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The peptide chain release factor methyltransferase PrmC is essential for pathogenicity and environmental adaptation of *Pseudomonas aeruginosa* PA14

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Summary

P. aeruginosa pathogenicity and its capability to adapt to multiple environments are dependent on the production of diverse virulence factors, controlled by the sophisticated quorum sensing (QS) network of *P. aeruginosa*. To better understand the molecular mechanisms that underlie this adaptation we searched for novel key regulators of virulence factor production by screening a PA14 transposon mutant library for potential candidates acting downstream of the unique 2-alkyl-4-quinolone (AQ) QS system of *P. aeruginosa*. We focused the work on a protein named HemK with high homology to PrmC of *E. coli* displaying a similar enzymatic activity (therefore also referred to as PrmC). In this study, we demonstrate that PrmC is an *S*-adenosyl-L-methionine (AdoMet)-dependent methyltransferase of peptide chain release factors (RFs) essential for the expression of several virulence factors, such as pyocyanin, rhamnolipids and the type III-secreted toxin ExoT. Furthermore, the PA14_*prmC* mutant strain is unable to grow under anoxic conditions and has a significantly reduced pathogenicity in the infection model *Galleria mellonella*. Along with transcriptomic and proteomic analyses, the presented data indicate that the methylation of RFs in *P. aeruginosa* seems to have a global effect on cellular processes related to the virulence of this nosocomial pathogen.

Introduction

The Gram-negative bacterium *Pseudomonas aeruginosa* inhabits a variety of environments including water and soil and has the capability to colonize animals, plants and humans. . Because of its exceptionally flexible mode of life and high adaptability, *P. aeruginosa* can be found in industrial and hospital settings and is one of the predominant Gram-negative pathogens in nosocomial infections of the urinary and respiratory tracts and in patients suffering from severe burns . In particular, it plays a critical role in the development and progression of life-threatening chronic lung infections in patients with the genetic disease cystic fibrosis (CF) . Acute and chronic infections are readily established by utilizing an extensive and interacting orchestra of virulence factors such as the redox active phenazine pyocyanin, secreted toxins, lipases, elastases and proteases .

The ability of *P. aeruginosa* to evoke various life-threatening infections and the flexibility to face the challenge of changing environments can partially be attributed to an intact quorum sensing (QS) system. . QS in *P. aeruginosa* is enabled by the hierarchically linked and *N*-acyl-L-homoserine lactone (AHL)-dependent *las* and *rhl* systems and the 2-alkyl-4-quinolone (AQ) signaling pathway . Out of over 50 AQ-congeners, two major AQs, 2-heptyl-3-hydroxy-4(1*H*)-quinolone (the '*P*seudomonas-quinolone-signal'; PQS) and its immediate precursor 2-heptyl-4-quinolone (HHQ) have been shown to function as QS signals with biological relevance. .

To unravel new genes involved in the regulation of virulence factor production we screened for *P. aeruginosa* PA14 mutants, unable to up-regulate the AQ-dependent virulence factor pyocyanin after exogenous addition of organic extract containing alkyl-quinolone signal molecules (PA14-extract) to the growth media. We identified several AQ-non responding mutants identified by the screen and focused our interest on a mutant carrying a transposon insertion within a gene, coding for the putative methyltransferase HemK.

The PA14 HemK homologue in *Escherichia coli,* PrmC, has been identified as an enzyme that post-translationally methylates a critical amino acid of the release factors RF-1 and RF-2. Inactivation of *prmC* leads to increased stop codon readthrough, growth retardation and induction of oxidative stress response . In *E. coli* and in *Porphyromonas gingivalis* it was shown, that *prmC* and its cognate homologue was up-regulated during infections or in host environment respectively . In this study we show, that HemK has a methyltransferase activity (and is therefore referred to as PrmC) and plays an essential role for *P. aeruginosa* motility, regulation of virulence factors and adaptation to anaerobic growth. Furthermore, we observed that PrmC is important for the type III secretion system (T3SS) and virulence of *P. aeruginosa* in the host infection model *Galleria mellonella*. Enhanced by transcriptomic and proteomic analyses our results indicate that PrmC exerts an intense effect on global regulatory processes and is significantly involved in calibrating pathogenicity *in P. aeruginosa*.

Results

Identification of AQ-non responding mutants

To identify new genes involved in AQ-dependent pyocyanin production, we screened all 5833 mutants of the PA14NR library for their capability to produce pyocyanin and analyzed their responsiveness towards the exogenous addition of concentrated PA14-extract. Excluding transposon mutants within genes, well-known to be involved in pyocyanin biosynthesis , we detected 26 AQ-non responder (without any growth limitation) with impaired pyocyanin production despite addition of PA14-extracts (Table S1).

Since pyocyanin production is strongly influenced by the activity of PqsE encoded from the last gene of the *pqsABCDE* operon , we next investigated whether the AQ-non responding mutants still display a reduced pyocyanin production, when complemented with plasmid borne *pqsE* (*pUC20pqsE*). Table S1 shows, that despite overexpression of PqsE, 4 out of 26 mutants were not capable of increasing the pyocyanin level in our experimental conditions. Those 4 genes were PA14_25100 and PA14_53980 (both coding for a hypothetical protein), PA14_72390 (the PA14 orthologue of *kinB* in *P. aeruginosa* PAO1), and the hypothetical methyltransferase PrmC. A detailed analysis of the latter mutant showed that PA14_*prmC* was not able to restore pyocyanin production despite overexpression of PqsE. However, also vice versa, overexpression of *prmC* (pUCP20*prmC*) in a PA14_*pqsE* mutant did not restore synthesis of the virulence factor (Fig. 1). These results clearly demonstrate that the presence of both proteins, PrmC and PqsE, is essential for *P. aeruginosa* PA14 to produce pyocyanin.

