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**Constitutive production of c-di-GMP is associated with
mutations in a variant of *Pseudomonas aeruginosa* with
altered membrane composition**

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Mutations in a clinical small colony variant of *Pseudomonas aeruginosa* alter its membrane composition to enable its attachment to surfaces

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Abstract

Most bacteria are capable of forming multicellular communities on biotic and abiotic surfaces. This multicellular response to surface contact correlates with an increased resistance to various adverse environmental conditions, including those encountered during infections of the human host and exposure to antimicrobial compounds. Despite increasing knowledge of the numerous stimuli that affect biofilm formation, the general mechanisms of surface perception and signal transduction that initiate bacterial adherence are still poorly defined. Here, we identified adaptive mutations within a clinical small colony variant (SCV) of *Pseudomonas aeruginosa* and correlated their presence with auto-aggregative growth behavior and an enhanced capacity to form biofilms. We present evidence that a point mutation in the 5'-untranslated region of the *accBC* gene cluster (which encodes the biotin carboxyl carrier protein (AccB) and the structural component of the biotin carboxylase (AccC)) is responsible for a stabilized mRNA structure that results in reduced translation efficiency and a shift in fatty acid composition towards shorter chain lengths. We propose a

model in which changes in the fatty acid composition of the plasma membrane of *P. aeruginosa* serve as a signal for a chemosensory-like surface sensing system (Wsp) to constitutively produce increased amounts of c-di-GMP, and thus to play a key role in the regulation of adhesion-stimulated bacterial responses.

Introduction

Many pathogenic bacteria efficiently adopt a protected mode of growth within multicellular, matrix-encased biofilms. Biofilm formation facilitates bacterial evasion of the host immune response and antimicrobial treatment, and it is recognized as an important factor in the progression and persistence of many infectious diseases (1, 2). The first step in biofilm formation occurs when freely swimming (planktonic) cells collide with a surface, which stimulates a cascade of physiological reactions leading to a genetic program that determines biofilm development. A key player in the regulation of the transition between a planktonic lifestyle and the biofilm mode of growth is the intracellular second messenger 3',5'-cyclic-di-guanosine monophosphate (c-di-GMP) (3). In general, high intracellular amounts of c-di-GMP promote adhesion, production of exopolysaccharides, and establishment of biofilms, and often a plethora of enzymes that synthesize or degrade c-di-GMP is used to adjust c-di-GMP concentrations in response to environmental signals (4, 5). c-di-GMP not only directs surface attachment and motility behavior, but it is also stimulated in the opportunistic pathogen *Pseudomonas aeruginosa* upon contact with surfaces. This is achieved through a chemosensory-like system (Wsp, wrinkly spreader phenotype), which senses a signal associated with the physical state of growth on a surface and stimulates the production of c-di-GMP (6–8); however, the exact nature of the signal is obscure.

A number of studies demonstrated a link between increased intracellular concentrations of c-di-GMP and a small colony variant (SCV) phenotype in *P. aeruginosa* (9–11). Those SCVs represent highly adapted subpopulations that are frequently isolated from the chronically infected lungs of cystic fibrosis (CF) patients (12). SCVs are characterized by a slow and auto-aggregative growth behavior, as well as an enhanced capacity to form biofilms (13, 14). Because of the critical roles of biofilms in chronic disease, a lot of effort has been made to gain new insights into the molecular mechanisms underlying the formation of auto-aggregative SCVs (15).

The rapid pace in technological advances in the post-genomic era provides a unique opportunity to foster our striving for the elucidation of critical biological processes. Genomics-based platforms are extensively used as a tool to uncover adaptive mutations. Thereby, the presence of adaptive mutations within, for example, SCV phenotypes that render cells proficient in biofilm formation, even under environmental conditions that do not induce biofilm formation, can be correlated with global responses. Because this approach holds promise to provide new information on the identity of the molecular mechanisms underlying signal transduction processes, we applied a whole-genome sequencing approach to unravel the genetic basis for the SCV phenotype of the clinical CF isolate of *P. aeruginosa*, SCV20265 (13). We showed that an adaptive translational control of the fatty acid composition of the bacterial cell membrane promoted a shift towards the production of fatty acids with shorter chains, which served as a signal to activate the chemosensory-like Wsp system and to produce constitutively large amounts of c-di-GMP in SCV20265. Our results shed light on the regulatory processes critical for the initial attachment of *P. aeruginosa* to surfaces, and thus will be useful to devise better treatments to combat devastating, biofilm-associated, nosocomial infections.

Results

Whole-genome sequence comparison between the clinical isolate SCV20265 and 10 non-aggregative, large colony-producing revertants uncovers adaptive mutations

The *P. aeruginosa* isolate SCV20265 was first described by Häussler *et al.* in 2003 and originated from the lung of a CF patient (13). Upon passage under rich medium conditions, SCV20265 gives rise to non-aggregative revertants that produce unusually large colonies on agar plates (13). Here, by sub-culturing SCV20265 *in vitro*, ten independently generated revertants were isolated (Fig. 1A) and subjected to whole-genome sequencing. In comparison to the genomic sequence of SCV20265 (15, 16), all ten revertants showed a single nucleotide polymorphism (SNP) in the 5'-untranslated region (5'-UTR) of the *accBC* gene cluster, which encodes the biotin carboxyl carrier protein (AccB) and the structural component of the biotin carboxylase (AccC), both of which are subunits of the acetyl coenzyme A (acetyl-CoA) carboxylase (17). Four of the ten revertants harbored a T to C exchange 31 base pairs (bp) upstream of the *accB* start codon, whereas the other six revertants exhibited a G to A exchange 12 to 16 bp upstream of the *accB* start codon (Fig. 1B).

