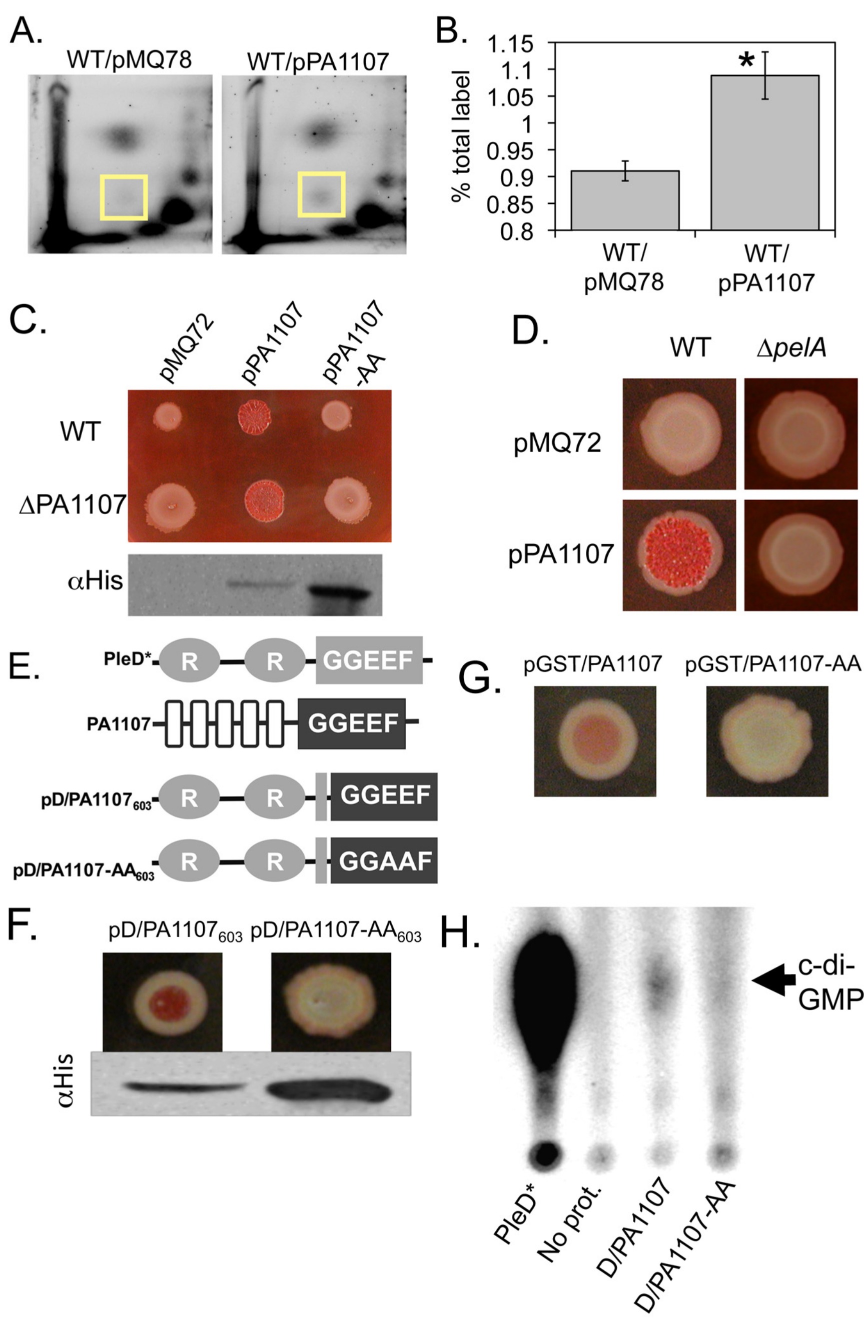

FIG 2 PA1107 is a DGC. (A) Levels of c-di-GMP were quantified after [^{32}P]orthophosphate labeling of bacterial cultures, followed by extraction of the nucleotides and analysis via 2D-TLC, for the WT strain expressing a vector control (pMQ78) or a His-tagged variant of PA1107 on a multicopy plasmid (pPA1107). (B) Graphical representation comparing the pixel densities of the c-di-GMP spots from the 2D-TLC plates in panel A normalized to the total pixel density ($n = 3$). Error bars indicate standard deviations. *, $P < 0.05$. (C) The WT or ΔPA1107 mutant strain carrying a vector control (pMQ72) or a plasmid expressing a His-tagged variant of WT PA1107 (pPA1107) or a mutant variant altered in the GGEEF domain (pPA1107-AA) was spotted onto CR plates supplemented with arabinose at 0.2%. Plates were imaged after incubation at 37°C for 24 h. This relatively short incubation period is not sufficient for the differences between the WT and Δ*roeA* mutant strains to become visible. (Bottom) Western blot assay of strains containing the vector control (pMQ72), pPA1107, or pPA1107-AA plasmid probed with anti-His antibody. Loading for the Western blot assay was normalized by OD_{600}. (D) WT or Δ*pelA* mutant strain carrying a vector control plasmid (pMQ72) or pMQ72 expressing a His-tagged variant of PA1107. CR plates were supplemented with 0.2% arabinose and incubated at 37°C for 24 h. Development of the red color depends on a functional *pel* locus. (E) Diagram of PleD* and PA1107 domain structure and chimera constructs used for DGC activity assays shown in panels F and H. PleD* (top) contains two N-terminal REC domains (grey ovals labeled "R," amino acids 3 to 116 and 154 to 265) and a GGDEF domain (box labeled "GGEEF," amino acids 271 to 452). PA1107 (second from the top) is predicted to have five transmembrane domains (white boxes, between amino acids 34 and 199) followed by a C-terminal GGDEF domain. The PleD*/PA1107 chimeras (bottom two constructs) contain the first 290 amino acids from PleD* and amino acids 202 to 