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Pseudomonas aeruginosa cupA encoded fimbriae expression is regulated by a GGDEF and EAL domain dependent modulation of the intracellular level of cyclic diguanylate

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Running title: Role of c-di-GMP in *P. aeruginosa* fimbriae expression

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Summary

Cyclic-diguanylate (c-di-GMP) is a widespread bacterial signal molecule that plays a major role in the modulation of cellular surface components, such as exopolysaccharides and fimbriae, and in the establishment of a sessile life style. Here, we report that intracellular c-di-GMP levels influence *cupA* encoded fimbriae expression in *Pseudomonas aeruginosa*. In an autoaggregative *P. aeruginosa* small colony variant (SCV) CupA fimbriae and the intracellular c-di-GMP concentration were found to be enhanced as compared to the clonal wild-type. The expression of CupA fimbriae were dependent on a functional PA1120 and *morA* gene both encoding a GGDEF domain. Overexpression of the GGDEF domain protein PA1120 complemented the PA1120 and the *morA* mutant with respect to CupA fimbriae expression. In agreement with these findings, overexpression of the EAL domain containing phenotypic variance regulator (PvrR) in the SCV resulted in a switch to wild-type colony morphology, a decreased intracellular level of c-di-GMP and reduced *cupA* fimbriae expression. Although a regulation of *cupA* encoded fimbriae expression via c-di-GMP was clearly demonstrated, *cupA* expression is also under transcriptional control that is independent of the global intracellular c-di-GMP levels.

Introduction

Biofilm formation by bacterial pathogens is recognized as an important factor in the progression and persistence of many infectious diseases (Donlan and Costerton, 2002; Hall-Stoodley, Costerton, and Stoodley, 2004; Watnick and Kolter, 2000). Bacteria have been shown to form biofilms on a number of surfaces, including abiotic surfaces and human tissues and one of the strongest links between biofilm formation and bacterial pathogenesis has been established for *Pseudomonas aeruginosa* (Costerton, Stewart, and Greenberg, 1999; Costerton, 2001). Recalcitrance of biofilm infections seems to be facilitated by the evolution of a genetic diverse bacterial population (Boles, Thoendel, and Singh, 2004). Accordantly, a very typical microbiological diagnostic finding is the recovery of various *P. aeruginosa* phenotypes from chronically infected respiratory tract specimens of cystic fibrosis (CF) patients. Apart from the best-studied mucoid *P. aeruginosa* phenotype (Govan and Deretic, 1996) it is known that dwarf colonies can be isolated from the chronically infected CF lung (Zierdt and Schmidt, 1964). These “small colony variants” (SCV) show increased antibiotic resistance to a broad range of antimicrobial agents and their recovery in CF patients could be correlated with parameters revealing poor lung function and inhaled antibiotic therapy (Haussler *et al.*, 1999). The observation that we do find biofilm forming auto-aggregative *P. aeruginosa* SCV phenotypes not only *in vivo* in the chronically infected CF lung (Haussler *et al.*, 2003), but also *in vitro* after exposure to antibiotics (Drenkard and Ausubel, 2002) or from biofilm cultures (Deziel, Comeau, and Villemur, 2001) makes those highly adherent SCV phenotypes particularly interesting and implicates a general mechanism for the emergence of biofilm phenotypes (Haussler, 2004).

Given the great medical importance of biofilm infections the identification of signals and regulatory factors required for phenotypic switching is a major research issue. Recently, a phenotype variant regulator (PvrR) was identified in *P. aeruginosa* that greatly enhanced the switch from a resistant auto-aggregative rough PA-14 SCV to a fast growing revertant. An

involvement of the two component response regulator PvrR in the regulation of phenotypic switching was postulated (Drenkard and Ausubel, 2002). Sequence analysis of the PvrR revealed an EAL domain, named for conserved amino acid residues.

Proteins containing a GGDEF or an EAL domain have been linked in genetic studies to c-di-GMP synthesis and degradation respectively (Tal *et al.*, 1998). Since then a diguanylate cyclase activity has been assigned to the GGDEF domain based on i) sequence alignment with eukaryotic adenylate cyclases (Pei and Grishin, 2001), ii) the ability of GGDEF domain proteins from other organisms to activate cellulose production in Rhizobium and Agrobacterium (Ausmees *et al.*, 2001), iii) direct evidence of the enzymatic activity of the GGDEF domain (Paul *et al.*, 2004;Ryjenkov *et al.*, 2005) and iv) on structural basis (Chan *et al.*, 2004). On the other hand, the EAL domain has been demonstrated to exhibit phosphodiesterase activity (Bobrov, Kirillina, and Perry, 2005; Christen *et al.*, 2005; Schmidt, Ryjenkov, and Gomelsky, 2005). Many of the proteins with GGDEF and/or EAL domains show a multimodular arrangement: a number contain both a GGDEF and an EAL domain and frequently the domains of the enzymatic activity are fused to signal receiver or transmission domains, suggesting that the c-di-GMP level is modulated by environmental cues (Gerstel and Romling, 2003; Merkel, Barros, and Stibitz, 1998; Romling *et al.*, 2000; Tischler, Lee, and Camilli, 2002). The fundamental work of M. Benziman and colleagues has identified c-di-GMP as an allosteric activator of the membrane bound cellulose synthase complex in *Gluconacetobacter xylinus* (formerly known as *Acetobacter xylinum)* (Ross *et al.*, 1987) and only recently the PilZ domain present in the BcsA subunit of the bacterial cellulose synthetase (Amikam and Galperin, 2006) as well as an *Escherichia coli* PilZ domain protein (YcgR) were shown to bind c-di-GMP (Ryjenkov *et al.*, 2006).

Proteins with GGDEF and/or EAL domains have previously been implicated in the generation of bacterial morphotypes characterized by an increased production of extracellular matrix components and by auto-aggregative growth behavior and biofilm formation and thus to

promote transition to a sessile lifestyle by the induction of surface-localized adhesive structures (Boles and McCarter, 2002; Bomchil, Watnick, and Kolter, 2003; Jones, Lillard, Jr., and Perry, 1999; Hoffman *et al.*, 2005; D'Argenio *et al.*, 2002; Hickman, Tifrea, and Harwood, 2005).