The translation of *accB* is reduced in SCV20265 because of the altered secondary structure of its mRNA

To investigate the effect of the mutations in the 5'-UTR of *accBC* (18) on the amounts of RNA and protein, we first performed quantitative reverse transcription polymerase chain reaction (RT-PCR) analysis and measured the expression of the *accB* and *accC* genes. With fold-changes of -1.23 ± 0.72 for *accB* and 1.01 ± 0.72 for *accC*, the extent of transcription of these genes was not substantially altered in SCV20265 as compared to one of its representative revertants (Rev 1, G-15A).

The sequence between the nucleotides at positions -16 and -12 of *accB* (GGGAG) harbored SNPs in six of ten revertants (Fig. 1B), and this sequence represents the Shine-Dalgarno (SD) sequence that determines the start point for the translation of *accB*. Prediction of mRNA secondary structures with CentroidFold (19) indicated that the SD sequence of *accB* in revertant 1 (GAGAG) is most likely accessible, whereas the SD sequence of *accB* in SCV (GGGAG) perfectly pairs with a CCCUC sequence located several bases downstream (at positions -28 to -32), thereby leading to the formation of a hairpin structure that includes the SD motif (Fig. 2A). All of the mutations in the revertants (at position -31 and from positions -16 to -12) disrupt this predicted hairpin. We hypothesized that blocking of the SD sequence through the formation of a hairpin in the 5'-UTR of *accBC* mRNA resulted in a decreased translation rate of *accBC*. Therefore, we examined the translation efficiency of *accB* in *Escherichia coli* DH5 α cells and *P. aeruginosa* PA14 wild-type cells transformed with a plasmid encoding an *accB-his₆* fusion and the upstream region of *accB* containing the sequence of SCV20265 or that of revertant 1. Western blotting analysis showed that the amount of AccB protein was reduced in PA14 wild-type cells and *E. coli* DH5 α cells when the SCV-based plasmid construct was used compared to the revertant 1 based constructs (Fig. 2B and fig. S1).

To provide experimental evidence for the altered mRNA secondary structure in the revertant strains, we used circular dichroism spectroscopy to determine the thermal unfolding of DNA oligonucleotides representing the respective *accBC* 5'-UTR of SCV20265, the clonal wild-type WT20265, and four representative revertants: Rev1 (G-15A), Rev4 (T-31C), Rev6 (G-12A), and Rev8 (G-16A) (fig. S2A). The melting temperature was statistically significantly increased ($P < 0.05$ according to one-tailed Mann-Whitney test) for the SCV oligonucleotide

($52.2 \pm 0.4^{\circ}\text{C}$) in comparison to the wild-type ($45.3 \pm 0.3^{\circ}\text{C}$) and the representative revertant 1 ($36.3 \pm 0.7^{\circ}\text{C}$) oligonucleotides (Fig. 2C). An increase in the melting temperature occurs when oligonucleotides contain a secondary structure which requires higher temperatures for the unfolding. In addition, we measured the melting points of oligonucleotides corresponding to revertants 4, 6, and 8, and observed a similar tendency with lower melting points (42.2 , 34.9 , and 44.5°C , respectively) compared to that of SCV (fig. S2B).

To demonstrate that a stabilized mRNA secondary structure prevents the binding of ribosomes to the SD sequence in SCV20265, we performed toeprint assays on mRNA fragments synthesized in vitro. Toeprint signals are a consequence of an inhibited primer extension reaction because of the binding of 30S ribosomes to the mRNA. A typical toeprint at position +17 with respect to the translational start site of *accB* was obtained with two representative revertant mRNAs (Rev1 and Rev4) (Fig. 2D). The much weaker toeprint signal obtained with SCV mRNA is consistent with a stabilized RNA structure that prevents ribosome binding. A pronounced signal corresponding to position -11 (Fig. 2D, asterisk) further indicates the formation of a stable hairpin structure that causes aborted reverse transcripts. Overall, the toeprint assays indicated that the ribosome had access to the 5'-UTRs of the *accBC* revertant mRNAs, whereas the hairpin structure in the SCV *accB* mRNA blocked binding of the ribosome. Together, these data suggest that the different mRNA structures caused by sequence variations in the 5'-UTR of *accBC* determined the translation efficiency of *accBC* in SCV20265 and its revertants.

The reduced translation of *accBC* mRNA leads to a shift in fatty acid composition towards shorter chain lengths

AccB and AccC form a subunit of the acetyl-CoA carboxylase complex, which catalyzes the formation of malonyl-CoA (17). We wondered whether a reduction in the amount of malonyl-CoA, which is the major precursor for the initiation of fatty acid biosynthesis and for each elongation cycle, led to an altered fatty acid metabolism in SCV20265. In addition to investigating the fatty acid composition in SCV20265 and revertant 1, we also analyzed the effect of different total amounts of AccB and AccC in two other strain backgrounds: PA14 and a large colony-producing clinical *P. aeruginosa* strain (WT20265), which was isolated from the same upper respiratory tract material of a CF patient as was SCV20265 (13). In comparison to SCV20265, both PA14 and WT20265 had a G at position -31 relative to the *accB* translational start site, which prevents stable hairpin formation in the 5'-UTR of *accBC* (compare to Fig. 1B).