Methyltransferase activity of PrmC

In the last decade it was demonstrated, that the PmrC homologues in *E. coli* (PrmC), *Yersinia pseudotuberculosis* (VagH) and in *Chlamydia trachomatis* (PrmC) showed *N*5-Glutamine-S-adenosyl-L-methionine-dependent methyltransferase activity. By methylation at the glutamine residue of a conserved GGQ motif of peptide chain release factors (RFs) the PmrC homologues were demonstrated to impact on translational termination and therefore on the global translational pattern . A protein alignment of PA14 PmrC with various previously described methyltransferases is depicted in Fig. S1 (suppl. material) and revealed that PA14 PmrC exhibits identity to the various homologues from 25.08 % (PrmC from *C. trachomatis*) and 48.55 % (VagH from *Y. pseudotuberculosis*) to 50.09 % (PrmC from *E. coli*).

Consequently, we wanted to investigate, whether PrmC also displays a methyltransferase activity. Since the knockout of *prmC* in *E. coli* (*E. coli* CK783) comes along with a significant growth defect , we analyzed if expression of the *P. aeruginosa* PA14 PrmC protein in *E. coli* CK783 can overcome the growth deficiency. The heterologous expression of PrmC restored the growth rate in *E. coli* CK783 to almost wildtype levels (Fig. 2), indicating that PrmC can complement the defective PrmC methyltransferase activity in *E. coli* CK783.

In addition, we measured the PrmC methyltransferase activity *in-vitro,* as previously described for *Y. pseudotuberculosis* . Purified PrmC was incubated with radioactive labeled [^{3}H-methyl]-SAM and cell lysate from *P. aeruginosa* PA14 wildtype and the respective *prmC* transposon mutant. When PrmC was incubated with PA14_*prmC* mutant lysate the incorporation rate was 2-fold higher as compared to PA14 wildtype lysate (Fig. 3), suggesting that in contrast to the PA14_*prmC* mutant, the putative PrmC targets, such as the peptide chain release factors PrfA and PrfB, are already methylated in the PA14 wildtype. To demonstrate that the methylation targets of PrmC are the RFs, we increased the RF concentration by generating cell lysates of *P. aeruginosa* PA14 wildtype and PA14_*prmC*, both overexpressing PrfA. As expected, cell lysates with high levels of PrfA remarkably increased the PrmC-dependent incorporation of radioactive ^{3}H-methyl (Fig. 3). Furthermore

PrmC-dependent methylation of PrfA in cell lysates of PA14_*prmC* pUCP20 and PA14_*prmC* pUCP20*prmC* was confirmed by SDS-gel autoradiography. The autoradiogram shown in Fig. S2 illustrates that PrmC specifically methylates PrfA since no incorporation of ^{3}H-methyl was detected in the control sample lacking PrmC. However, this method was not sensitive enough to visualize methlyation of endogenous RFs of PA14_*prmC* pUCP20. In addition we performed a parallel in-gel digestion and LC-MS/MS analysis of the fragment containing PrfA and the methylation at the glutamine residue of the GGQ motif (peptide sequence SSGAGGQHVNK, amino acids 231 to 241) was clearly confirmed (data not shown).

The influence of PrmC on the* P. aeruginosa *quorum sensing system

The production of pyocyanin is highly complex and influenced by various different regulatory genes including those involved in the QS circuit of *P. aeruginosa* . Since pyocyanin production was almost abolished in the PA14_*prmC* mutant and could not be restored by *pqsE* overexpression, we aimed at investigating, whether PrmC is modulating the interconnected QS network of *P. aeruginosa.* First we measured the PrmC-dependent protein levels of the AQ-effector protein PqsE and the transcriptional regulator RhlR in late stationary phase. In comparison to the wildtype the *prmC* mutation had just a marginal effect on RhlR protein level and no significant difference in extracellular C4-HSL levels could be detected by performing a bioreporter analysis (data not shown). Interestingly complementation with *prmC* increased the RhlR production about 2-fold. Overexpression of *pqsE* in the *prmC* mutant background clearly increased the RhlR protein level; however, as demonstrated before (Fig. 1) this does not lead to a restored pyocyanin production in the *prmC* mutant.

Unlike RhlR, the protein level of PqsE was significantly decreased in a PA14_*prmC* mutant and complementation with *prmC* or *pqsE* restored wildtype PqsE protein levels (Fig. 4). These results indicate that PrmC affects pyocyanin production independent of the level of

RhlR and PqsE. The *pqsE* gene is encoded by the *pqsABCDE* operon involved in PQS biosynthesis, so we were interested in whether a reduction in PqsE level is a direct consequence of a decreased expression of the entire operon. We compared the PQS production of a *prmC* mutant with the wildtype strain but could not detect any significant differences in quinolone signal molecule level (data not shown). Therefore it is likely, that PrmC affects expression of PqsE posttranscriptionally.

Global identification of PrmC-regulated genes and proteins in* P. aeruginosa *PA14

To identify genes regulated by PrmC, we used RNA sequencing technology as recently described by Dötsch and colleagues . Under the experimental conditions employed, the transcriptomic data revealed that the expression of 147 genes (P-value ≤ 0.01) equivalent to 2.3 % of all annotated *P. aeruginosa* genes, was affected more than 4-fold by the loss of PrmC (Table S2). About one third of genes was up-regulated (52 genes) and mainly comprised hypothetical and putative proteins. Among the 95 down-regulated genes, we found many genes involved in the production of virulence determinants, such as pyocyanin (*phzC1D1E1F1G1, phzC2D2E2F2G2, phzM, phzS*), the chitinolytic enzyme ChiC (*chiC*) and genes involved in pyochelin biosynthesis or uptake respectively (*pchA, pchB, pchC, pchD, pchE, pchF, pchG, fptA*) . Furthermore we found many genes involved in T3SS and the production of secretion factors (*pscB, pscE, pscF, pscG, pscH, pscK, pscN, pscQ, pscU, popB, popD, popN, pcrH, pcrG, pcrV, exsC, exoT, exoY, exoU*), a key gene enabling denitrification (*norB*- nitric-oxide reductase subunit B) and genes associated with resistance (*opmD, mexI, mexH, mexG*) .