In this study we demonstrate that the expression of fimbriae encoded by the chaperone usher pathway (*cupA*) gene cluster in *P. aeruginosa* is involved in evolution of an auto-aggregative SCV phenotype and that CupA expression is modulated by the intracellular level of c-di-GMP via proteins encoding for EAL and GGDEF domain proteins, respectively.

Results

The auto-aggregative P. aeruginosa *SCV phenotype is linked to fimbria expression encoded by the* cupA *gene cluster*

In pursuit of our interests in the etiological factors involved in the emergence of the auto-aggregative biofilm-forming SCV phenotypes (Fig. 1 A, B), we generated a *P. aeruginosa* SCV 20265 transposon insertion mutant library and screened 10.000 mutants for a switch of the small colony phenotype to the wild-type phenotype after growth on Columbia blood agar plates for 48 h. 67 large surface colony mutants were identified. Among these, 21 mutants had lost their autoaggregative growth behavior as determined by macroscopic examination after growth in minimal medium (Fig. 1a, b). Sequencing of the affected genes was successful for 18 mutants, 8 of these were demonstrated to be clonal isolates and the transposon insertion site of the remaining 10 mutants corresponded to 7 gene loci (Table 1). Among these SCV mutants we found two mutants with transposon insertions within the `chaperone usher pathway´ (*cupA*) gene cluster, one mutant with an insertion in the gene encoding for an ATP-dependent RNA helicase (RhlB), one insertion within the motility regulator *morA* and three mutants with insertions in genes encoding hypothetical proteins.

Since it has previously been demonstrated that the *cupA* gene cluster is essential for biofilm formation and probably encodes for fimbriae at the bacterial surface (Vallet *et al.*, 2001), we monitored CupA expression using immunoblots developed with a highly specific polyclonal serum directed against the structural fimbrial subunit encoded by the *cupA1* gene. As depicted in Fig. 2A the auto-aggregative SCV 20265 exhibited an increased CupA expression as compared to its clonal wild-type, whereas in the transposon mutants with an insertion in the *cupA* gene cluster (*cupA2* and *cupA3*) no CupA expression could be detected. Moreover, CupA expression seemed to be weaker in all other mutants which exhibited a switch to a

wild-type phenotype (Fig. 2A). These results implicate that an auto-aggregative SCV phenotype of *P. aeruginosa* is linked to the expression of CupA.

To further confirm that *cupA* encodes for fimbrial structures we applied electron microscopy, which revealed that the SCV 20265 but not the wild-type expressed abundant fimbrial structures when grown at 28°C. These structures were not restricted to the cellular poles and the environment of the cells were surrounded by fimbrial fragments (data not shown).

Since the anti-CupA1 polyclonal serum was not suitable for immunomicroscopy, we designed a second peptide of the CupA1 protein and generated a monoclonal anti-CupA1 antibody. This monoclonal antibody detected *cupA* encoded fimbriae of the SCV 20265 in immunofluorescence microscopy (data not shown) and in immunogold electron microscopy (Fig. 3A). For the visualization of the fimbria the probes had to be formalin fixed and heat treated which probably accounts for the low labeling yields by field emission scanning electron microscopy. A *cupA* mutant served as a control and showed no labeling (Fig. 3B).

Interestingly, temperature seems to be one environmental determinant that is of importance for CupA expression and thus for the auto-aggregative *P. aeruginosa* phenotype and biofilm formation. Western blot analysis revealed a markedly enhanced CupA expression at 28°C compared to 37°C. We found that CupA1 expression in the SCV 20265 was clearly enhanced after the bacteria had been cultivated at 28°C instead of 37°C and even in the wild-type a slight CupA1 expression could be visualized in Western blot analysis after cultivation at 28°C (Fig 2B).

GGDEF domains are involved in CupA expression

There is growing evidence that attachment of various bacteria to surfaces is regulated via the modulation of the newly identified bacterial signal molecule, cyclic di-GMP. A rise in the intracellular c-di-GMP level is thought to be mediated by activated GGDEF domain containing proteins, whereas proteins encoding an EAL domain seem to be responsible for the

decrease of intracellular c-di-GMP. Our screen for SCV mutants that showed a reversion to the wild type phenotype revealed two insertions within the putative membrane proteins PA1120 and MorA. Both of these proteins possess a transmembrane domain, a central sensory domain (PAS-PAC motifs in MorA and a HAMP domain in PA1120) and have a C-terminal GGDEF domains while MorA harbors an additional EAL domain. To test whether the GGDEF domain encoding protein PA1120 can restore CupA expression in both of these mutants we cloned PA1120 into pUCP20, transformed the SCV 20265 PA1120 mutant and the SCV 20265 *morA* mutant with the vector and monitored CupA expression using western blots. Overexpression of PA1120 restored *cupA* encoded fimbriae expression in both mutants (Fig. 4A/B). therefore, we concluded that c-di-GMP is important for CupA fimbriae expression.

Influence of the c-di-GMP level on fimbriae expression

Recently, high intracellular levels of c-di-GMP have been demonstrated to activate cellulose and curli fimbriae biosynthesis in *Salmonella enterica* serovar Typhimurium (Simm *et al.*, 2004; Zogaj *et al.*, 2001; Romling, Gomelsky, and Galperin, 2005). We therefore were interested in whether the c-di-GMP level of the *P. aeruginosa* SCV 20265 was elevated compared to the wild-type and determined the c-di-GMP level by analyzing bacterial extracts by reversed phase high pressure liquid chromatography (HPLC) coupled with MALDI TOF analysis. The results clearly show an enhanced c-di-GMP level in the autoaggregative, CupA fimbriae over-expressing SCV 20265 as compared to its wild-type (Fig. 5). A decrease in the level of c-di-GMP was observed in the transposon mutant of the SCV 20265 with an insertion in the GGDEF domain protein PA1120. Moreover, growth at low temperature revealed not only an increased *cupA* encoded fimbriae expression in the wild-type, but also an elevated c-di-GMP level (Fig. 5).