398 from PA1107. The two chimeras contain a small portion of the GGEEF domain from PleD, as well as the complete GGEEF domain of PA1107. Light grey indicates PleD, while the black box indicates the GGEEF domain derived from PA1107. (F) Phenotypes of PleD* chimera constructs in the ΔPA1107 background on CR plates supplemented with 0.2% arabinose. Plates were incubated as described for panel C. (Bottom) Western blot assay of strains containing the pD/Roe_{603} or pD/Roe-AA_{603} plasmid. Strains were normalized by OD_{600} before analysis by Western blotting. (G) Chimeras similar to those described for panel F were built with GST replacing the N terminus of PleD*. Expression of the GST/PA1107 fusion, but not the mutant PA1107-AA variant with a mutation in the GGEEF domain, stimulated CR binding in the WT genetic background. (H) *In vitro* DGC assay with PleD*/PA1107 chimeras shown in panel F (D/PA1107, D/PA1107-AA), including a PleD* positive control and a no-protein (No prot.) negative control. Reaction products were separated by TLC, followed by exposure to a PhosphorImager plate. The arrow indicates the position of c-di-GMP on the TLC plate.

mera stimulating CR binding but the GST-GGAAF chimera showing no such stimulation (Fig. 2G).

We next assessed the function of the PleD* chimeras *in vitro*. Both of these chimeras could be purified, but only the construct with the WT GGEEF domain demonstrated *in vitro* DGC activity (Fig. 2H).

Given the role of PA1107 in the synthesis of c-di-GMP *in vitro* and *in vivo*, its impact on Pel polysaccharide production, and additional information presented below, we renamed the PA1107 ORF *roeA* for *r*egulator *o*f *e*xopolysaccharide *A*.

RoeA and SadC differentially contribute to biofilm formation. The biofilm-defective phenotypes of the Δ*sadC* and Δ*roeA* mutants led us to ask whether the SadC and RoeA DGCs might impact known factors involved in the formation of these communities. An extracellular matrix is a regular feature of biofilms, and the presence of EPS is generally considered to be a defining feature