Since we expected PrmC to affect translation rather than transcription we performed a proteomic analysis which partially overlapped with the transcriptomic data. We found 163 proteins to be differentially regulated in PA14_*prmC*, but were just able to identify 26 candidates (Table S1). Among them we detected several down-regulated proteins of which

the coding genes were already identified in the transcriptomic analysis, such as PhzS, PhzB2, PhzD1, PhzE1 and PhzF1, all involved in phenazine biosynthesis and the chitinolytic enzyme ChiC. GroEL, described as the heat shock 60 kD chaperonin, was also significantly down-regulated in PA14_*prmC*. GroEL is known to be involved in the folding, assembly and transport of newly synthesized proteins in *E. coli* . The proteome data shows that up-regulated proteins identified by the analysis were mainly involved in translation such as the 30S ribosomal protein S2, energy metabolism, nucleotide biosynthesis (PpiB, PurT, Tal) and iron acquisition (PA14_64520).

PrmC-dependent growth, virulence factor production and motility

The growth rate of PA14_*prmC* in various defined media displayed no significant differences in comparison to the wildtype strain (data not shown). But we observed an intense growth defect in an anaerobic environment. PA14_*prmC* was not able to grow in the absence of oxygen (Fig. 5), probably due to the diminished production of key proteins involved in denitrification such as NorB (Tab. S2).

Since PrmC exerts an intensive effect on pyocyanin production and positively influences the RhlR protein concentration, we examined if PrmC has a more global effect on bacterial virulence factor production, motility and biofilm formation. The *rhl* system is essential for the regulation of rhamnolipid production (*rhlAB*), which occurs during stationary phase of growth . Therefore we first measured the influence of the *prmC* mutation on rhamnolipid levels. Figure 6 shows that loss of PrmC is attended by a reduction of rhamnolipids, whereby complementation of *prmC* restores rhamnolipid levels. These results demonstrate that the presence of *prmC* influences the RhlR-dependent production of rhamnolipids.

In addition to the results revealed by the trancriptomic analysis, a recent report by Garbom *et al*. (2007) showed that the PrmC homologue VagH in *Yersinia pseudotuberculosis* is involved in the regulation of the T3SS. Hence, we determined the PrmC-dependent

expression of the secreted adenylatecyclase ExoT by introducing the reporter plasmid pUC20*PexoT-gfp* into PA14 wildtype and PA14_*prmC* mutant respectively. Indeed, absence of PrmC led to decreased *exoT* expression as compared with the wildtype strain (Fig. 7).

It is known that all three modes of motility in *P. aeruginosa* are dependent on the *rhl* system . Since our results indicate that PrmC affects RhlR-dependent rhamnolipid production, we analyzed if PA14_*prmC* is as well attenuated in swimming, swarming or twitching. As expected, PA14_*prmC* displays a significant impaired swimming and twitching phenotype (Fig. 8B;C). PrmC was also essential for swarming motility, as the *prmC* mutant completely lost the ability to swarm under the experimental conditions used (Fig. 8A). Interestingly, overexpression of PrmC in the wildtype and the *prmC* mutant did not lead to an increased swarming and twitching phenotype. Conversely we observed an enhanced swimming motility once PrmC was overproduced in both, the wildtype and the *prmC* mutant strain.

Swarming motility was reported to be inversely related to the capability to form biofilms . Thus we investigated, if PrmC also effects biofilm formation in a static 96well assay. We were not able to detect any differences in biofilm formation between the wildtype and the PA14_*prmC* mutant (data not shown).

The role of PrmC in pathogenicity of P. aeruginosa *PA14*

Considering the fact, that PrmC is essential for pyocyanin, rhamnolipid and ExoT production and effects motility, we investigated the impact of *prmC* mutation on *P. aeruginosa* pathogenicity, using the vertebrate infection animal model *Galleria mellonella* (greater wax moth). The larvae of *G. mellonella* are sensitive to *P. aeruginosa* infections and extensively used as a mini-host model for pathogenic bacteria and fungi, that are responsible for severe human diseases such as: *Bacillus cereus* , *Candida albicans* , *Cryptococcus neoformans* , *Enterococcus faecalis* , *Francisella tularensis* , *Listeria monocytogenes* , *Staphylococcus aureus* and *Yersinia pseudotuberculosis* . It was also reported, that the T3SS in *P.*

aeruginosa plays a significant role in *G. mellonella* killing . As expected, in *G. mellonella* the PA14_*prmC* mutant was significantly attenuated in virulence. With a median survival of 50 hours, PA14_*prmC* infected *G. mellonella* survived about 4 times longer than *G. mellonella* infected with PA14 wildtype which died after 14 hours. Plasmid-mediated complementation of *prmC* mutation restored the pathogenicity of PA14, as the median survival with 20 hours was close to wildtype levels (Fig. 9A). To scrutinize the significant higher survival rate of *G. mellonella* infected with PA14_*prmC*, we investigated, if the decreased pathogenicity was a possible consequence of growth deficiency *in-vivo*. The results obtained from the CFU analysis showed that PA14_*prmC* is not able to proliferate after injection into the host organism (Fig. 9B), which in turn could reflect a restrained growth effect correlating with the growth deficiency observed in oxygen limiting conditions (Fig. 5).