GGDEF and EAL domains influence the c-di-GMP level and fimbriae expression in an antagonizing way

Recently, the phenotype variant regulator (PvrR) that harbors an EAL domain was shown to enhance the switch of the auto-aggregative rough *P. aeruginosa* PA-14 SCV to a fast growing revertant (Drenkard and Ausubel, 2002). The authors proposed that PvrR was not directly involved in the modifications required for phenotypic switching but may regulate the factors that are required.

Although *pvrR* is present in the genome of strain 20265 we were not able to identify a mutant harboring an insertion in *pvrR* within a screen of 12.000 wild-type 20265 transposon mutants for a switch to the small colony phenotype nor did we identify any transposon insertion within a gene encoding an EAL domain. Since we were interested in whether not only GGDEF but also EAL domains had an effect on fimbrial expression in *P. aeruginosa* via the modulation of the intracellular level of c-di-GMP, we introduced the *pvrR* gene on the pED202 plasmid (kindly provided by F. Ausubel) into the SCV 20265. The PvrR overexpressing SCV exhibited a reversion to a wild-type phenotype and a decreased fimbriae expression was detected by Western blot analysis (Fig. 4C). Moreover, we performed a quantitative RT-PCR for *cupA1* and determined the relative level of its expression as normalized to the expression level of the housekeeping PA1580 (citrate synthase) gene (*gltA*). We were able to show a 2.97-fold ($\pm$ 0.56) relative down-regulation of *cupA1* in the PvrR over-expressing SCV at the transcriptional level and as shown in Fig. 5, compared to the vector control, the PvrR over-expressing SCV exhibited a decreased level of c-di-GMP.

CupA *encoded fimbriae expression independent of the global c-di-GMP level*

Within the screen of 12.000 wild-type 20265 transposon mutants for a switch to the small colony phenotype, 32 mutants were identified that exhibited a SCV phenotype on Columbia blood agar plates and on Müller-Hinton agar plates and 28 were successfully sequenced.

Among the set of 32 wild-type 20265 transposon mutants exhibiting a SCV phenotype, 13 mutants showed an autoaggregative growth behavior but only one mutant demonstrated an increased expression of CupA. This wild-type 20265 mutant harbored a transposon insertion within the lipoamide dehydrogenase (*lpdG*) gene and expressed significantly increased CupA fimbriae at the protein level, as determined by Western blot analysis (Fig. 4D). Moreover, we performed a quantitative real-time PCR and determined the relative levels of *cupA* expression as normalized to the level of expression the housekeeping PA1580 (citrate synthase) gene (*gltA*). We were able to show a 115-fold (± 97.71) relative up-regulation of the genes coding for the CupA on the transcriptional level. Nevertheless, the global c-di-GMP level of this transposon mutant was not increased (Fig. 5). Overexpression of the *lpdG* gene in trans in the WT 20265 *lpdG* transposon mutant led to a decreased transcription of *cupA1* (13.41-fold ± 9.1), a decreased fimbriae expression (Fig. 4D), whereas the c-di-GMP levels were even increased (Fig. 5). These results indicate that *cupA* expression is also under transcriptional control that is independent of the global intracellular c-di-GMP levels.

Phenotype and motility

Both SCV 20265 GGDEF domain mutants (PA1120 and *morA*) exhibited a non-autoaggregative growth behavior in liquid culture and a large colony phenotype on agar plates. However, the structure of the colonies could be distinguished from one another suggesting that *morA* and PA1120 might function in a convergent manner to coordinate fimbria development. Moreover, we observed that overexpression of PA1120 in the two SCV 20265 GGDEF domain mutants restored CupA expression but did not restore the small colony phenotype on agar plates. The PA1120 overexpressing strains even produced surface colonies that were larger than those of the wild-type. This lead us to survey the motility phenotype of the various mutants. As shown in Table 1 all SCV 20265 mutants exhibiting a switch to a wild-type phenotype expressed low CupA fimbriae and all of them exhibited an

increased twitching motility and a increased capability to swim as compared to the parent SCV 20265. The fact that even the *cupA2* and *cupA3* mutants exhibited an increased swimming and twitching motility implicates that CupA fimbriae may disturb swimming and type IV pili mediated twitching motility. However, overexpression of the GGDEF containing protein PA1120 in the *morA* and PA1120 membrane protein mutants only restored *cupA* expression but was not sufficient to fully restore the reduced swimming and twitching motility phenotype of the parental SCV 20265 (Table 1).

Discussion

Bacterial cell-surface organelles play a key role in attachment of microorganisms to surfaces and have been shown to be necessary for biofilm development (Dunne, Jr., 2002; O'Toole and Kolter, 1998; Klausen *et al.*, 2003b; Klausen *et al.*, 2003a). Recently, the assembly of *P. aeruginosa* fimbrial adhesins other than type IV pili have been considered to be important for biofilm formation in *P. aeruginosa* (Kulasekara *et al.*, 2005; Vallet *et al.*, 2001; Vallet *et al.*, 2004). It was proposed that the three gene clusters *cupA, cupB and cupC* of the chaperon/usher family encode the components of a new class of *P. aeruginosa* fimbrial adhesins, related to the adhesins in other microorganisms (Sauer *et al.*, 2000; Soto and Hultgren, 1999; Wu and Fives-Taylor, 2001). *P. aeruginosa* mutants devoid of a functional *cupA* gene locus were defective in the formation of biofilms, in a manner that was shown to be independent of the presence of type IV pili (Vallet *et al.*, 2001).