We generated mutant strains of WT20265 and PA14 (referred to as *WTaroQIaccBC_{scv}* and *PA14aroQIaccBC_{scv}*, respectively) by replacing the chromosomal region containing *accBC* and the upstream gene *aroQI* with that of SCV20265 to introduce the stable SCV20265 hairpin in the 5'-UTR of *accBC*. Gas chromatography was then used to analyze the cellular fatty acid compositions of SCV20265, *WTaroQIaccBC_{scv}*, and *PA14aroQIaccBC_{scv}* in comparison to their respective reference strains, which do not exhibit hairpin formation (Rev1, WT20265, and PA14) (Fig. 3). All of the strains that contained the SCV *aroQIaccBC* region showed a shift towards generating fatty acids with shorter chain lengths. Cis-vaccenic acid (C18:1 ω 7), the most abundant fatty acid of *P. aeruginosa* grown in planktonic and biofilm cultures (20), was reduced in abundance in the SCV20265 and *WTaroQIaccBC_{scv}* strains compared to that in WT20265 and revertant 1 (Fig. 3). In contrast, the relative amount of unsaturated cis-palmitoleic acid (C16:1 ω 7) was substantially increased in SCV20265, *WTaroQIaccBC_{scv}*, and the *PA14aroQIaccBC_{scv}* strains compared to that in the respective

wild-types WT20265 and PA14 wild-type (Fig. 3). We furthermore used the plasmids pHERD20T::*accB* and pHERD20T::*accBC* to induce the overexpression of these genes. Only the presence of both *accB* and *accC* restored the extent of production of C18:1 ω 7 and C16:0 in the SCV20265 and WT*aroQIaccBC*_{scv} strains to that in the wild-type strains (fig. S3). These results are consistent with those of a previous study that demonstrated that *accB* and *accC* are co-transcribed (21).

The reduced translation of *accBC* affects colony morphologies and c-di-GMP concentrations

Next, we wondered whether the introduction of the hairpin-inducing SNP in the 5'-UTR of *accBC* in the WT20265 and PA14 strains not only affected fatty acid composition, but also led to differences in colony morphology as was observed for SCV20265 compared to its revertant. Introduction of the *aroQIaccBC*_{scv} allele (with only one G to T SNP in the 5'-UTR of the *accBC* transcript) led to a switch of the WT20265 colony to an SCV phenotype (Fig. 4), whereas complementation of SCV20265 and also WT*aroQIaccBC*_{scv} with pHERD20T::*accBC* led to an increase in colony size (fig. S4). However, the differences in colony size were not as prominent as for SCV20265 and its revertants. In PA14, chromosomal replacement of *aroQIaccBC* with *aroQIaccBC*_{scv} did not result in SCV colony morphologies (Fig. 4B), indicating that the effect of the reduced translation of *accBC* on colony morphology was strain specific.

SCV20265 is characterized by high intracellular concentrations of the second messenger c-di-GMP. In the revertant 1, restoration of wild-type AccBC production—as a result of the acquisition of a secondary mutation in the 5'-UTR of *accBC*—seemed to affect not only fatty acid composition and colony morphology, but also c-di-GMP production, because the

amounts of c-di-GMP in the Rev1 strain were substantially lower than those in SCV20265 (Fig. 4). In contrast, although we observed a change in fatty acid composition (Fig. 3), we did not detect an increase in the c-di-GMP concentration in the PA14*aroQIaccBC*_{SCV} strain compared to that in the PA14 strain (Fig. 4B). Similarly, the WT*aroQIaccBC*_{SCV} strain, despite exhibiting smaller colony morphology and a change towards the synthesis of shorter-chain fatty acids (Fig. 3), only showed a trend towards a small increase in the amount of c-di-GMP, which overall remained lower than that of SCV20265 (Fig. 4).

Increased c-di-GMP concentrations in strains with the SCV20265 background depend on the Wsp signaling system

It was previously speculated that mechanical perturbations of the cell membrane are sensed by the Wsp signaling system (6, 8), and that activation of the Wsp system leads to the production of c-di-GMP through the enzyme WspR (6, 22). We wondered whether the Wsp system is activated in SCV20265, which exhibits a shift towards the synthesis of shorter-chain fatty acids. We constructed *wspR* deletion mutants in SCV20265, revertant 1, WT20265, and PA14 and measured their c-di-GMP concentrations. Whereas deletion of *wspR* did not substantially affect the c-di-GMP concentration in Rev1, WT20265, or PA14 cells, it led to a marked reduction in the c-di-GMP concentration in the SCV20265 strain (Fig. 5A), suggesting that the diguanylate cyclase WspR is responsible for the increased concentrations of c-di-GMP in SCV20265 cells. Consistent with this, deletion of *wspR* also reverted the small-colony phenotype of SCV20265 to one of large and smooth colonies, similar to those of revertant 1, whereas no change occurred in WT20265 and PA14 upon deletion of *wspR* (Fig. 5A).

A SNP within *wspF* is required for the increased concentrations of c-di-GMP in the SCV20265 strain

Despite the effect of low AccB abundance on the WspR-dependent production of c-di-GMP and colony morphology in the SCV20265 strain, decreased AccB production in the PA14 strain was not sufficient to affect colony morphology or c-di-GMP abundance, and it only partly had an effect in the WT20265 strain. We therefore searched for additional mutations in SCV20265 that might explain the specific influence of the altered translation of *accBC*. Thus, we found an additional T to A point mutation in *wspF* in SCV20265, which led to a Leu²⁰Gln (L20Q) amino acid exchange in the response regulatory domain of the methylesterase WspF, which is a component of the Wsp system. To examine whether the T59A SNP in *wspF* contributed to the increased c-di-GMP concentrations in the SCV20265 strain, we replaced the *wspF*_{SCV} gene with the *wspF*_{WT} allele and measured the concentration of c-di-GMP in this mutant strain. This replacement substantially reduced the amount of c-di-GMP produced in SCV20265 (Fig. 5B), and it also led to an increase in colony size, although the colonies of SCV_{wspF}_{WT} were still smaller than those of Rev1 or those of the SCV20265 *wspR* knockout strain (Fig. 5A). Consistent with those results, replacement of the *wspF* gene in WT20265 with *wspF*_{SCV} (which contained the SNP T59A) increased c-di-GMP concentrations and reduced colony size (Fig. 5B).