Discussion

In this study we looked for new genes affecting QS and virulence factor production in *P. aeruginosa* PA14, such as the phenazine pigment pyocyanin. Pyocyanin synthesis is facilitated by an intact QS system and induced by exogenous addition of AQs. By screening the transposon mutant library towards AQ-responsiveness, we identified a new set of AQ-non responding mutants with a decreased pyocyanin production.

We focused our studies on one mutant affected in the *prmC* gene. PrmC was demonstrated to be essential for pyocyanin production even in the presence of exogenously added extract containing QS-signal molecules and independent of the AQ effector protein PqsE. By western blot analysis we obtained evidence that in the PA14_*prmC* background PqsE is still active since overexpression leads to increased RhlR level. PrmC exhibits high homology to the *S*-adenosyl-L-methionine (AdoMet)-dependent methyltransferase in various Gram-positive and Gram-negative bacteria including *E. coli.* Various proteins of the HemK family were shown to methylate RFs modulating translational regulation *in-vitro* and *in-vivo* .

In this study we demonstrate that heterologous expression of PA14 PrmC in *E. coli* can overcome and complement the growth defect in the *E. coli prmC* knockout mutant CK783 suggesting a similar enzymatic activity as *E. coli* PrmC. By autoradiography and LC-MS/MS analysis it was clearly shown, that PrmC specifically methylates the peptide chain release factor PrfA and that the methylation occurs at the conserved GGQ motif.

Since previous reports indicate that proteins of the PrmC family exhibit a decisive role in gene expression and posttranscriptional regulation, we analyzed the global regulatory effects of PrmC by generating a transcriptional profile and by conducting a proteomic study of *P. aeruginosa* PA14_*prmC* . Our results showed, that loss of PrmC affects ~2.3 % of the annotated genes in *P. aeruginosa* with 95 genes down-regulated (P-value ≤ 0.01), including a high proportion of virulence associated genes and genes involved in the T3SS. However, this

impact of PrmC on gene expression may not in all cases be a direct consequence of inefficient translation due to non-methylation of RFs, but may likewise represent an indirect result of a decrease in levels of positive regulators. Our proteomic analysis revealed just 26 proteins to be differentially regulated in PA14_*prmC*. This was a surprising finding since PrmC-dependent methylation of RFs strongly affects their translational termination efficiency and thus, we expected to see major differences in protein expression levels. However, one interesting protein negatively affected by the loss of PrmC was GroEL, a chaperon involved in the correct assembly of expressed proteins. Thus, some phenotypic effects triggered by PrmC might be assigned to the impaired expression of GroEL. Among the proteins which were up regulated in PA14_*prmC* we found the flagellar filament structural protein FliC. Since PA14_*prmC* displayed a non-motile phenotype, the accumulation of FliC might reflect a defective flagellar assembly leading to an increased intracellular protein level. The marginal effect of PrmC on the proteome has raised the question of whether the few identified proteins are directly affected through under-methylation of RFs and share any commonalities on the gene level. To address this question we analyzed the 26 genes with regard to similarities in their termination codon sequence context but could not identify any common features.

During infection and colonization it is crucial for *P. aeruginosa* to adapt to various environmental changes and to circumvent hostile conditions by moving towards beneficial places. Loss of *prmC* comes along with a non-motile phenotype and the bacterium is no longer able to grow in an anaerobic atmosphere. Despite the obvious functional similarities of PrmC (*E. coli*) and PrmC (*P. aeruginosa*), their regulation of genes involved in respiration and denitrification is different. The loss of *prmC* in *E. coli* leads to repression of genes related to aerobic respiration and induction of genes involved in anaerobic growth, whereas in this study we demonstrate that the presence of PrmC in *P. aeruginosa* PA14 conversely is essential for anaerobic growth .

The inability of the PA14_*prmC* mutant to adapt in a sufficient way to various environmental changes, is reflected in restrained infection efficiency in the *G. mellonella* host infection model. Pathogenicity of *P. aeruginosa* PA14_*prmC* in this infection model was impaired and fully restored after *prmC* complementation. Hereby PrmC plays a significant role in proliferation since *P. aeruginosa* PA14_*prmC* rarely achieves a critical bacterial mass (CFU of ~$1x10^5$) to kill the larvae. The reduced growth rate within the host may be explained by the lack of virulence factors important for nutrition utilization, or it may be a result of the previously described importance of PrmC for survival in the absence of oxygen considering the bacteria face a microaerophilic or anaerobic environment during infection.

Taken together, in this study we demonstrate that the methyltransferase PrmC in *P. aeruginosa* PA14 is essential for virulence, plays an important role in adaptation to various environmental conditions, and the functional activity of PrmC is crucial for *P. aeruginosa* pathogenicity. Thereby, the regulation of various virulence factors such as pyocyanin, ExoT and rhamnolipids can partially be explained by a reduced quorum sensing activity. Although the absence of PrmC led to pronounced changes in motility and anaerobic growth, we were not able to detect a detrimental effect on biofilm formation. Nevertheless, further investigations may help to understand, if RF-methylation creates a bias to affect preferential genes important for the bacterium to survive under certain stress conditions and if PrmC is a potential target for anti-virulence drug development.

Experimental procedures

Bacterial strains, media and growth conditions

Unless otherwise noted, bacterial strains, listed in Tab. S3 were routinely grown in Luria broth (LB) medium at 37°C and shaking at 180 rpm. Growth kinetics were monitored by taking OD_{600} measurements. Anaerobic growth of *P. aeruginosa* was monitored in an anaerobic workstation (D. Whitley) at 37°C in PYG-KNO_3 [DSMZ_Medium 104 (http://www.dsmz.de/microorganisms/medium/pdf/DSMZ_Medium104.pdf) supplemented with 100 mM KNO_3]. Antibiotics were added at the following final concentrations [µg/ml]: for *E. coli*, kanamycin 50; ampicillin 100; tetracycline 12.5; for *P. aeruginosa*, carbenicillin 400; tetracaycline 100. Isopropyl-β-D-thiogalactopyranosid (IPTG) was added to the medium at a concentration of 1 mM (*E. coli*) and 0.1 mM (*P. aeruginosa*).