The function and regulation of the Cup systems in response to environmental cues have recently been studied in more detail by analyzing the regulatory networks that control the expression of *cup* genes. *CupB* and *cupC* were demonstrated to be influenced by the two-component system RocS1-RocA1-RocR. The finding that RocR which repressed *cupC* expression harbors an EAL domain (Kulasekara *et al.*, 2005) suggested that the modulation of intracellular c-di-GMP is critical for Cup expression. Interestingly, the GGDEF containing

response regulator WspR has previously been shown to be required for *P. aeruginosa* biofilm formation and was suggested to induce the expression of *cupA* encoded putative fimbrial adhesin (D'Argenio *et al.*, 2002). In accordance, a recent comprehensive analysis of *P. aeruginosa* genes encoding the enzymes of c-di-GMP metabolism revealed that a PA-14 transposon mutant with a non-functional WspR (PA3207) exhibited a decreased capability to form biofilms whereas overexpression of *wspR* enhanced biofilm formation (Kulesekara *et al.*, 2006). Apart from *wspR*, 4 other gene loci were identified in that study that affected biofilm formation in the same way: the membrane proteins PA1120, PA1107 and PA1727 and PA5487. All of these genes encode for proteins with confirmed di-guanylate cyclase activity. PA1727 harbors an additional EAL domain without detectable phosphodiesterase activity. These results demonstrated that an increase in the levels of di-guanylate activity and presumably in c-di-GMP results in enhanced production of factors that promote biofilm formation in *P. aeruginosa.*

In this study we provide evidence that at least part of these biofilm promoting factors are *cupA* encoded fimbriae which are regulated via a modulation of the intracellular level of c-di-GMP by proteins encoding for EAL and GGDEF domains, respectively. This is supported by several lines of evidence. The autoaggregative SCV 20265 overexpressed *cupA* encoded fimbriae and exhibited an increased intracellular level of c-di-GMP as opposed to its wild-type. In a screen for transposon mutants that have lost the autoaggregative SCV biofilm phenotype, 7 independent non-aggregative wild-type phenotype mutants were identified, all of them exhibited a reduced expression of CupA fimbriae. Among these mutants two harbored transposon insertions within the *cupA* gene cluster and two within genes encoding membrane proteins harboring GGDEF domains. The first, PA1120, was among the genes identified before to exhibit di-guanylate cyclase activity and to influence biofilm formation (Kulesekara *et al.*, 2006); the second, *morA*, was previously not identified in PA-14 to neither

influence biofilm formation nor to exhibit guanylate cyclase activity. However, *morA* was shown to affect flagella development in *P. putida* and biofilm formation in *P. aeruginosa* PAO1 (Choy *et al.*, 2004). Finally we have shown that similar to the two mutants harboring a transposon insertion in membrane GGDEF domain proteins, overexpression of the PvrR EAL family protein in the SCV 20265 revealed a reduced c-di-GMP level, a reduced expression of CupA and a switch to the wild-type colony morphology.

Our overexpressing experiments demonstrate that altering the global c-di-GMP level is sufficient to induce CupA expression. Overexpression of the GGDEF domain protein PA1120 could complement the PA1120 and the *morA* SCV 20265 mutant strain for CupA expression implicating that the guanylate cyclase activity of the GGDEF domain and not the phosphodiesterase activity of the EAL domain is the relevant enzymatic activity in MorA. However, as the phenotypes of the two GGDEF domain mutants differed, it also implicates that the *P. aeruginosa* GGDEF containing proteins do not appear to be redundant. Nevertheless, overexpression of PA1120 in the PA1120 or *morA* mutant did not fully restore the (motility) phenotype probably due to an elimination of c-di-GMP signaling specificity. It has been shown before in *S. enterica* serovar Typhimurium and *E. coli* that overexpression of GGDEF domain proteins allow elevated concentrations of c-di-GMP in the whole cytoplasm probably leading to a saturation of different c-di-GMP pools that activate various c-di-GMP dependent pathways (Kader *et al.*, 2006; Weber *et al.*, 2006). 38 GGDEF and/or EAL containing proteins have been found in *P. aeruginosa* PAO1 (Croft *et al.*, 2000; Kulesekara *et al.*, 2006) all of which potentially impact on the intracellular c-di-GMP level creating a complex network of regulatory interactions (Kulesekara *et al.*, 2006; Simm *et al.*, 2004). Thus in overexpression experiments response genes directed by different GGDEF domain proteins might be activated which are originally functionally and spatially separated. It seems that overproduction of GGDEF domain proteins are sufficient to enhance *cupA* expression but

overrun the complex interrelation by which c-di-GMP signaling affects swimming and twitching motility in *P. aeruginosa*. This is corroborated by our finding that in the *cupA* overexpressing *lpdG* mutant which exhibited a switch to the small colony variant phenotype, the global c-di-GMP level was not elevated. We suggest that a distinct c-di-GMP pool may be responsible for the enhanced *cupA* expression in this mutant.

Our results contribute to the dissection of the various regulation levels of *P. aeruginosa* fimbriae expression and the motility phenotype. Further experimental characterization will significantly advance our understanding of bacterial adaptation as an inevitable precondition for bacterial pathogenicity.

Experimental procedures

Bacterial strains, plasmids and culture conditions

The clinical *P. aeruginosa* strain SCV 20265, isolated from the respiratory tract of a CF patient who attended the cystic fibrosis clinic at Hanover Medical School, Hanover, Germany and the clonal wild-type WT 20265 isolated from the same CF patient were used in this study (Haussler *et al.*, 1999). *P. aeruginosa* was routinely cultured at 37°C or 28°C on sheep blood based Columbia (Becton, Dickinson and Company) or Luria-Bertani (LB) agar. Autoaggregation was monitored by macroscopic inspection of bacterial cultures after 48 h of growth in mod. Vogel-Bonner medium (3.3 mM $MgSO_4$, 10 mM citric acid, 28 mM $NaNH_4HPO_4$, 37 mM K_2HPO_4, 214 mM potassium D-gluconate; pH 7.2).