High salt concentrations and surface-attached growth restore c-di-GMP concentrations in the revertant

Previous studies demonstrated that both growth on surfaces and the presence of salt in LB medium is required for Wsp signaling (6, 8). We therefore tested whether the Wsp system could be activated in the revertants if they were grown under appropriate conditions. We extracted c-di-GMP from bacteria grown in planktonic cultures (Fig. 6A) and attached on LB

agar plates that contained different salt concentrations (Fig. 6B). Under planktonic growth conditions, high salt concentrations stimulated the production of an increased amount of c-di-GMP in the revertant (Fig. 6A). Furthermore, in attached cells, the c-di-GMP concentration in the revertant was similar to that of SCV20265 both in LB alone and in LB containing 300 mM NaCl, but it remained lower on plates completely lacking NaCl (Fig. 6B). These results suggest that both attached growth and high salt concentrations restore intracellular c-di-GMP concentrations in the revertant such that they are comparable to those of SCV20265. To validate that the Wsp system was responsible for the observed increase in c-di-GMP, we measured c-di-GMP concentrations in a revertant with a *wspR* deletion. The c-di-GMP concentration was substantially reduced upon growth under high-salt conditions in a *wspR* deletion mutant (fig. S5), indicating that c-di-GMP production in the revertant was conditional and that it could be induced under conditions that activated the Wsp system.

We next analyzed whether the conditional activation of the Wsp system in the revertant was mediated by a change in its fatty acid composition. We therefore analyzed the fatty acid composition of attached cells under various salt concentrations and compared it to that of planktonic cultures. Consistent with a previous study that demonstrated that more short-chain and unsaturated fatty acids are produced upon osmotic stress (23), we observed an increase in the amounts of the C16:1 ω 7 and C18:1 ω 7 fatty acids in SCV20265 and revertant 1 upon growth on agar plates containing 120 or 300 mM NaCl (fig. S6).

The presence of the *wspF*_{SCV} allele and hairpin formation in the 5'-UTR of *accBC* act additively on an SCV phenotype

Our data suggest that because of a mutation in *wspF*, the activated Wsp system might enhance the bacterial response to positive stimuli of the Wsp signaling cascade. We therefore

speculated that the introduction of the *wspF_{scv}* allele into the PA14 and WT20265 strains should also sensitize the bacteria to alterations in AccB abundance. We introduced the *wspF_{scv}* allele into PA14 and WT20265 strains singly and in combination with the SCV20265 SNP in the *accBC* 5'-UTR. Introduction of the *wspF_{scv}* allele led to smaller colony morphologies in both the PA14 and WT20265 strains as compared to those of the respective wild-types (Fig. 5B and fig. S7), which indeed could be further enhanced by introducing a stable hairpin in the 5'-UTR of the *accBC* transcript (fig. S7). Note that introduction of the *wspF_{scv}* allele into WT20265 led to c-di-GMP concentrations comparable to those of SCV20265 (Fig. 5B). Because of the high variability in the increased c-di-GMP amounts ~~standard deviations of elevated c-di-GMP levels~~, testing for additional increases in c-di-GMP concentration in the double mutant could not be performed.

Discussion

The initial interactions of bacteria with surfaces or host tissues are the first and essential steps in biofilm-generating, gene expression programs, and thus determine the initiation of problematic biofilm-associated bacterial infections (24). Although it is well-established that bacteria adjust their physiology during adhesion, little is known about how cellular or abiotic surfaces are perceived or how appropriate adaptive responses are mediated (25). Identification of global responses in a mutant strain that has gone through adaptive evolution provides a unique opportunity to associate the presence of adaptive mutations with the activation of a specific regulatory process and thus to gain information on the molecular mechanisms underlying an adapted phenotype (26, 27). During chronic persistent infections, the emergence of a number of mutations in *P. aeruginosa* strains reflects bacterial adaptation to the habitat of the human host (28). Among those, mutations that affect the activity of key regulators of c-di-GMP production drive the formation of SCV phenotypes, which render

cells proficient in biofilm formation even under non-biofilm-inducing environmental conditions (9, 29–31).

Here, we uncovered causative adaptive mutations of the clinical *P. aeruginosa* SCV20265 isolate, which stimulated constitutively increased concentrations of c-di-GMP. We isolated fast-growing revertants, and, by applying whole-genome sequencing, we identified a point mutation in the 5'-UTR of the *accBC* gene cluster as being responsible for the phenotypic switch between the SCV20265 strain and its revertants. Prediction of the secondary structure of the 5'-UTR of the *accBC* mRNA, oligonucleotide melting experiments, and toeprinting assays provided evidence to suggest that this SNP causes the formation of a hairpin structure that hinders ribosome access to the SD sequence of *accB* in SCV20265. As a consequence of the reduced translation of *accB*, and consistent with previous reports (17), we found a shift towards the synthesis of fatty acids with shorter chain lengths.

It is well known that the formation of a hairpin structure in an mRNA can reduce its translational efficiency if the SD sequence is involved, and that mutations in the SD sequence can decrease the rate of translation (32). Single SNPs have also been described to be sufficient to alter the translation of target genes if they occur either in regulatory or small anti-sense RNAs or in the target mRNAs and lead to a loss of binding specificity (33, 34). Here, we present evidence that one single SNP in the 5'-UTR of the *accBC* mRNA modulates the production of an essential protein through alteration of the secondary structure of the mRNA independently of any additional factors. The roadblock imposed by the hairpin structure in SCV20265 was relieved by various suppressor mutations. Only G to A mutations were found, most likely to maintain the integrity of the SD sequence. Because SNPs in the UTR of an mRNA do not alter the protein sequence, their selection as an adaptation process

of protein translation is an efficient way to initiate an invertible on-off state of protein production, and, as in the case of SCV20265, of colony morphology and increased amounts of c-di-GMP. Thus, the adaptive mutations within the 5'-UTR of the *accBC* mRNA enable this clinical strain to readily respond to the current environmental challenge and either increase c-di-GMP abundance during chronic colonization of the CF lung, or decrease it if exposed to environmental conditions that favor fast growth.