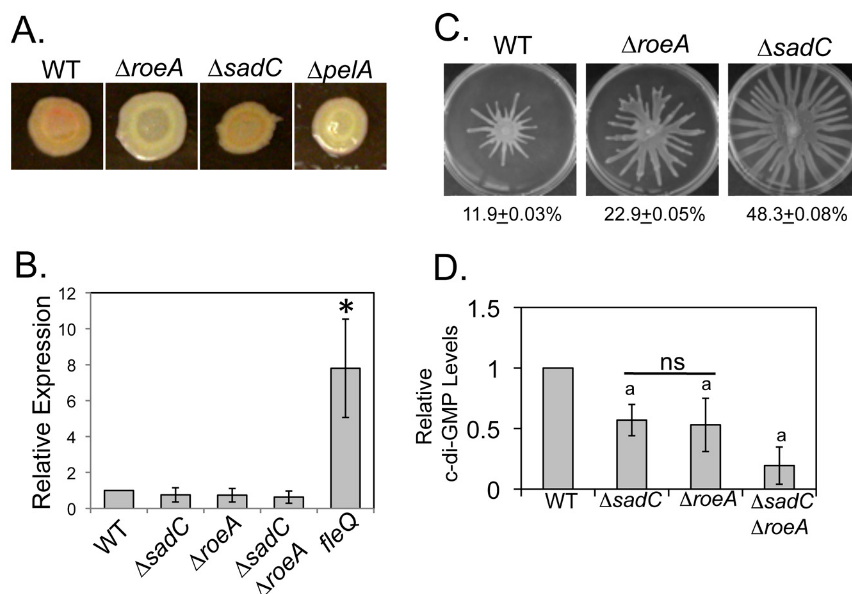

FIG 3 Mutation of *sadC* or *roeA* yields a distinct phenotype. (A) CR plates inoculated with an overnight liquid culture of the WT strain or the Δ*roeA*, Δ*sadC*, or Δ*pelA* mutant strain and incubated for 24 h at 37°C and then at room temperature for 3 days. (B) β-Galactosidase assays of cultures of the indicated strains grown on agar plates with M63 medium supplemented with glucose and CAA for 24 h at 37°C. These are the same conditions used for CR assays, except that the dyes were not added to the growth medium. The relative expression, compared to that of the WT (set at 1), is shown for each strain. Each bar depicts the average of two individual cultures, assayed in triplicate, and the error bars represent the standard deviation (*, $P = 0.019$ compared to the WT). (C) Swarm plates (0.5% agar) inoculated with liquid cultures of the WT strain or the Δ*roeA* or Δ*sadC* mutant strain. Plates were incubated for 16 h at 37°C. (Bottom) Surface area of a plate covered by the swarms (± standard deviation), which was calculated by averaging data from four individual swarm plates. (D) Relative c-di-GMP measurements of the WT (set to a value of 1) and the indicated mutant strains by LC-MS. Assays were performed as described in Materials and Methods. A lowercase letter a above a bar indicates a statistically significant difference from the WT level ($P < 0.05$). The abbreviation ns indicates that the values below the horizontal bar are not significantly different from each other.

of biofilms (18, 19). EPS produced by the Pel proteins is known to play a role in biofilm formation in *P. aeruginosa* PA14 (20, 21) and has been shown previously to be regulated by c-di-GMP (5, 14, 16, 17). Thus, we tested whether the *roeA* gene might also have a role in regulating EPS production.

Agar plates supplemented with the dye Congo red (CR) are a standard method for examining EPS synthesis, and the red color accumulated over time by a colony due to EPS-dependent CR binding serves as a useful surrogate for assessment of EPS production (20, 22). The Δ*roeA* mutant produces colonies that are nearly white on a CR plate and never develop the pink/red coloring associated with EPS production. This is in contrast to the light red color that accumulates in the WT strain after several days (Fig. 3A). Indeed, the Δ*roeA* mutant phenotype bears a close resemblance to that of the Δ*pelA* mutant, which cannot synthesize the Pel polysaccharide (20) (Fig. 3A). In contrast, the Δ*sadC* mutant exhibits a CR binding phenotype only marginally reduced compared to that of the WT, as was observed previously (14).

To assess whether the decreased Pel polysaccharide production is a consequence of reduced levels of the *pel* transcript in the Δ*roeA* mutant, we utilized a reporter system consisting of a *lacZ* transcriptional fusion to the *pel* promoter (23). The *pel-lacZ* fusion was integrated into strains of interest, and β-galactosidase activity was determined as a relative measure of *pel* transcript levels (2). We found that in cells grown on agar plates for 24 h (conditions chosen to correspond to those of CR assays), there was no significant change ($P > 0.05$) in *pel-lacZ* expression assessed in the WT compared to that in the Δ*roeA*, Δ*sadC*, or Δ*sadC* Δ*roeA* mutant background (Fig. 3B). Similar results were observed when these same strains were grown statically in microcentrifuge tubes to mimic biofilm growth conditions (not shown). These data indicate that the impact of RoeA on Pel EPS production is not at the level of transcription. As a control, similarly testing a strain carrying a mutation in the *fleQ* gene, which encodes a known repressor of *pel* gene expression in *P. aeruginosa* PAO1 (16), resulted in the expected increase in *pel* gene expression (Fig. 3B).

Motility is a second phenotype of *P. aeruginosa* related to biofilm formation, and thus, we also investigated the effects of RoeA and SadC on motility. While the Δ*roeA* mutant swims and twitches similarly to the WT, the Δ*roeA* mutant swarms ~2-fold better than the WT (Fig. 3C and data not shown). In contrast to the Δ*roeA* mutant, and as reported previously (14), the Δ*sadC* mutant shows an almost 5-fold increase in swarming motility, as judged by coverage of the agar surface area (Fig. 3C).

In previous studies, we found that strains altered for swarming motility show a distinct phenotype with regard to flagellar function; that is, these strains display a viscosity-dependent change in the frequency of flagellar reversals (14, 24, 25). Our studies have established that changes in swarming motility act as a robust surrogate for this change in flagellar function. Flagellar reversals are determined by monitoring the direction of movement of individual cells while traveling through high-viscosity liquid medium, a condition analogous to the environment encountered by the bacteria during swarming motility (24, 26). Strains that act as hyperswarmers, for example, tend to reverse their flagellar rotation more frequently than the WT (14, 24) and are thus observed to change their direction of movement through viscous liquid medium more frequently.

We assessed the Δ*roeA* mutant for a direction reversal phenotype and found that the Δ*roeA* mutant (0.9 ± 0.4) was not statistically significantly different from the WT (1.2 ± 0.5) in the measured number of reversals/cell ($P = 0.12$). The phenotype of the Δ*roeA* strain differs from that of the *sadB* mutant (24), which undergoes significantly more reversals than the WT and was included here as a control (1.75 ± 0.5 reversals/cell, $P = 0.01$). We also reported a similar increased reversal rate for the hyperswarming Δ*sadC* mutant (14). The linear movement of individual bacteria was also measured under the same conditions utilized to assess flagellar reversals. We observed no significant difference in linear swimming speed between the Δ*roeA* mutant (27.0 ± 5.9 μm/s) and the WT (24.7 ± 5.4 μm/s, $P = 0.23$). Thus, it