A transposon mutant library of the SCV 20265 and the wild-type 20265 were generated using the transposon construction vector EZ:TN pMOD-3 (Epicentre) (harboring a gentamycin cassette derived from the plasposon pTnMod-Ogm (AF061920)) according to the manufacturers instructions. Transformants of the WT 20265 were selected on LB medium

supplemented with 50 µg/ml gentamycin and the colony morphology was evaluated after 48 h of growth at 37°C. Small colonies were re-streaked on Columbia and Muller Hinton agar plates to re-evaluate the small colony phenotype. Transformants of the SCV 20265 were also selected on LB medium supplemented with 50 µg/ml gentamycin, however, for the evaluation of colony morphology these clones had to be transferred onto Columbia agar. Therefore approximately 1000 clones were pooled and appropriate dilutions were plated onto Columbia agar plates. Large colony phenotypes were re-streaked an Columbia agar plates to re-evaluate the large surface phenotype. Transposon insertion sites were identified using a Y linker as described previously (Kwon and Ricke, 2000). The pED202 plasmid kindly provided by F.M. Ausubel, harbored a 3.5 kb *Pst*I fragment containing *pvrR* from *P. aeruginosa* PA-14 cloned into pUCP19 (Drenkard and Ausubel, 2002).

Plasmid construction and transfer

Routine molecular biological techniques were performed using established protocols. Complementation plasmids were constructed by PCR amplifying the complete open reading frame of *lpdG* and PA1120. Primer sequences that were used to amplify *P. aeruginosa* DNA are available upon request. The PCR fragments were cloned using the pGEM-T Easy kit (Promega). The resulting clones of *E. coli* JM109 were selected on LB agar plates containing 100 µg/ml ampicillin and verified by sequencing the inserts. Subsequently, restricted and purified fragments were cloned into pUCP20, transformed into *P. aeruginosa* by electoporation and selected on LB agar plates containing 300 µg/ml carbenicillin.

Generation of a polyclonal serum and a monoclonal antibody directed against CupA1

As deduced from the DNA sequence of *cupA1* from *P. aeruginosa* PAO1, a synthetic HPLC-purified peptide was kindly provided by Dr. W. Tegge, Helmholtz Centre for Infection Research (C-V-A-P-T-A-D-E-H-F-T-T-L-F-Q-A-T-N-P-S-amide). The Imject Maleimide

Activated Immunogen Conjugation Kit (Pierce) was applied to couple the peptide to ovalbumin. For the generation of polyclonal antibodies, 2 to 6 months old rabbits ('SPF, specific pathogen free') (Eurogentec (Seraing, Belgium) and Biogenes (Berlin)) were used. Two rabbits were immunized in parallel. The injections of the respective antigen was carried out at days 90, 73, 59 and 31 prior to the bleeding.

For affinity purification of the polyclonal antiserum an affinity column was generated by coupling of the respective peptide to CNBr-Sepharose 4 B (Amersham Biosciences) according to the manufacturers' recommendations. 25 ml of the rabbit serum were passed three times on the column. The resin was washed with 100 ml PBS and pre-wash buffer (0.1 M sodium acetic acid, 0.5 M NaCl, pH 4.8) was applied to the column until the OD_{280} became stable. Antibodies were eluted in elution buffer (0.2 M sodium acetic acid, 0.5 M NaCl pH 2.5). 1 ml fractions were collected and immediately neutralized by addition of 100 µl 1.5 M Tris-HCl (pH 8.8). The concentration of each fraction was measured photometrically and fractions containing protein were pooled and dialyzed overnight against PBS. Antibodies were supplemented with 0.02% sodium azide and stored at 4°C.

Monclonal antibodies was generated by standard protocol of Synaptic Systems (Göttingen) in addition using ELISA-, western- and immunofluorescence screening (see also www.sysy.com/mabservice.html). Briefly, we designed a peptide of the CupA1 protein (C-G-A-T-S-T-S-Y-D-Y-A-V-Q-Y-amide) which was coupled to KLH (Pierce). Three 8 to 10 weeks old Balb/c females were subcutaneously immunized over a period of 17 days. Cells from the knee lymph nodes were fused with the mouse myeloma cell line P3X63Ag.653 (ATCC CRL-1580). The clone used in this study (126D1) was cloned two times by limiting dilution and the subclass was determined to be IgG1 (Caltag Laboratories).

Western Blot Analysis

For Western Blot analysis of CupA expression bacteria were grown for 48h in LB broth at 28°C or 37°C respectively. Bacteria were harvested, suspended in loading buffer and separated on a 16.5 % Tris-Tricine gels with a 10 % spacer gel according to the procedure described by Schägger and von Jagow (Schagger and von Jagow, 1987). For the determination of the total protein concentration gels were stained with silver blue coomassie (Neuhoff *et al.*, 1988). After washing the gels overnight in water the respective lanes were cut out and destained in 5 ml destaining solution (30% ethanol, 10% acetic acid). Total protein amount was measured photometrically at a wavelength of 595 nm. The proteins were adjusted to the OD_{595} of the sample exhibiting the lowest values (OD_{595} between 0.2-0.4). The adjusted proteins were transferred to a polyvinyldene difluoride (PVDF) membrane (Millipore) and incubated in blocking buffer containing 5% slim milk powder and 0,1% Tween. The polyclonal serum directed against CupA1 was used as the primary antibody and a peroxidase-conjugated goat anti rabbit antibody as the secondary antibody (IgG(H+L), Dianova). Bound antibodies were visualized using chemoluminescence (Roche) and detected with a CCD camera (Intelligent Darkbox with Fujifilm LAS100). Western blots of whole bacterial extracts with the polyclonal serum revealed one single band. To proof the specificity of the serum the band was excised and validated to be CupA1 by LC-MS analysis followed by a MASCOT database search (Wehmhoner *et al.*, 2005).

Real-time quantitative RT-PCR

Total RNA was isolated from approx. 3×10^9 bacteria, suspended in RNAProtect (Quiagen) using RNeasy columns (Qiagen). The nucleic acids were treated with DNaseI (Roche) and the yield of total cellular RNA was determined by UV-absorption. In order to quantify mRNA levels of selected genes, quantitative real-time PCR was performed with a GeneAmp 5700 Sequence Detection System (Applied Biosystems, Weiterstadt, Germany). Amplifications

were run in 50 µl assays with the QPCR Core Reagent Kit (Applied Biosystems) containing SYBR-Green and PCR core reagents, according to the manufacturers protocol. The cycling program was as follows: Initial activation of Taq polymerase for 10 min at 95 °C, then, 40 amplification cycles of a 15 sec denaturing interval at 95 °C, a 1 min annealing step at 60 °C and a 1 min amplification step at 72 °C were run. Standard curves were generated using serially diluted cDNA. The PA1580 housekeeping gene (coding for citrate synthase) was used for normalization.