Mutational studies performed with strains on different backgrounds demonstrated that the extent of the effect of the mutation in the 5'-UTR of *accBC* on colony morphology and c-di-GMP abundance varied substantially. The cell membrane composition differs among various *P. aeruginosa* isolates, and thus a modulation of AccBC protein concentrations might have different effects on the fatty acid composition, and thus the activation, of the Wsp system. Furthermore, although reduced AccBC concentrations resulted in a shift towards the synthesis of shorter-chain fatty acids in the WT20265 and PA14 strains, only a small effect on colony morphology was observed in WT20265 cells, and no effect was detected in the laboratory strain PA14. A second mutation within *wspF* activates the Wsp system in SCV20265 and sensitizes the bacteria to reduced concentrations of AccBC.

Note that the revertants exhibited large colony sizes although they contained a *wspF* mutation that, when introduced into the wild-type strain, induced c-di-GMP production; however, the SCV Δ *wspR* strain exhibited a colony size comparable to those of the revertants. This finding suggests that the Wsp system is very effectively silenced in the revertant because of the formation of a mutation-inhibiting hairpin in the 5'-UTR of *accBC*. In contrast, inactivation of *wspR* in the WT20265 strain did not lead to the large colony sizes of the revertants, indicating further Wsp-independent effects on colony size in WT20265.

The *wspF* mutation in the SCV20265 strain is located in the response regulatory domain of the methylesterase. Mutations in *wspF* (which encodes this enzyme) are well known to lead to the constitutive activation of the Wsp system (22), and they are one of the most frequent genetic adaptations of *P. aeruginosa* during chronic infections of the lung in CF patients (9, 35). The predicted role of WspF is to maintain WspA in an inactive form; thus, inactivating mutations within *wspF* are expected to lock the Wsp signaling complex in a conformation in which it constitutively phosphorylates the WspR response regulator, which catalyzes the synthesis of c-di-GMP (6, 22).

The Wsp signaling cascade is activated by high salt concentrations and attachment to surfaces (6–8). Indeed, in the revertant 1, high salt concentrations and surface attachment complemented for the missing hairpin formation in the 5'-UTR of *accBC* and restored c-di-GMP concentrations to those of SCV20265 (Fig. 6). A study showed that osmotic stress changes the fatty acid profile of *P. aeruginosa* through an alternative sigma factor (SigX)-dependent influence on *accAB* transcription (36). However, high salt concentrations might also stimulate the Wsp cascade through an alteration of the mechanical properties of the cell membrane because of the introduction of unsaturated fatty acids (Fig. 7). A study showed that ethanol activates the Wsp system, and, consistent with our data, plasma membrane perturbations were speculated to activate the WspA sensor in the membrane (37).

In summary, our analysis of a highly adapted *P. aeruginosa* subpopulation that emerged during chronic infection and that exhibits a strong biofilm-forming phenotype revealed that alterations in the fatty acid composition of the plasma membrane are sensed by the chemosensory-like Wsp system to increase the concentration of intracellular c-di-GMP and to

promote surface adherence and growth within protected biofilms. Our study demonstrated that although the Wsp system is crucial in modulating c-di-GMP concentrations within the cell, its activity is regulated by various means. The Wsp system is activated by changes in the membrane composition. These changes are observed when AccBC abundance is low (resulting in the synthesis of shorter, more unsaturated fatty acids) or when cells grow attached to a surface and under conditions of high salt concentrations (which result in an increased proportion of unsaturated fatty acids in the plasma membrane). However, sequence variations (commonly within *wspF*) also affect the overall activity of the Wsp system and modulate its responsiveness. Our results shed light on the molecular mechanisms that enable bacteria to process micro-environmental information and to adapt by generating appropriate surface-associated biological responses. Knowledge of the key role of surface-sensing and signal transduction may enable the development of effective treatment strategies that target biofilm-associated chronic infections that are largely refractory to antimicrobial treatment.

Materials and Methods

Bacterial strains and growth conditions

The bacterial strains and plasmids used in this study are listed in table S1. The sequences of primers used are listed in table S2. Unless otherwise stated, all strains of *P. aeruginosa* and *E. coli* were cultivated in LB broth at 37°C with shaking at 180 rpm. To generate revertants in vitro, ten independent pre-cultures from the clinical isolate SCV20265 were inoculated in 25 ml of fresh LB, with a start OD₆₀₀ of 0.01. The bacteria were transferred twice daily to a new culture such that the cultures always started with an OD₆₀₀ of 0.01. In parallel, an aliquot of each culture was streaked on Columbia blood agar plates (BD) to monitor changes in colony morphology. After approximately 14 days of serial passages, stable revertant phenotypes were observed for all ten clones.