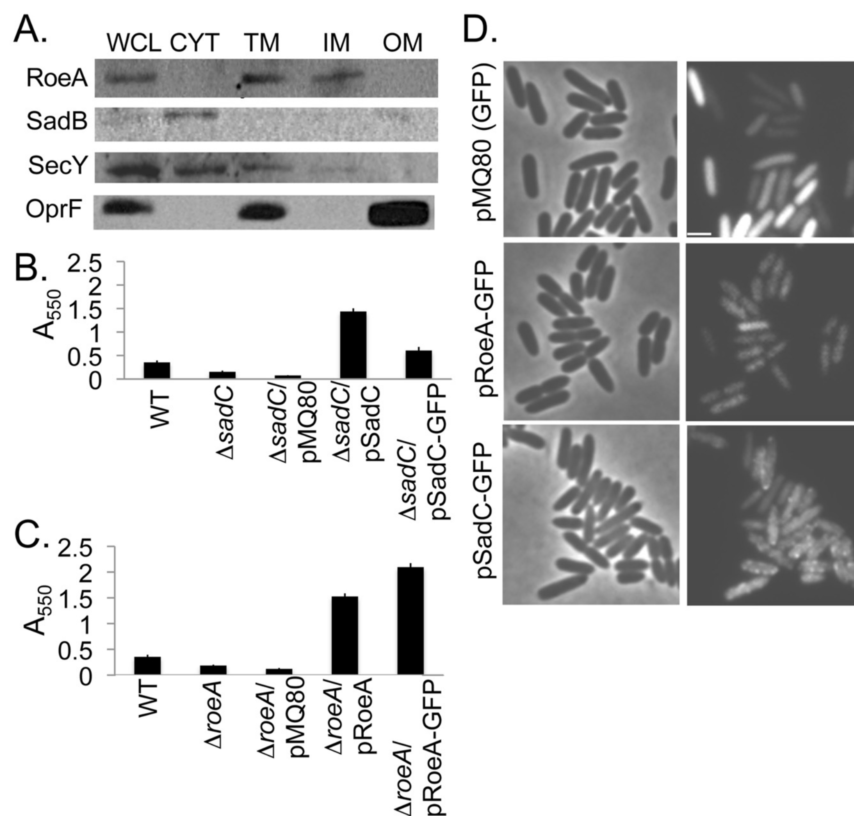

FIG 4 SadC and RoeA are differentially localized. (A) Western blot assays of cellular fractions representing the whole-cell lysate (WCL), cytoplasm (CYT), total membrane (TM), inner membrane (IM), and outer membrane (OM) of the WT strain. SadB, SecY, and OprF are provided as cytoplasmic, IM, and OM controls, respectively, and each protein was detected with a polyclonal antibody. RoeA was detected using anti-His antibody to detect the RoeA-His protein expressed from a plasmid with a P_{BAD} promoter induced with 0.2% arabinose. (B) Control experiments indicating that pSadC-GFP is functional for complementing the Δ*sadC* mutation in a static biofilm formation assay in the presence of 0.2% arabinose. (C) Control experiments indicating that pRoeA-GFP is functional for complementation of the Δ*roeA* mutation, performed as described for panel B. (D) Phase-contrast (left) and epifluorescence micrographs (right) of WT *P. aeruginosa* expressing the vector control pMQ80-GFP (top), pRoeA-GFP (middle), or pSadC-GFP (bottom).

appears that mutating the *roeA* gene has no detectable impact on flagellar function.

In summary, the data presented here, together with previous work regarding SadC (14), indicate that while mutating the *sadC* gene strongly impacts flagellar motility and has a minimal effect on Pel EPS production, mutating the RoeA DGC results in a severe defect in EPS production and a small increase in swarming but no observable alteration in flagellar function.

Mutating *sadC* or *roeA* has similar effects on total levels of c-di-GMP. Given that SadC and RoeA are both c-di-GMP DGCs, we next explored whether they contribute measurably to the cellular level of this signaling molecule. To assess the amounts of c-di-GMP present in the WT and the Δ*roeA* and Δ*sadC* mutants, we analyzed strains carrying the individual mutations and the combined mutations via LC-MS. For these studies, we extracted c-di-GMP from planktonic cells grown in the same medium used in our biofilm assays. We chose to use planktonic cultures because mutations in the *sadC* and *roeA* genes negatively impact biofilm formation; thus, it is not possible to measure c-di-GMP in these mutant backgrounds using biofilm-grown cells. In addition, planktonic cells provided the most uniform population for analysis and were free of the clumping that is common in plate-grown cells. Finally, *roeA* and *sadC* both impact the earliest stages of the transition from planktonic growth to attachment to a surface; thus, the levels of c-di-GMP measured in planktonic cells are indeed quite relevant. However, we cannot discount the possibility that growth in a biofilm, on agar plates, or in swarming medium might impact the total and relative c-di-GMP levels compared to those produced by planktonic growth.