Preparation of concentrated organic extract including AQs (PA14-extract)

A volume of 250 ml BHI medium was inoculated with an overnight *P. aeruginosa* PA14 culture and incubated in an orbital shaker for 24 h with 180 rpm at 37°C. To extract AQs, equal amounts of dichloromethane (250 ml) and culture (250 ml) were mixed, shaken for 1 minute and added to a separating funnel. The lower organic phase was collected and filtered by a paper filter to eliminate slimy interphase residues followed by evaporation under a hood (stirring speed up the process). The dried concentrate was resuspended in 10 ml methanol and frozen in 2 ml aliquots at -20° C.

AQ screen of the PA14NR library

96 well plates with 200 µl LB per well were inoculated from the deep-frozen 96 well stock of the non-redundant PA14 transposon mutant library (in total 63 plates) by use of a 96 pin replicator . The microtiter plates were inserted in a box with humid atmosphere and incubated

in an orbital shaker for approx. 4 h with 180 rpm at 37°C. Following a defined schema, the 96 samples were split to eight 24 well plates: 5 µl of each mutant preculture was used to inoculate both 500 µl LB and 500 µl LB containing 1 µl/ml PA14-extract. The 24 well plates were inserted in boxes with humid atmospheres and incubated in an orbital shaker with 180 rpm for approx. 16 h at 37°C. After incubation pyocyanin levels were analyzed by eye to judge the differences between cultures with and without exogenously added AQs.

Immunoblotting

Bacterial cultures were grown for 20 h in BM2 medium [7 mM $(NH_4)_2SO_4$, 40 mM K_2HPO_4 and 22 mM KH_2PO_4 with 0.5 % CAS amino acids] at 37°C to an OD_{600} of 3.0. Whole cell lysates were normalized for protein content and 10 µl of an OD_{600} of 10.0 were separated by SDS-PAGE (10 % acrylamide) after 15 min incubation at 95°C. Primary antibodies: Rabbit polyklonal antisera α-RhlR and a polyclonal antibody α-PqsE (Biogenes) were used at dilutions of 1:20.000 and 1:5.000 respectively. B4c goat anti-rabbit IgG (Dianova) was used as the secondary antibody at a dilution of 1:4.000. The blot was developed using Lumi-Light Western Blotting Substrate (Roche) and chemiluminescence was detected using Las-3000 Imager (Fujifilm).

Galleria mellonella *infection assay*

Bacterial strains were grown to exponential growth phase in LB supplemented with carbenicillin. Cells were harvested by centrifugation at 13.000 rpm for 4 min, resuspended in sterile phosphate buffered saline (10 mM PBS, pH 7.5) to an OD_{600} of 1.0 and 10-fold serially diluted in PBS. *G. mellonella* larvae were inoculated with 20 µl of a 1:20.000 dilution containing $5x10^2 \pm 40$ colony-forming units (CFUs) by injection into the haemocoel of the hindmost proleg with a 100 µl Hamilton syringe and a 30G needle. The larvae were placed in Petri-dishes and incubated in the dark at 37°C. Mortality rates of 30 larvae per treatment were

monitored for 65 h. Larval death was assessed by the lack of movement of larvae in response to stimulation together with melanization of the cuticle. To determine bacterial growth in infected larvae, five larvae of each treatment were homogenized individually in eppendorf tubes at seven different time points in 500 µl PBS by vortexing for 30 s. A Volume of 10 µl drops of serial dilutions in PBS were plated on *Pseudomonas* isolation agar (Fluca Analytical/Sigma-Aldrich) containing carbenicillin to select for pUCP20-carrying *P. aeruginosa*. CFUs were determined after 14 – 24 h incubation at 37°C. PBS was used as a negative control in the experiment.

Transcription analysis

Preparation of RNA and comparative analysis of gene expression was performed as previously described by Dötsch *et al*. (2012). RNA was extracted from *P. aeruginosa* cultures grown in BM2 medium at 37°C to late exponential growth phase (OD_{600} of 1.9 – 2.1). For each strain two biological replica were used.

Proteomics

P. aeruginosa PA14 wildtype and PA14_*prmC* mutant were grown in LB medium at 37°C. Cultures were harvested at an OD_{600} of 2.0, washed twice with PBS and the pellet was resuspended in 10 ml lysis buffer (7 M Urea, 2 M Thio-urea, 4 % Chaps, 20 mM Tris base, DTT) and protease inhibitors (Complete mini, EDTA free, Roche). Disruption of the cells was performed by sonication. Lysate was precipitated using the 2D Clean-Up Kit (GE Healthcare) and pellets were resuspended in urea buffer. The protein concentration was determined using a Bradford reagent (BIO-RAD). Samples were passively rehydrated for 12 h and run on IPG strips (pH 3-10, 18 cm) using an Ettan IPGpfor system (GE Healthcare). Each sample contained in total 150 µg of proteins. After first dimension, proteins distributed on IPG strip were reduced with DTT and alkylation was performed with idodoceamide. The second

dimension separation was performed using gradient SDS-PAGE gels (10-15 %). Gels were fixed with 10 % TCA, stained with Commassie brilliant blue, scanned with ImageScanner III (GE Helathcare) and matched using a Compugen Z3 software. Differentially expressed proteins were cut out from the gel and digested with trypsin and further sequenced using MALDI-TOF.