Electron microscopy

For transmission electron microscopic analysis of negatively stained samples, bacteria were grown in liquid culture at 28°C, centrifuged and resuspended in TE buffer (10 mM Tris/HCl, 2 mM EDTA, pH 6.9). A carbon-coated Formvar copper grid (300 mesh) was floated on a drop of the resuspended bacteria for 1 min, rinsed with TE buffer and distilled water. Negative-staining was performed with a 2 % aqueous solution of uranyl acetate, pH 4.5. Samples were examined in a Zeiss transmission electron microscope EM910 at an acceleration voltage of 80 kV.

Immunogold electron microscopy was performed on formalin fixed and heat treated samples incubated with a monoclonal anti-CupA1 antibody (cell culture supernatant; 1:10 dilution) for 1 h at 30°C, washed twice with PBS and incubated with protein A/G gold complexes (1:50 dilution of the stock solution, 15 nm in diameter) for 30 min at 30°C. After washing with PBS samples were adsorbed onto poly-L-lysine coated cover slips with a diameter of 12 mm for 10 min, placed without washing in 2% glutaraldehyde in cacodylate buffer (0.1M cacodylate, 0.01 M $CaCl_2$, 0.01 M $MgCl_2$, 0.09 M sucrose, pH 6.9) and fixed for 5 min at room temperature and subsequently washed with TE-buffer (20 mM TRIS, 1 mM EDTA, pH 6,9) before dehydrating in a graded series of acetone (10, 30, 50, 70, 90, 100%) on ice for 15 min for each step. Samples in the 100% acetone step were allowed to reach room temperature

before another change in 100% acetone. Samples were then subjected to critical-point drying with liquid CO_2 (CPD 30, Balzers, Liechtenstein). Dried samples were covered with a gold film by sputter coating (SCD 40, Balzers Union, Liechtenstein) before examination in a field emission scanning electron microscope Zeiss DSM 982 Gemini using the Everhart Thornley SE detector and the inlens detector in a 50:50 ratio at an acceleration voltage of 5 kV.

C-di-GMP Synthesis

C-di-GMP was synthesized according to previously published protocols (de Vroom *et al.*, 1987;Hsu and Dennis, 1982) using precursor substances that were kindly provided by W. Pfleiderer and C. Ramamurthy (University of Konstanz, Germany). C-di-GMP was purified by reversed-phase HPLC and the identity of the structure was verified by nuclear magnetic resonance (NMR) spectroscopy and electrospray-mass spectrometry (ESI-MS) as described (Simm *et al.*, 2004).

Quantification of c-di GMP from P. aeruginosa

C-di-GMP isolation and detection were performed as described previously (Simm *et al.*, 2004). Briefly, bacterial pellets of a 48 h old 25 ml LB culture were resuspended in 300 µl water and heated at 100°C for 10 min. Subsequently nucleotides were extracted twice with 700 µl of ice cold 70% ethanol and lyophilized. Nucleotide extracts equivalent to 10mg cells (wet weight) were subjected to HPLC-separation using a reversed phase column (Hypersil ODS 5µ; Hypersil-Keystone). Runs were carried out in 0.1 M tri-ethyl-ammonium acteate (TEAA) pH 6.0 at 1ml min^{-1} using a multistep gradient of acetonitrile. Relevant fractions were collected, lyophilized and resuspended in 10µl water. MALDI-TOF was used to pinpoint all fractions containing c-di-GMP. Thereby the samples were applied to the target as described (Simm, 2004). Quantification by MALDI-TOF was carried out after pooling all fractions, which contained c-di-GMP. A standard curve was established using fractions spiked

458 with a known amount of c-di-GMP and c-di-AMP as internal control. The isotope area of c-
459 di-GMP and c-di-AMP was calculated and the ratio determined.

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630

Fig. 1. As compared to the SCV 20265 (A), the wild-type 20265 (B) produces larger surface colonies on Columbia agar plates. When grown in mod. Vogel Bonner medium for 48 h in the cultures of the SCV 20265 (a) but not in the wild-type 20265 (b) bacterial aggregates become visible.

Fig. 2. Western blots developed with a polyclonal serum directed against the structural subunit of *P. aeruginosa* fimbriae encoded by *cupA1*. **A.** The wild-type 20265 as well as the SCV 20265 mutants - exhibiting a switch to a non-autoaggregative wild-type phenotype - express reduced CupA1 as compared to the SCV 20265. **B.** CupA1 expression is temperature dependent.

Fig. 3. Immunogold field emission scanning electron microscopy using a monoclonal anti-CupA1 antibody on formalin fixed and heat treated SCV 20265 (A) and the isogenic *cupA2* mutant (B). Gold-labeling was detected bound to the exterior of the SCV 20265 (arrowheads), no labeling was detected in the *cupA* mutant.

Fig. 4. Western blots developed with a polyclonal serum directed against the structural subunit of *P. aeruginosa* fimbriae encoded by *cupA1*. Overexpression of PA1120 encoding the GGDEF domain protein enhances CupA1 expression in the PA1120 and the *morA* mutant (A, B), whereas overexpression of *pvrR* encoding an EAL domain led to a decreased CupA1 expression (C). The *lpdG* mutant expressed significantly increased CupA1 (D).

Fig. 5. Detection of c-di-GMP in *P. aeruginosa* as determined by HPLC analysis. All strains harbored the pUCP20 vector either as a control or as the cloning vehicle for *pvrR* or *lpdG*.