Mutating either *sadC* or *roeA* did, indeed, produce a significant reduction in the levels of c-di-GMP of ~50% compared to those of the WT (Fig. 3D). Interestingly, the decreases in c-di-GMP found in the Δ*sadC* and Δ*roeA* single mutants were not significantly different from each other. The Δ*sadC* Δ*roeA* double mutant showed amounts of c-di-GMP that were significantly reduced compared to those of the WT. While the Δ*sadC* Δ*roeA* double mutant had reduced amounts of c-di-GMP versus those of the single mutants, these differences were not statistically significant. These findings suggest that SadC and RoeA both contribute to the c-di-GMP present under these growth conditions.

SadC and RoeA are differentially localized. Our findings indicate that SadC and RoeA contribute to different phenotypes that depend on c-di-GMP, but these phenotypes are independent of total c-di-GMP levels. One possible explanation for these observations is that the two enzymes could differentially localize such that they produce unique local c-di-GMP pools while having the same effect on the total c-di-GMP level. To better understand the basis of these findings, we explored the possibility that these DGCs, while both predicted to be integral inner membrane proteins, might be differentially localized.

We demonstrated previously that SadC is localized to the inner membrane (14). Here, we also tested the cellular localization of RoeA using the same methodology employed for SadC (14, 17) and detected RoeA specifically in the inner membrane fraction (Fig. 4A).

We next made green fluorescent protein (GFP) translational fusions to the C termini of SadC and RoeA and observed their subcellular localization. These fusion proteins were shown to complement the biofilm defect of the respective mutants (Fig. 4B and C) and thus are functional.

We find that SadC and RoeA are not equivalently distributed throughout the cell. RoeA-GFP localization is somewhat patchy, with a diffuse background (Fig. 4D, middle) compared to that of the GFP-only control, which is uniformly diffuse (Fig. 4D, top). We also attempted to construct several different GFP fusions to

PelD, the c-di-GMP binding component of the Pel system (5); however, these constructs either were toxic to the cell or did not make sufficient signal to visualize. Thus, we were not able to address whether RoeA colocalizes with the Pel system.

In contrast to the RoeA-GFP construct, the SadC-GFP fusion formed distinct foci around the cell periphery (Fig. 4D, bottom), in a pattern reminiscent of that of proteins known to form helical structures, such as the bacterial actin homolog MreB (27). Thus, the differential subcellular localization pattern of these proteins may reflect the differential roles of the SadC and RoeA DGCs in swarming and EPS production, respectively.

Biofilm-related phenotypes do not correspond to total c-di-GMP concentration. We previously reported that BifA is an inner membrane c-di-GMP PDE, the deletion of which results in an elevated cellular level of c-di-GMP, a hyperbiofilm phenotype, increased EPS production, and loss of swarming motility (17). Previous epistasis studies indicated that SadC functions upstream of BifA with regard to biofilm formation (14). Similarly, deleting the *roeA* gene in the Δ*bifA* mutant partially relieved the hyperbiofilm phenotype caused by the Δ*bifA* mutation (Fig. 5A, row 1; see Fig. S2 in the supplemental material). This finding is also consistent with RoeA functioning upstream of BifA. Furthermore, the Δ*sadC* Δ*roeA* Δ*bifA* triple mutant strain showed a reduced-biofilm phenotype compared to both the Δ*bifA* mutant and the WT. These genetic data support a model wherein both the SadC and RoeA DGCs operate upstream of BifA and likely provide much or all of the c-di-GMP that is degraded by the BifA PDE, at least under the conditions tested here.

We were struck by the observation that despite the clear phenotypic differences between the Δ*sadC* or Δ*roeA* single mutants, there was no significant difference in the total levels of c-di-GMP measured between these strains (Fig. 3). To explore this point further, we examined the phenotypes of the Δ*bifA* mutant carrying mutations in the *sadC* and/or *roeA* genes and compared these phenotypes to the levels of c-di-GMP in these strains.

As mentioned above, the Δ*bifA* mutant displays increased EPS production, as judged by its CR hyperbinding phenotype, and this mutant is unable to swarm (Fig. 5A, rows 2 and 3) (17). Introduction of the Δ*sadC* mutation into the Δ*bifA* mutant strain had little impact on the CR phenotype but reduced biofilm formation and restored swarming to nearly WT levels (14). In contrast, the Δ*roeA* Δ*bifA* double mutant displayed a marked reduction in CR binding but was still unable to swarm. Finally, the Δ*sadC* Δ*roeA* Δ*bifA* triple mutant showed less EPS production than the WT and is a hyperswarmer. Thus, as was described above in the WT background (Fig. 3), mutating Δ*sadC* versus Δ*roeA* in the Δ*bifA* mutant background resulted in conspicuously distinct phenotypes.

Analysis of these mutants with regard to c-di-GMP production also showed that total levels of c-di-GMP could not predict the phenotypes observed. As shown previously, the level of c-di-GMP in the Δ*bifA* mutant was increased compared to that of the WT (Fig. 5B). Strikingly, despite the robust phenotypic differences observed in the Δ*sadC* Δ*bifA* and Δ*roeA* Δ*bifA* double mutants, the total c-di-GMP levels in these two strains are not significantly different.