Pyocyanin quantification

Pyocyanin levels were determined as previously described using cell-free supernatants of *P. aeruginosa* cultures grown in BM2 medium for 24 h at 37°C. After centrifugation at 13.000 rpm for 10 min, 1 ml of cell-free supernatant was mixed with an equal volume of chloroform. Samples were centrifuged at 13.000 rpm for 5 min, and the organic phase was mixed with 1 ml of 0.2 M HCl by brief vortexing. After centrifugation at 13.000 rpm for 1 min, the pink/red top layer was used for spectrophotometrical analysis at 520 nm. Pyocyanin concentrations were calculated by multiplication with 17.072 and standardized by dividing the OD_{520} through the respective OD_{600} of the cultures.

Rhamnolipid quantification

Relative rhamnolipid levels in the supernatant of *P. aeruginosa* cultures grown in BM2 medium for 48 h at 37°C were quantified indirectly using a 1.6 % orcinol assay previously described . After centrifugation at 13.000 rpm for 10 min, a volume of 600 µl of the culture supernatants was mixed with 3.4 ml diethylether by brief vortexing. Samples were centrifuged at 13.000 rpm for 5 min and 2 ml of the upper fraction was evaporated to a final volume of 1 ml. After addition of 600 µl 20 mM HCl and vigorous vortexing, samples were centrifuged for 3 min at 13.000 rpm and 500 µl of the organic phase was evaporated to dryness. The remainder was dissolved in 100 µl 1.6 % orcinol and 800 µl of 60 % H_2SO_4 and after 30 min incubation at 80°C rhamnolipids were measured spectrophotmometrically at an OD_{421}.

Extraction and quantification of P. aeruginosa AQ metabolites

AQs were extracted from *P. aeruginosa* cultures grown in BM2 medium at 37°C for 24 h with dichlormethane as described previously . Briefly, the bacterial cultures were normalized to an OD_{600} of 3.0 and mixed with 2 volumes of dichlormethane by vigorous shaking. After centrifugation at 13.000 rpm for 5 min, the lower organic phase was evaporated to dryness, before being dissolved in methanol.

TLC was perfomed using a silica gel 60 F254 plate which had been previously soaked for 30 min in 5 % KH_2PO_4 and activated at 85°C for 1 h. The *P. aeruginosa* extracts were separated by TLC using a 95:5 dichlormethane-methanol mobile phase until the solvent front reached the top of the plate. Fluorescent spots were visualized under UV light and photographed. Synthesized PQS (2 µl of a 2 mg/ml standard) was used as standard.

GC-MS analysis was performed by derivatization with trimethylsilylation (50 % pyridine, 50 % BSTFA [bistrimethylsilyltrifluoroacetamide] containing 1 % TMC [trimethylchlorosilane]), (70°C, 1 h) with a Thermo-Finnigan GCQ ion trap mass spectrometer (Finnigan MAT Corp., San Jose, CA) running in the positive-ion electron impact (EI) mode equipped with a 30-m DB5 capillary column as described by Bredenbruch *et al*. (2005). Quantification was performed by electronic integration of the most abundant fragment ion traces at m/z 304 (PQS) and correction of the integrals by the relative intensities of the respective fragment ions

Motility assay

Swimming motility of *P. aeruginosa* was evaluated by seeding stationary-phase cells (1 µl of an OD_{600} of 2.0) onto the agar surface of swimming agar plates (BM2, 1.5 % agar, 0.4 % glucose, 2 mM $MgSO_4$ and 10 µM $FeSO_4$), which were air dried 15 min directly before use. Plates were incubated at 37°C, and the diameter of the circular turbid zone formed by bacterial cells migrating from the point of inoculation was measured 16 h postinoculation.

Swarming migration was analysed by inoculating precultured bacteria (1 µl of an OD_{600} of 2.0) carefully onto the surface of swarming agar plates [BM2 without $(NH_4)_2SO_4$, 1.5 % agar, 0.4 % glucose, 2 mM $MgSO_4$ and 10 µM $FeSO_4$]. The plates were incubated at 37°C for 12 h. Swarming motility was assessed by examining the colony sizes and the branch-spreading patterns on the semisolid agar medium.

Twitching motility was assessed by stab inoculating cells to the bottom of LB-agar plates (1.5 %) with a toothpick and subsequent 24 h incubation at 37°C. Strains capable of twitching motility form a light haze zone of growth at the interface between the agar and the petri plate surrounding the colony, whereas strains defective in twitching motility are supposed to remain clustered in the area of initial inoculation. Attached cells were stained with 1 % crystal violet and the characteristic flat, spreading colony morphology was used as a measure of twitching motility.

Fluorescence assay

To analyze the *in-vivo* expression level of ExoT in *P. aeruginosa*, the vector p*exoT-gfp* was digested with EcoRI and cloned in the opposite orientation to the *lac* promoter in pUCP20. The resulting plasmid pUCP20*PexoT-gfp* was transformed into the PA14 wildtype and PA14_*prmC* mutant strain both harboring the empty vector pME6032, or pME6032*prmC* respectively. *P. aeruginosa* cultures were grown in SM-Medium supplemented with the respective antibiotics and IPTG at 37°C and shaking at 180 rpm to stimulate the T3SS. After 6 h growth, nine aliquots of 200 µl of each strain were transferred to the wells of a 96-well

plate. Growth and fluorescence kinetics were monitored using a Varioskan Flash (Thermo Scientific) by with an excitation λ 488nm and an emission λ 508nm.