Table 1 Phenotypic characteristics of SCV 20265 transposon mutants exhibiting a switch to a non-autoaggregative wild-type phenotype as compared to the parental strain SCV 20265 and the wild-type 20265

Strain	Transposon Insertion site (Tn5 position)	Auto-aggregation	CupA expression (Western Blot)	Phenotype on agar	Twitching motility cm+/-SD	Swimming motility cm+/-SD
SCV 20265	-	↑↑	↑↑	SCV	1,4 +/-0,28	0,7 +/- 0,09
WT 20265	-	-	↓	WT	Neg.	4,8 +/- 0,73
SCV-tn5-1L	*lptA* (421)	-	↓	WT	2,0 +/-0,25	1,2 +/- 0,05
SCV-tn5-3F	*cupA2* (560)	-	↓	WT	1,7 +/-0,35	1,4 +/- 0,12
SCV-tn5-3D	*cupA3* (1469)	-	↓	WT	2,1 +/-0,12	1,9 +/- 0,28
SCV-tn5-1C	*rhl* (74)	-	↓	WT	2,3 +/-0,06	1,3 +/- 0,05
SCV-tn5-2E	PA1119 (329)	-	↓	WT	1,7 +/-0,45	2,7 +/- 0,16
SCV-tn5-2L	*morA* (2474)	-	↓	WT	2,0 +/-0,10	2,6 +/- 0,33
SCV-tn5-6E	PA1120 (1203)	-	↓	WT	2,0 +/-0,46	3,3 +/- 0,28
SCV-tn5-6F	PA1120 (1224)	-	↓	WT	n.d.	n.d.
SCV-tn5-6G	PA1120 (1193)	-	↓	WT	n.d.	n.d.
SCV-Tn5-8A	PA1120 (975)	-	↓	WT	n.d.	n.d.
SCV PA1120 pUC20:PA1120	PA1120 (1203)	n.d.	↑	Larger than WT	2,0 +/-0,55	3,0 +/- 0,17
SCV *morA* pUC20:PA1120	*morA* (2474)	n.d.	↑	Larger than WT	1,9 +/-0,20	2,6 +/-0,05

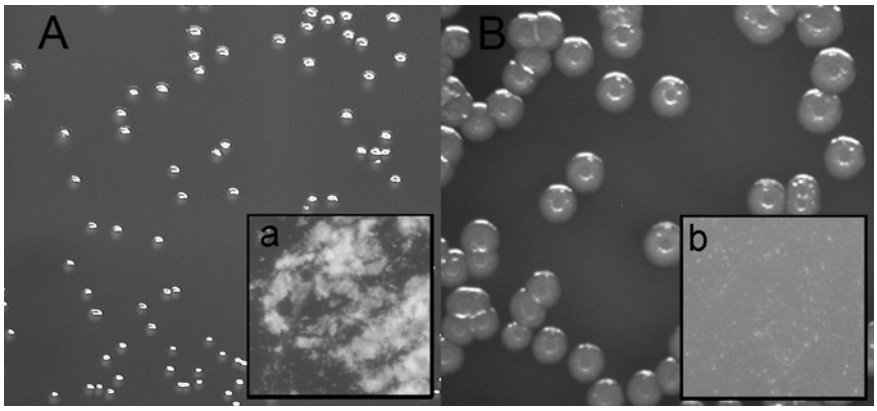
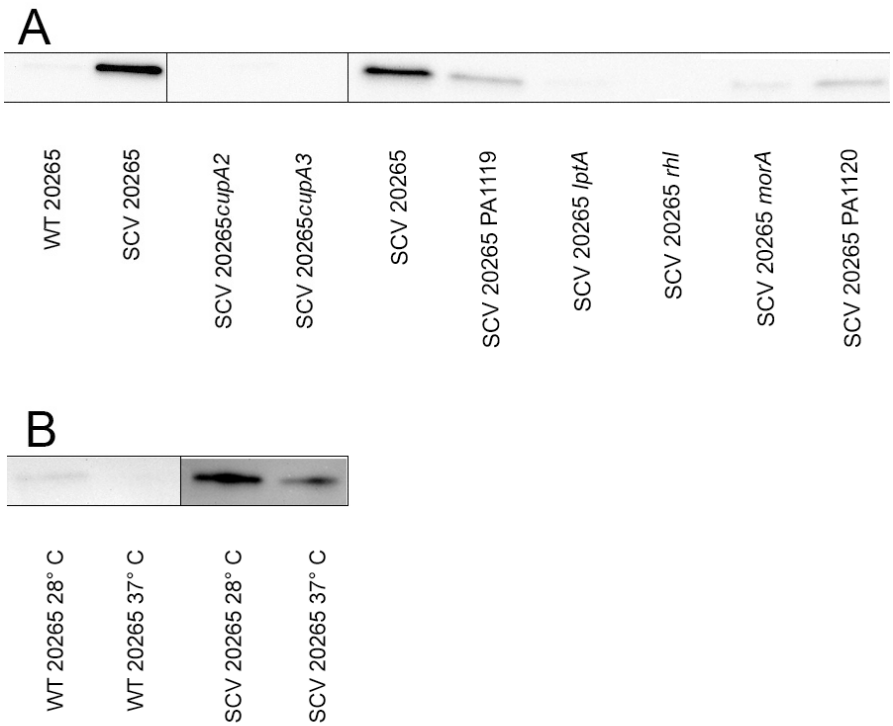