Construction of knockout mutants and replacement of genes

To generate knockout mutants and replace genes in SCV20265, WT20265, and PA14 wild-type strains, we used an adapted version of a published protocol (38). In-frame deletions of *wspR* were constructed by overlap extension polymerase chain reaction (PCR) assays, as described previously (39). Briefly, the regions from 0.5 kb upstream and 0.5 kb downstream of *wspR* were amplified from SCV20265 and revertant 1 chromosomal DNA with Herculase II Fusion Polymerase (Agilent Technologies) and the primers indicated in table S2. The upstream and downstream PCR products were mixed and used in a second PCR reaction with the primer pairs KO_Up_fw1 and KO_Down_rev1. The resulting 1000-bp fragment was subcloned into the Eco RI and Hind III restriction sites of pEX18Ap. To replace *accBC* and *wspF*, these genes were amplified from SCV20265 or WT20265 with the HotStar HiFidelity Polymerase (Qiagen) and primers that contained Eco RI and Hind III restriction sites (listed in table S2) for subsequent cloning into pEX18Ap (38). *E. coli* S17-1 or *E. coli* WM3064 cells were transformed with the pEX18Ap constructs for gene knockout and replacement, and these were then used as donor strains during the conjugation process. The WTaro*Q1accBC*_{scv} mutant and Rev1Δ*wspR* were generated with the help of the diaminopimelic acid (DAP) auxotroph *E. coli* WM3064. To cultivate *E. coli* WM3064, 300 μM DAP was added to the medium during the conjugation process. For conjugation, donor strains were cultivated at 37°C and the recipient strain was cultivated at 42°C overnight, shaking at 180 rpm, and mixed at a ratio of 10:1 (donor:recipient), and then 50 μl of the mixed culture was spotted onto an LB agar plate and incubated for 6 hours at 37°C. After the first recombination, cells were harvested by scraping, resuspended in 1 ml of LB medium, and dilutions of 1:10 and 1:100 were plated on LB agar plates supplemented with carbenicillin. Carbenicillin-resistant clones were checked for sucrose sensitivity, and then sucrose-sensitive, carbenicillin-resistant

clones were re-cultivated in LB broth overnight to perform the second recombination. Subsequently, dilutions of the obtained culture were plated on LB agar plates supplemented with 10% sucrose. Sucrose-resistant clones were then selected in parallel on 10% sucrose and carbenicillin plates to select for the loss of the *sacB* gene and the loss of the pEX18Ap vector mediating carbenicillin resistance. The replacement and knockout of genes in carbenicillin-sensitive and sucrose-resistant clones was confirmed by sequencing. For plasmid or mutant selection, carbenicillin (400 µg/ml) was used for *P. aeruginosa*, whereas ampicillin (100 µg/ml) was used for *E. coli*.

Cloning of His₆-tag constructs and Western blotting analysis

The translation efficiency of *accB* was analyzed by fusing six histidine residues to the C-terminus of AccB. Constructs including the *accB*-his-tag fusion gene and 467 upstream nucleotides containing the respective SNP of SCV20265, one representative revertant (Rev1, G-15A), and WT20265 were cloned into pUCP20, which was transferred in *E. coli* DH5α and PA14 wild-type. To prepare samples for Western blotting analysis, *E. coli* and PA14 strains were cultivated for 24 hours. To ensure loading of equal amounts, the protein concentration of the cell lysates was determined using Roti Nanoquant solution for Bradford protein assays according to the manufacturer's instructions (Roth). For each sample a total protein amount of 50 µg was separated by SDS-PAGE. To detect His-tagged proteins, samples were analyzed by Western blotting with a monoclonal anti-His-tag antibody (mouse IgG₁, Novagen) and the Amersham ECL Plus Western Blot detection System (GE Healthcare). The intensities of the bands were quantified by densitometry with ImageJ 1.48v software, and mean intensities from three independent blots were plotted.

Construction of complementation vectors

To generate pHERD20T::*accB* and pHERD20T::*accBC* complementation constructs, the cDNAs encoding *accB* and *accBC* were amplified with HotStar HiFidelity Polymerase (Qiagen) and the primers listed in table S2, which contained restriction sites for subsequent cloning into pHERD20T (40). The pHERD20T::*accB* and pHERD20T::*accBC* plasmids were transferred to the respective *P. aeruginosa* strains, and expression of their encoded genes was induced with 0.2% arabinose. Congo Red agar plates were prepared as described previously (41). Briefly, LB medium without NaCl was solidified with 1.6% of agar and supplemented with Congo Red dye (40 µg/ml) and carbenicillin (400 µg/ml). 5 µl of bacterial cell cultures that had an OD₆₀₀ of 0.025 were spotted onto the agar plates and then were incubated at room temperature for 7 days.

Genome sequencing

The chromosomal DNA of the clinical isolate SCV20265, WT20265, and the in vitro-generated revertants was isolated from exponentially growing cells (with an OD₆₀₀ of 1.0). DNA was isolated with the DNeasy Blood & Tissue Kit (Qiagen) according to the manufacturer's instructions. Libraries of 250 bp were prepared according to the manufacturer's instructions entitled "Preparing Samples for Paired-End-Sequencing." Cluster generation was performed with the Illumina cluster station, sequencing for read 1 and read 2 on the Genome Analyzer according to a standard protocol. All templates were sequenced for 76 cycles from both ends on the Illumina GAII platform, producing 14- to 35-fold coverage for SCV, WT, and revertant strains. The fluorescent images were processed to sequences with Genome Analyzer Pipeline Analysis software 1.6 (Illumina). The sequence reads (76 bp) were quality-trimmed with the perl script Trim.pl by Nik Joshi (obtained from: <http://wiki.bioinformatics.ucdavis.edu/index.php/Trim.pl>) in the "windowed adaptive trimming" mode, removing any sequence with a quality score <10. On average, between 3.8

and 5.7% of the reads were discarded after the quality trimming. The raw reads were assembled into contigs with the AMOS pipeline, taking independently PAO1 and PA14 as reference sequences, which produced 249 and 527 contigs, respectively. Reference-guided, de-novo assembly was performed with Velvet version 1.0.14 and the 249 contigs as reference, thus reducing the number to 66 contigs. Finally, manual contig alignment with GAP4 resulted in a total of 14 contigs that were aligned to the PAO1 genome with the software package Mauve version 2.3.1. The estimated size of the 14 SCV20265 contigs is 6,233,956 bp with an average G and C content of 66.6%. This places the newly sequenced genome very close in size to PAO1, which is of 6,264,404 bp length. The 5688 genes covering 89% of the genome were annotated with GLIMMER and BLAST according to the PAO1 gene nomenclature. Detection of SNPs was performed with MAQ software (42, 43).