PrmC purification

A volume of 500 ml LB supplemented with kanamycin was inoculated 1:25 with an overnight culture of *E. coli* BL21 cells harboring the *prmC* expression vector pET28a*prmC*. The culture was grown at 37°C and PrmC expression was induced at OD_{600} of 0.5 – 0.8 by addition of 1 mM IPTG. Subsequently, the culture was shaken overnight at 20°C before harvesting the cells by centrifugation. Bacterial cells were resuspended in lysis buffer (50 mM NaH_2PO_4, pH 8.0, 300 mM NaCl, 10 mM imidazole) containing 1 mM DTT, 1 mg/ml lysozyme, protease inhibitors (Complete mini, EDTA free, Roche) and Benzonase Nuclease (Novagen). After ribolysing the cells for 60 s and subsequent centrifugation at 13.000 rpm for 15 min at 4°C, the supernatant was incubated with nickel-nitrilotriacetic acid agarose resin (Qiagen) for 1 h at 4°C. The resin was washed with lysis buffer and proteins were eluted with 50 mM NaH_2PO_4, pH 8.0, 300 mM NaCl containing 1 mM DTT with a stepwise increase in imidazole concentration (50 mM, 100 mM, 150 mM, 250 mM). After SDS-PAGE analysis, the fraction containing pure protein PrmC-His_6 (elution with 250 mM imidazol) was dialyzed for 16 h at 4°C in 50 mM NaH_2PO_4, pH 8.0 and 300 mM NaCl.

Methylation assay

In-vitro methylation assays were performed as previously described with minor modifications 22. For the preparation of PA14 cell lysates, the wild type and *prmC* mutant strain, each carrying the PA14 *prfA* expression vector pME6032*prfA*, and the empty vector as control, were grown overnight in LB supplemented with tetracycline. Expression of the release factors was induced by addition of 1 mM IPTG. The cultures were equalized to an OD_{600} of 20.0 in a volume of 1 ml, centrifuged and cells were washed once in reaction buffer (50 mM Tris, 100

1mM NaCL, 10 mM EDTA, 20% (v/v) glycerol, pH 8.0) containing protease inhibitors 2(Complete mini, EDTA free, Roche). Bacterial pellets were finally resuspended in an equal 3volume of the same buffer. Cells were ribolyzed for 60 s and after centrifugation at 13.000 4rpm for 15 min at 4°C the supernatants were directly used for the methylation assay. Protein 5concentrations were determined using a Bradford reagent (BIO-RAD). The reaction mixture 6was adapted to a final concentration of 30 µg/ml PA14 cell lysates, 6 µg/ml purified PrmC-7His_6, 0.6 µM [^{3}H-methyl]-SAM (specific activity 1 mCi/ml, Hartman analytics) and incubated 8at 37°C. At several time points, 10 µl aliquots were removed, spotted on presoaked and dried 9filter paper (1 cm^2, Rotilabo, Roth) and quenched with 10 % (w/v) trichloroacetic acid (TCA). 10The filters were washed twice with 10 % (w/v) TCA for 15 min and once with 95 % EtOH for 1110 min. Air-dried filters were transferred into a 24-well sample plate, covered with 1 ml 12scintillation fluid and radioactivity was quantified using a microplate liquid scintillation 13counter (1450 MicroBeta TriLux, Wallac). For autoradiography analysis 20 µl aliquots were 14removed after 30 min incubation and directly transferred to a NuPAGE 10% Bis-Tris gel. 15After electrophoresis and coomassie staining (acetic acid 10%, methanol 25% coomassie 16brilliant blue R250 0.2%), proteins were washed for 90 min and incubated for 30 min in 17amplification reagent (Amersham Amplify™ Fluorographic Reagent, GE Healthcare), dried 18and exposed to a High performance autoradiography film (Amersham Hyperfilm™ MP, GE 19Healthcare) for 24h at -70°C before developing. In parallel, protein samples were separated 20by SDS-PAGE and bands corresponding to PrfA were digested with trypsin and further 21analyzed by MALDI-TOF.

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Tab.1: List of differentially expressed proteins in PA14_*prmC* as compared to the wildtype.

PA14 number	PAO1 orthologs	Protein name	Fold change	Description
PA14_05230	PA0400		- 5.3	cystathionine gamma-lyase
PA14_09150	PA4236	KatA	- 9.4	Catalase
PA14_09400	PA4217	PhzS	- 18.2	phenazine biosynthesis protein PhzS
PA14_09420	PA1904	PhzF1	- 10.4	phenazine biosynthesis protein phzF1
PA14_09440	PA1903	PhzE1	- 30.3	phenazine biosynthesis protein PhzE
PA14_09450	PA1902	PhzD1	--	phenazine biosynthesis protein PhzD1
PA14_09470	PA4211	PhzB1	--	phenazine biosynthesis protein phzB1
PA14_11810	PA4022		- 7.0	putative aldehyde dehydrogenase
PA14_15890	PA3751	PurT	+ 5.9	phosphoribosylglycinamide formyltransferase 2
PA14_17060	PA3656	RpsB	+ 7.179	30S ribosomal protein S2
PA14_27960	PA2796	Tal	+ 4.705	transaldolase B
PA14_25390	PA2991	Sth	- 10.4	soluble pyridine nucleotide transhydrogenase
PA14_34870	PA2300	ChiC	--	chitinolytic enzyme
PA14_35490	PA2250	lpdV	- 2.7	dihydrolipoamide dehydrogenase (Energy metabolism)
PA14_35500	PA2249		- 4.9	branched-chain alpha-keto acid dehydrogenase subunit E2
PA14_39960	PA1900	PhzB2	- 20.8	phenazine biosynthesis protein PhzB2
PA14_41390	PA1793	PpiB	+ 2.4	peptidyl-prolyl cis-trans isomerase B
PA14_50290	PA1092	FliC	+ 2.3	flagellin type B
PA14_54660	PA0744		- 3.8	enoyl-CoA hydratase/isomerise
PA14_56240	PA4329	PykA	- 4.4	pyruvate kinase
PA14_57010	PA4385	GroEL	- 12.34	chaperonin GroEL
PA14_57275	PA4407	FtsZ	+ 5.232	cell division protein FtsZ
PA14_60800	PA4595		- 4.3	putative ABC transporter ATP-binding protein
PA14_61780	PA4671		- 3.6	50S ribosomal protein L25/general stress protein Ctc
PA14_64520	PA4880		+ 5.4	putative bacterioferritin
PA14_68340	PA5172	ArcB	- 9.6	ornithine carbamoyltransferase

In PA14_*prmC* 26 proteins were identified to be differentially expressed. A positive value indicates a higher expression in the absence of PrmC as compared to the wildtype. When the fold change was indicated with a dash (--) the protein was not identified in PA14_*prmC*.