Furthermore, comparing c-di-GMP levels (Figs. 3D, 5B) further illustrates the lack of correspondence between the total amount of c-di-GMP and the phenotypes observed. For example, despite the observation that there is ~5-fold more c-di-GMP present in the Δ*roeA* Δ*bifA* mutant than in the WT, the Δ*roeA* Δ*bifA* mutant strain shows markedly less CR binding than the WT strain (Fig. 5B; see Fig. S2 in the supplemental material). Additionally, despite the ~10-fold difference in c-di-GMP levels between the Δ*roeA* single mutant and the Δ*roeA* Δ*bifA* double mutant, these two strains have comparable CR binding phenotypes (Fig. 2), suggesting that it is c-di-GMP synthesized specifically by RoeA, rather than the total c-di-GMP, that is important for this phenotype. A similar lack of correlation between c-di-GMP and swarming phenotypes is observed when comparing the WT to the Δ*sadC* and Δ*sadC* Δ*bifA* mutants. Together, these data show that c-di-GMP levels alone are not sufficient to predict the phenotype of a strain and strongly argue against a model in which total levels of c-di-GMP are sufficient to regulate specific phenotypes in *P. aeruginosa*.

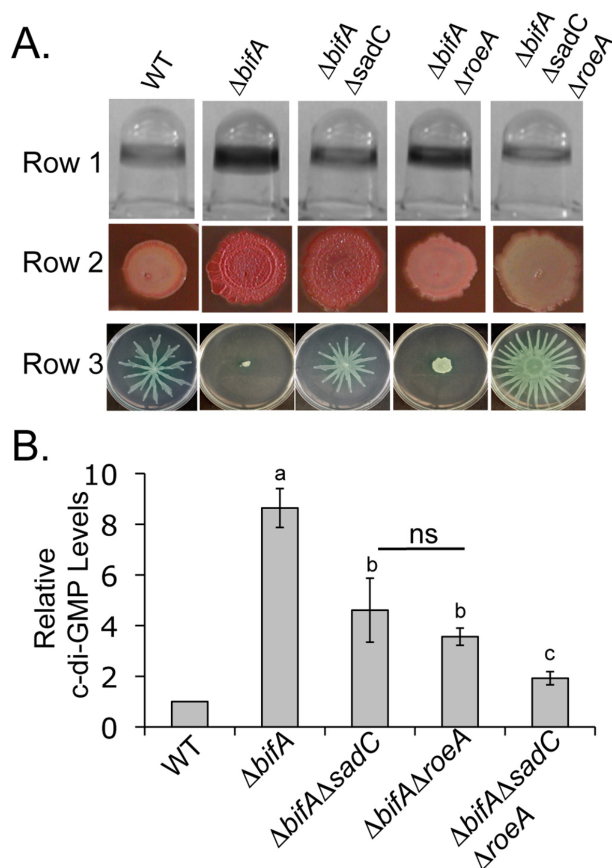

FIG 5 Observed phenotypes do not correlate with total c-di-GMP levels. (A) Biofilm formation (row 1), CR binding (row 2), and swarming (row 3) assays of the strains indicated. CR plates were incubated at 37°C for 24 h, followed by 3 days at room temperature. (B) Relative levels of c-di-GMP in the WT (set to a value of 1) and the mutant strains indicated. Assays were performed as described in Materials and Methods. Letters above bars: a, statistically significant difference from the WT level ($P < 0.05$); b, statistically significant difference from the Δ*bifA* mutant ($P < 0.05$); c, statistically significant difference from the Δ*bifA* and Δ*bifA* Δ*sadC* mutants ($P < 0.05$). The abbreviation ns indicates that the values below the horizontal bar are not significantly different from each other.

DISCUSSION

In *P. aeruginosa*, modulation of c-di-GMP has been associated with control of biofilm formation and additional group behaviors (14, 16, 17). A puzzling aspect of *P. aeruginosa* c-di-GMP regula-

tion is that it encodes close to 40 DGCs and PDEs that are predicted to function in the synthesis and/or degradation of this molecule. Thus, an important unanswered question is whether c-di-GMP-mediated regulation is driven by the coordinated activity of multiple proteins modulating the cellular levels of c-di-GMP. Controlling the total level of a signaling molecule is thought to be the means for regulation via cyclic AMP or acylhomoserine lactones, although bacteria typically utilize a limited number of enzymes to control signal levels in these cases (28, 29). Alternatively, it has also been postulated that localized pools of c-di-GMP might play a key role in mediating specific outputs regulated by this nucleotide signal (30).

The work presented here highlights the apparent disconnect between the total levels of c-di-GMP measured in various mutant backgrounds and the observed phenotypes of these mutants, and thus, at least for the early biofilm formation events driven by the SadC and RoeA DGCs, our findings argue against the model wherein changes in total levels of c-di-GMP can explain the regulation of surface-associated behaviors by *P. aeruginosa*. For example, deleting either the *sadC* or the *roeA* gene results in an ~50% reduction in the c-di-GMP level compared to that of the WT, and the c-di-GMP levels are not significantly different between these two mutants. However, while the Δ*sadC* mutant shows a 5-fold increase in swarming and a corresponding alteration in flagellar function, this mutant has only a minimal change in EPS production compared to that of the WT. In contrast, the Δ*roeA* mutant shows an almost complete loss of Pel EPS production and only a 2-fold increase in swarming motility. The reason for the ~2-fold increase in the swarming motility of the *roeA* mutant is unclear, but our data do show that RoeA has no detectable impact on flagellar function. Thus, mutating the *sadC* and *roeA* DGC genes individually results in strains with similar reductions in measured c-di-GMP levels but distinct phenotypes.