Quantitative real-time PCR analysis

For total RNA extraction, 2 ml of bacterial culture with an OD₆₀₀ of 2 were mixed with the same amount of RNA-Protect buffer (Qiagen). After incubation for 10 min at room temperature, the cell suspension was centrifuged for 10 min at 3488 g, the supernatant was removed, and the pellet was stored at -80°C. RNA was extracted with the RNeasy Mini Kit and Qias shredder columns (both obtained from Qiagen) according to the manufacturer's instructions, with some modifications. The RNA was treated with the DNA-free kit (Ambion), and the concentration was determined by spectrophotometry (Nanodrop, Thermo Scientific). To quantify *accB* and *accC* mRNAs, we used the LightCycler480 RNA Master Hydrolysis Probes kit (Roche) in combination with the Light Cycler 480 instrument (Roche) according to the manufacturer's instructions. The primers used are listed in table S2. The following cycling conditions were applied for all primer combinations in a reaction volume of 20 µl: 30 s at 95°C for denaturation; 45 cycles of amplification at 95°C for 15 s and at

55°C for 30 s; and a final cooling step at 40°C. The abundances of the mRNAs of interest were normalized to that of *rpsL* mRNA. The relative abundances were calculated with the C_T method from at least three biological samples, with two technical replicates of each (44). The relative abundances are given as the fold-changes with standard deviations.

CD spectroscopy analysis of DNA oligonucleotides and toeprinting assays

CD spectra of 27-nucleotide DNA oligonucleotides (table S3) were recorded on a JASCO J-815 CD spectrometer at a concentration of 32 μ M in 50 mM sodium phosphate (pH 7.2) with 1 mM MgSO₄. Thermal unfolding of secondary structures was detected as a decrease in molar ellipticity at 277 nm as a function of temperature in intervals of 0.5 μ C and a ramp rate of 1°C per minute. To determine the melting temperatures, raw data were fitted with an equation for a single-step unfolding of a monomer with corrections for linear changes of the CD signal before and after the unfolding transition (45). Toeprinting (primer extension inhibition) assays were performed as described previously (46). Plasmids used as templates for mRNA synthesis (which are listed in table S1) were constructed by blunt-end cloning. The respective *accB* 5'-UTR with 60 nucleotides of coding sequence was amplified with the primers indicated in table S2, thereby adding a T7 promoter sequence (GAAATTAATACGACTCACTATAGG) to the 5'-end and an *EcoRV* site to the 3' end into the *Sma* I site of pUCP20. In the reverse transcription reaction, the primer *accB_TOE_R-10* (CCGGACTCTTCCAGCAGC) was used for primer extension. The experiments were performed at 28°C and 37°C for mRNA representing the 5'-UTR of *accBC* from SCV20265, Rev1 (G-15A) and Rev4 (T-31C) in the presence and absence of *E. coli* 30S ribosomal subunits, respectively.

Fatty acid composition analysis

Bacteria were grown for 24 hours on Columbia blood agar plates (BD) at 37°C, collected by scraping, and the cell material was washed once with 1 ml of LB medium, centrifuged for 5 min at 6797 g, and the supernatant was discarded. Extraction of cellular fatty acids was performed as described previously (47) with 2 g of wet cells from the cell pellet to extract the total lipids and to prepare the fatty acids for separation by gas chromatography. The fatty acid composition is provided as the percentage of the total lipid fraction and the measurements were performed in triplicate.

Quantification of c-di-GMP

To quantify intracellular c-di-GMP concentrations, 5 ml of bacterial suspensions were harvested after 24 hours of cultivation in LB at 28°C. Alternatively, bacteria were grown as a lawn for 20 hours at 37°C on LB agar plates alone or supplemented with 120 or 300 mM NaCl or under the corresponding planktonic conditions. The extraction of c-di-GMP and its quantification by HPLC-coupled tandem mass spectrometry was performed as described previously (48); however, isotope-labeled $^{13}\text{C}^{15}\text{N}$ -c-di-GMP was used as internal standard. The c-di-GMP extracts were stored overnight at -20°C to enable complete protein precipitation. The c-di-GMP concentrations are given as pmol of c-di-GMP/mg protein and as mean values from three biological samples, each analyzed in duplicate. The protein concentration was determined using RotiNanoquant solution for Bradford protein assays according to the manufacturer's instructions (Roth). For samples obtained from agar plates, the c-di-GMP concentrations are given as pmol c-di-GMP/mg cells (wet weight), and are mean values from three biological samples, each analyzed in duplicate.

Statistical analysis

Statistical analysis was performed with GraphPad Prism 6 software using the non-parametric one- or two-tailed Mann-Whitney test. Data from the analysis of fatty acid composition, Western blots, and CD spectroscopy were analyzed with the one-tailed Mann-Whitney test because we expected a reduction in the length of the fatty acid chains, decreased concentrations of AccBC, and an increase in the melting temperature because of the mutation in the 5'-UTR of *accBC*. The two-tailed Mann-Whitney test was used to evaluate c-di-GMP measurements because the effect of the mutation in the 5'-UTR of *accBC* and of different cultivation conditions could not be predicted. For statistical calculations, at least three independent experiments or biological replicates were used.

Supplementary Materials

Fig. S1. Analysis of AccB production with His₆ translational fusion constructs.

Fig. S2. Prediction of the secondary structures of DNA oligonucleotides and CD spectroscopy.

Fig. S3. Analysis of cellular fatty acid composition.

Fig. S4. Colony morphologies of complemented strains.