Figure legends

Fig. 1: Pyocyanin production by PA14 wildtype and the *prmC* and *pqsE* transposon mutants.

All strains harbored the empty plasmid vector pUCP20 (white), pUCP20*pqsE* (grey) or pUCP20*prmC* (dark grey). Bacterial cultures were grown in BM2 medium and pyocyanin was extracted after 24 h growth (late stationary phase). Error bars represent one standard deviation of the mean value from three independent experiments, * $P \leq 0.05$ PA14 pUCP20 versus PA14 pUCP20*pqsE*.

Fig. 2: Growth of the *E. coli_prmC* knockout mutant CK783 complemented with PrmC from PA14.

Growth of *E. coli* wildtype strain CA293 (square), the *prmC* mutant strain CK783 (filled triangle) both harboring the empty plasmid vector pET28a and CK783 overexpressing *prmC* via pET28a*prmC* (grey circle). The growth media LB broth was supplemented with 1 mM IPTG. Data represent the mean from three independent experiments, * $P \leq 0.05$ and *** $P \leq 0.001$ CK783 pET28a versus CA293 pET28a.

Fig. 3: SAM-dependent methyltransferase activity of PrmC.

Cell lysates from PA14 wildtype and PA14*_prmC* carrying the empty plasmid pME6032 or pME6032*prfA* were incubated with purified PrmC and [^{3}H-methyl]-SAM. Methylation was quantified using a microplate liquid scintillation counter (1450 MicroBeta TriLux, Wallac). Data represent the mean from three independent experiments, ** $P \leq 0.01$ and *** $P \leq 0.001$ PA14 wildtype pME6032 versus PA14*_prmC* pME6032 and PA14 wildtype pME6032*prfA* versus PA14*_prmC* pME6032*prfA*.

Fig. 4: Western blot analysis of PrmC-dependent RhlR and PqsE production.

Proteins were extracted from (1) PA14 pUCP20, (2) PA14*_rhlR* pUCP20, (3) PA14*_pqsE* pUCP20, (4) PA14*_prmC* pUCP20, (5) PA14*_prmC* pUCP20*prmC* and (6) PA14*_prmC* pUCP20*pqsE*. Cultures were grown for 20 h (stationary phase of growth) in BM2 medium. Extracts from (2) PA14*_rhlR* and (3) PA14*_pqsE* were used as negative controls.

Fig .5: PrmC-dependent growth of PA14 in anaerobic environment.

Anaerobic growth of PA14 wildtype (square), *prmC* mutant (filled triangle) both harboring the empty plasmid pUCP20 and PA14_*prmC* complemented with pUCP20*prmC* (circle) was measured in PYG-KNO_3. Data is reported with standard deviation of the mean from three independent experiments. * $P \leq 0.05$ and ** $P \leq 0.01$ PA14_*prmC* versus PA14_*prmC* pUCP20*prmC*.

Fig. 6: Rhamnolipids production by PA14 and *prmC* mutant.

PA14 wildtype and PA14_*prmC* harbored the empty plasmid pUCP20 (grey bars) and pUCP20 constitutively expressing *prmC* (black bars). PA14_*rhlR* pUCP20 was used as negative control. Bacterial cultures were grown in BM2 medium and rhamnolipids were extracted after 48 h growth (late stationary phase). Error bars represent one standard deviation of the mean value from three independent experiments. ** $P \leq 0.01$ PA14 and PA14_*prmC* carrying the empty plasmid pUCP20 versus pUCP20*prmC*.

Fig. 7: Effects of PrmC on *exoT* activity

All strains harbored the *exoT* reporter plasmid pUCP20*PexoT-gfp* and activation of the *exoT* promoter was measured by fluorescence (Ex. 488nm / Em. 506nm) using a microplate reader (Synergy4; Bio-Tek) and fluorescence was divided by the respective OD_{600} of the cultures (relative luminescence). PA14 wildtype (white bars) and PA14_*prmC* (grey bars) were both transformed with the empty plasmid pME6032 and PA14_*prmC* was complemented with pME6032*prmC* (black bars). Bacterial cultures were grown in SM medium to stimulate secretion of the exotoxin T (ExoT). The average of three independent replicates is reported with standard deviation. * $P \leq 0.05$ and ** $P \leq 0.01$ PA14_*prmC* pUCP20*PexoT-gfp* / pME6032 versus PA14_*prmC* pUCP20*PexoT-gfp* / pME6032*prmC*.

Fig. 8: PrmC-dependent modulation of PA14 motility

Motility assays were performed with PA14 and PA14_*prmC* harboring pUCP20*prmC* and the respective empty vector. (A) Swarming motility; (B) Swimming motility; (C) Twitching motility.

Fig. 9: *Galleria mellonella* pathogenicity assay.

G. mellonella larvae were inoculated with PA14 and PA14_*prmC* both harboring the empty plasmid pUCP20 and PA14_*prmC* complemented with pUCP20*prmC*. PBS was used as a negative control. (A) Survival rates of the infected *G. mellonella* larvae; 20 larvae for PBS and 30 larvae for each strain. (B) Bacterial growth within each larvae was monitored for 1 day; 5 larvae for each timepoint, error bars represent one standard deviation of the mean, * $P \leq 0.05$ and ** $P \leq 0.01$ PA14_*prmC* pUCP20 versus PA14_*prmC* pUCP20*prmC*.