Even more striking is the comparison of the Δ*sadC* and Δ*roeA* mutants in the Δ*bifA* mutant background. Despite the absence of a detectable difference in c-di-GMP levels between the Δ*sadC* Δ*bifA* and Δ*roeA* Δ*bifA* double mutants, these strains have substantially disparate EPS production and swarming phenotypes. We also showed that mutating *roeA* results in complete loss of Pel EPS production even in the Δ*bifA* background, despite the fact that the ΔroeA Δ*bifA* double mutant has levels of c-di-GMP ~5-fold higher than those measured in the WT. Similarly, loss of SadC function results in increased swarming in the WT or the Δ*bifA* mutant, despite the ~10-fold difference in c-di-GMP levels measured for the Δ*sadC* mutant versus the Δ*sadC* Δ*bifA* double mutant. Taken together, these data demonstrate that there is no correlation between the total levels of c-di-GMP and the observed phenotypic output.

How do we interpret such a finding? One possibility is that there are subcellular pools of c-di-GMP in the cell. Such pools could be generated in several ways: by specific localization and/or localized activation of DGCs to produce c-di-GMP, by limiting diffusion of c-di-GMP through the action of proteins that efficiently bind and/or degrade c-di-GMP, or by the availability/activity of c-di-GMP receptors that are specific for a particular phenotype (30). Indeed, recent data obtained using a fluorescence resonance energy transfer sensor for c-di-GMP do indicate the possibility of subcellular pools of this signal, although the functional significance of these pools has not been addressed (31). Our data are consistent with the hypothesis that the differential localization of SadC and RoeA may contribute to their distinct contributions to biofilm formation. Future studies will focus on exploring the very challenging question of how individual DGCs differentially impact processes that control key microbial behaviors such as biofilm formation.

The possibility should not be discounted that physiologically relevant changes in the total level of c-di-GMP may act as a control mechanism under some circumstances; however, the difficulty in measuring the real-time subcellular localization of signaling molecules and proteins has limited the studies that have attempted to address this question. Thus, it is likely that a combination of mechanisms, including total changes in c-di-GMP levels, as well as local changes and/or responses to this signal, regulate bacterial group behaviors in pseudomonads.

MATERIALS AND METHODS

Growth media and molecular techniques. *P. aeruginosa* and *E. coli* strains were routinely cultured in LB medium in the presence of antibiotics, when appropriate, using antibiotic concentrations as previously reported (14). Recombineering with *Saccharomyces cerevisiae* was performed as previously reported (32). M63 minimal medium supplemented with glucose (0.2%), Casamino Acids (CAA; 0.5%), and $MgSO_4$ (1 mM) was used as the base for biofilm, CR, and swim reversal measurements and nucleotide analysis, as described previously (14). Swarming medium contained M8 salts (33) with glucose, CAA, and $MgSO_4$ as described above. A morpholinepropanesulfonic acid (MOPS)-based medium (50 mM NaCl, 40 mM MOPS [pH 7.4], 10 g/liter NH_4SO_4, 1 mM $MgSO_4$, 30 mM succinate, 0.15 mM K_2HPO_4) was used to culture *P. aeruginosa* for assessment of whole-cell levels of c-di-GMP by thin-layer chromatography (TLC) as described previously (16). The strains, plasmids, and primers used in this study are listed in in the supplemental material.

Plasmids constructed during the course of this study were prepared using homologous recombination in S. *cerevisiae* (32) or standard ligation of digested DNA. Constructs made with yeast were then electroporated into *E. coli* for confirmation by PCR or sequencing. Restriction enzymes were obtained from New England Biolabs unless otherwise noted. pKO1107 contains DNA flanking the PA1107 gene and was constructed using the primers indicated in Table S3 in the supplemental material. pKO1107 was utilized to introduce a deletion into the PA1107 (*roeA*) gene by an allelic replacement strategy (34). For construction of plasmids with genes under arabinose regulation, the DNA of interest was amplified by PCR and inserted in place of the *gfp* ORF in pMQ80 or pMQ78. These were then transferred to *P. aeruginosa* by electroporation (35).