Fig. S5. Intracellular c-di-GMP concentrations for revertant 1

Fig. S6. Fatty acid composition under different growth conditions.

Fig. S7. Colony morphologies on Columbia blood agar plates.

Table S1. Strains and plasmids.

Table S2. Primers.

Table S3. DNA oligonucleotides used for CD spectroscopy.

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Fig. 1. Phenotypic and genetic characteristics of SCV20265 and its revertants. (A)

Representative photographs of colonies of the indicated strains grown on Columbia blood agar plates at 37°C for 18 hours. Scale bar: 1mm. **(B)** Organization of the *aroQIaccBC* gene region. The 3'-end sequence of *aroQI* is depicted in light gray and the intergenic region is in black. The ATG start codon of *accB* is displayed in italics. The mutations identified in the

indicated revertants are underlined and the SD sequence is shaded gray. Numbering of nucleotides is relative to the transcriptional start site.

Fig. 2. Effects of point mutations in the 5'-UTR of *accBC*. (A) Predicted mRNA secondary structures (19) of 27-nucleotide fragments of mRNA representing the 5'-UTR of *accBC* of SCV20265 and revertant 1 (Rev1, G⁻¹⁵→A). Numbers indicate nucleotide positions as referred to in Fig. 1B, and the color shading indicates the probability of base-pairing, from low (0.0) to high (1.0), as indicated. (B) Top: Western blotting analysis of AccB protein in PA14 wild-type cells expressing *accB-his₆* translational fusion constructs containing the *accBC* 5'-UTR of SCV20265 or Rev1. The Western blot is representative of three independent experiments. Bottom: Densitometric analysis of the amounts of AccB proteins in the indicated cells relative to that in WT cells. Data are means ± SD of three independent experiments. **P* < 0.05, according to the one-tailed Mann-Whitney test. (C) Thermal stability of DNA oligonucleotides (table S3) representing the respective 5'-UTR of *accBC* from SCV20265 and revertants monitored by circular dichroism at 277 nm. Circles represent the raw data recalculated to the fraction of unfolded DNA. Continuous lines represent the data fits used to calculate the melting temperatures displayed in the table as means ± SD from three independent measurements. (D) Toeprint assays were performed with mRNA representing the 5'-UTR of *accBC* from SCV20265, Rev1 (G⁻¹⁵→A), or Rev4 (T⁻³¹→C) and a radioactively labeled primer in a reverse transcription reaction at 28→C and 37°C. The presence (+) or absence (-) of 30S ribosomal subunits (30S) is indicated. The AUG start codon and the SD sequence are indicated on the right, and full-length products and toeprint transcripts are indicated on the left. A truncated reverse transcription product of the SCV20265 mRNA is marked by an asterisk in the representative toeprint image. Data are representative of two independent experiments.

Fig. 3. Analysis of the cellular composition of fatty acids. Comparison of the amounts of the three major fatty acids, unsaturated palmitoleic acid (C16:1 ω 7), saturated palmitic acid (C16:0), and cis-vaccenic acid (C18:1 ω 7) as percentages of the total lipid fraction for the indicated strains grown on Columbia blood agar plates. Data are means \pm SD from three independent experiments. * P < 0.1; ns, not significant according to the one-tailed Mann-Whitney test as compared to Rev1, WT, and PA14, respectively.

Fig. 4. Phenotypes of wild-type and mutant strains. (A and B) Top: Intracellular c-di-GMP concentrations were measured after planktonic cultivation of (A) strains with a 20265 background and (B) strains with a PA14 background for 24 hours in LB at 28°C. Data are means \pm SD from three independent experiments, each performed in duplicate. * P < 0.05; ** P < 0.01; ns, not significant according to the two-tailed Mann-Whitney test. Bottom: Images of the colony morphologies of the indicated strains after growth on Columbia blood agar plates at 37°C for 18 hours. Scale bar: 1 mm.

Fig. 5. Phenotypes of *wsp* mutants and their respective wild-types. (A and B) Intracellular c-di-GMP concentrations were measured after planktonic cultivation of (A) *wspR* deletion mutants and (B) *wspF* mutants for 24 hours in LB at 28°C. Data are means \pm SD from three independent experiments, each performed in duplicate. * P < 0.05; ** P < 0.01; ns, not significant according to the two-tailed Mann-Whitney test. Images beside the bar graphs show the colony morphologies of the indicated strains after growth on Columbia blood agar plates at 37°C for 18 hours. Scale bar: 1 mm.

Fig. 6. Measurement of intracellular c-di-GMP concentrations under different growth conditions. (A) Intracellular c-di-GMP concentrations of the indicated strains grown (A) under planktonic conditions in liquid LB medium or (B) attached on agar plates at 37°C for 20 hours. LB medium contained 0 (dark gray), 120 mM NaCl (black), or 300 mM NaCl (light gray). Data are means \pm SD from three independent experiments, each performed in duplicate. ** $P < 0.01$; ns, not significant according to the two-tailed Mann-Whitney test.

Fig. 7. Proposed model for the activation of Wsp signaling. The reduced length of fatty acid chains caused by decreased translation of *accBC* or the increased amount of unsaturated fatty acids caused by exposure to high salt concentrations and attachment to surfaces activates the membrane-bound chemoreceptor WspA. WspA stimulates the enhanced phosphorylation of the response regulator WspR by the sensor kinase WspE. Activated WspR then produces c-di-GMP through its GGDEF domain. WspB and WspD function as linker proteins. WspC, a methyltransferase, and WspF, a methylesterase, modify WspA and thus promote (WspC) or inhibit (WspF) the kinase activity of WspE. Mutations that reduce or abolish the activity of WspF lead to an imbalanced methylation of WspA, constitutive activation of WspE, and enhanced phosphorylation of WspR, which results in the overproduction of c-di-GMP.