Figure 1



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663 Figure 2

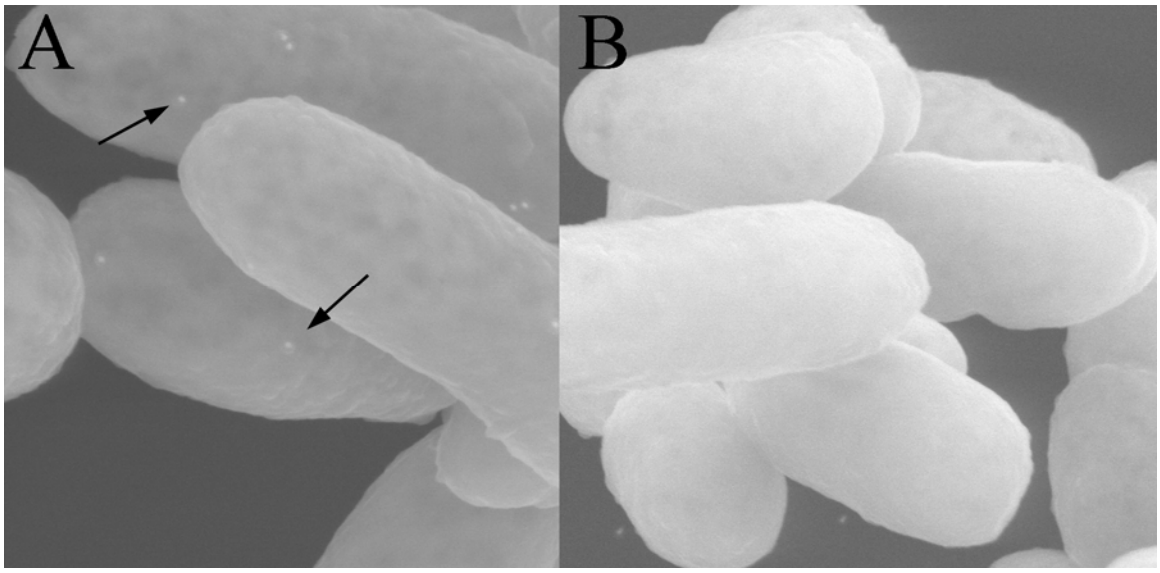


Figure 3

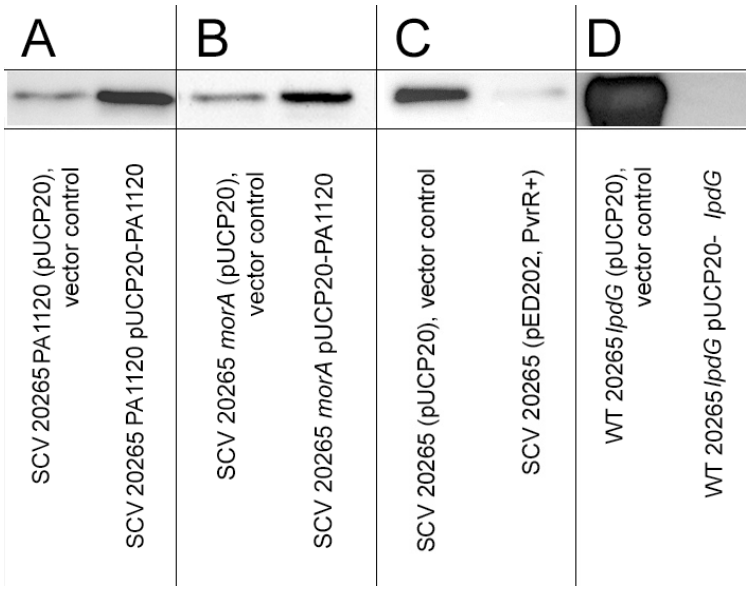
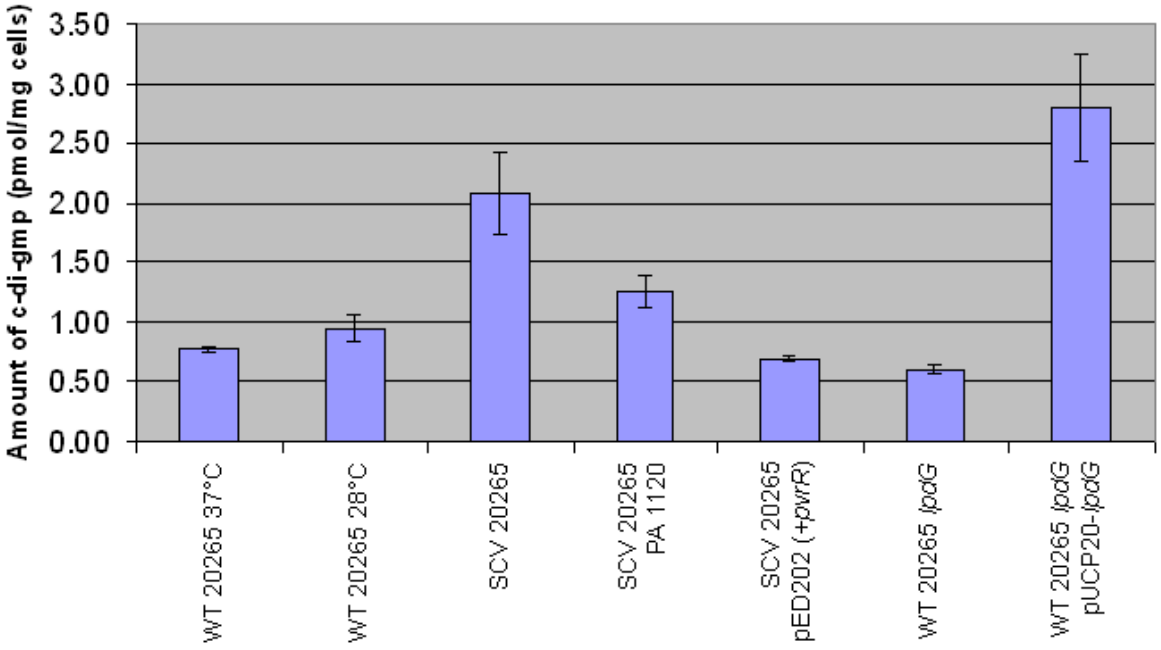

A
B
C
D
SCV 20265 PA1120 (pUCP20), vector control
SCV 20265 PA1120 pUCP20-PA1120
SCV 20265 morA (pUCP20), vector control
SCV 20265 morA pUCP20-PA1120
SCV 20265 (pUCP20), vector control
SCV 20265 (pED202, PvrR+)
WT 20265 lpdG (pUCP20), vector control
WT 20265 lpdG pUCP20- lpdG

Figure 4

668



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670 Figure 5