Biofilm, motility, and EPS assays. Static biofilm assays were performed with 96-well microtiter plates as previously described (36), with incubation at 37°C for 24 h unless otherwise indicated. All biofilm assays were reproduced a minimum of three times. Swarm assays were conducted as previously described by Toutain et al., with 0.5% agar plates (37). ImageJ software (NIH) was used to determine the area of the plate surface covered by the swarming bacteria as previously described (24). Directional reversals in a viscous medium were quantified as described by Caiazza et al. (24). For directional reversal assays, cells were diluted in M63 glucose CAA medium as for biofilm assays with the addition of Ficoll to 15% to provide the desired viscosity. M63 glucose CAA medium was solidified with 0.3% agar for swimming motility plates (38). Twitching motility assays were performed with LB medium solidified with 1.5% agar (39). To monitor EPS production via CR binding, M63 salt solution was supplemented as for biofilm assays with the addition of CR (40 μg/ml), Coomassie brilliant blue (20 μg/ml), and 1% (wt/vol) agar (20). A 2.5-μl volume of an overnight culture grown in LB broth, with antibiotics as appropriate, was used to inoculate CR plates. CR plates were incubated at 37°C for approximately 24 h. Plates were further incubated for 1 or 2 days at room temperature to improve color development, as indicated.

***In vitro* DGC assays.** *In vitro* DGC assays were conducted essentially as reported by Paul et al. (10) and modified by Monds et al. (40), with an incubation time of 24 h at room temperature. Reactions were stopped by the addition of 10 μl 0.5 M EDTA and the addition of an equal volume of running buffer (1:1.5 saturated NH_4SO_4 and 1.5 M KH_2PO_4, pH 3.6). Reaction products were resolved by TLC and analyzed as previously described (40).

Measurement of c-di-GMP levels. To measure *in vivo* levels of c-di-GMP using two-dimensional TLC (2D-TLC), radiolabeled c-di-GMP was generated *in vivo* from [^{32}P]orthophosphate and separated from other labeled species in a formic acid extract by 2D-TLC on polyethylenimine cellulose plates (Selecto Scientific) as described by Hickman et al. (16). TLC plates were exposed to a phosphor screen and analyzed as previously described (40).

Alternatively, c-di-GMP levels were analyzed via LC-MS. Strains of interest were grown to stationary phase in LB medium and subcultured to an optical density at 600 nm (OD_{600}) of 0.04 in 50 ml of M63 G/CAA. When the cultures attained an OD_{600} of 0.4, the culture volume was normalized by OD_{600} and approximately 40 ml of each culture was harvested by centrifugation at 25°C for 3 min at 10,000 × *g*. Pellets were resuspended in 250 μl of extraction buffer (methanol-acetonitrile-water [40:40:20] plus 0.1 N formic acid at −20°C) and incubated at −20°C for 30 min. The cell debris was pelleted for 5 min at 4°C, and the supernatant containing the nucleotide extract was saved. Samples were immediately adjusted to a pH of ~7.5 with 15% $(NH_4)_2HCO_3$ and stored on dry ice prior to analysis. The resultant extract was analyzed via LC-MS using the LC-20AD high-performance LC system (Shimadzu, Columbia, MD) coupled to a Finnigan TSQ Quantum Discovery MAX triple-quadrupole mass spectrometer (Thermo Electron Corp., San Jose, CA) as previously described (41). For each experiment, the average value of the c-di-GMP peak for the WT was set to 1 and the c-di-GMP measurements from additional strains are presented relative to the WT value.

Microscopy. *P. aeruginosa* PA14 carrying pMQ80 (GFP alone), pSadC-GFP, or pRoeA-GFP was grown overnight in M63 glucose (0.2%), CAA (0.5%), $MgSO_4$ (1 mM), and gentamicin (50 μg/ml), subcultured 1:50 into fresh medium, grown for 2 h at 37°C, and then induced with 0.2% arabinose for an additional 1 h at 37°C. To collect single images of protein localization, bacterial cells were placed on 1% agarose pads made from the same M63 medium and visualized using a 100× 1.4 numerical aperture objective on a Nikon 90i microscope equipped with a Rolera XR camera and NIS Elements software as reported previously (42).

Protein localization and detection. Strains were grown in LB medium supplemented with gentamicin (50 μg/ml) and arabinose (0.2%) and harvested as previously reported in lysis buffer containing 50 mM Tris (pH 8), 150 mM NaCl, 5% glycerol, and a complete EDTA-free protease inhibitor cocktail tablet (Roche) (14). Lysis was carried out by passage through a French pressure cell. Unbroken cells were pelleted by centrifugation at 13,000 × *g*, and cellular fractionations were performed on the resulting supernatant based on the method of Nunn and Lory (43) as modified by Hinsa and O'Toole (44). Fractions were normalized based on protein concentration and analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis on a gradient (4 to 15%) gel, followed by Western blotting. Proteins were detected using antibodies that recognize the His epitope (Qiagen), SadB (45), SecY (45), and OprF (46) to distinguish the His-tagged RoeA protein and cytoplasmic and inner and outer membrane fractionation controls, respectively.

Statistical analyses. Pairwise comparisons were performed with the two-tailed Student *t* test. For multiple comparisons, we compared groups using analysis of variance in R (http://www.r-project.org). *P* values are adjusted for multiple comparisons using Tukey's honest significant difference.

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SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at http://mbio.asm.org/lookup/suppl/doi:10.1128/mBio.00183-10/-/DCSupplemental.

Figure S1, PDF file, 1.779 MB.
Figure S2, PDF file, 0.771 MB.
Table S1, PDF file, 0.063 MB.
Table S2, PDF file, 0.058 MB.
Table S3, PDF file, 0.048 MB.

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