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A calcium-responsive kinase induces the acute-to-chronic lifestyle switch in *Pseudomonas aeruginosa*

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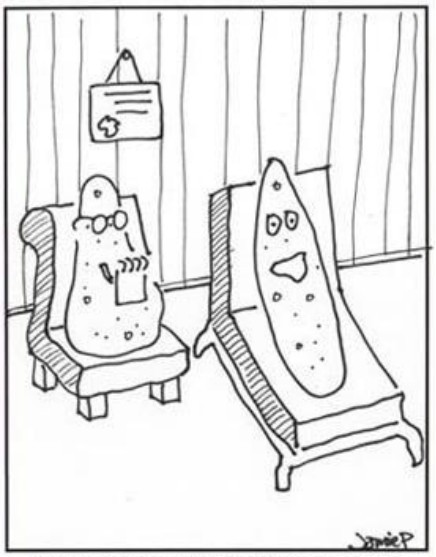

I just can't go with the flow anymore.
I've been thinking about joining a biofilm.

Credits: Jamie Pennington

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SUMMARY

As an opportunistic pathogen, *Pseudomonas aeruginosa* is frequently involved in nosocomial infections and represents the leading cause of morbidity and mortality in cystic fibrosis (CF) patients. Early stages of disease usually carry the signature of acute infections, which are associated with motile planktonic cells expressing a diverse set of virulence factors. Prolonged infections trigger adaptation processes towards reduced virulence and increased biofilm formation, the latter being a hallmark of chronic infections. Several regulatory components involved in this lifestyle switch were identified recently. Next to the ubiquitous second messenger bis-(3',5')-cyclic dimeric guanosine monophosphate (c-di-GMP), the global Gac/Rsm signaling cascade has been shown to be key for the transition between acute and chronic infections. The two-component system GacS/GacA positively controls the expression of two small regulatory RNAs. They in turn bind to and inactivate the translational regulator RsmA, which directly represses genes involved in the community-associated lifestyle and indirectly stimulates acute virulence traits. Signals associated with high cell density have been found to activate the Gac/Rsm cascade, however their exact nature remains unclear.

To study the regulatory network underlying the Gac/Rsm cascade, its associated kinases and the corresponding activating signals we developed dual Gac/Rsm-responsive reporter systems, which allow analyzing the behavior of single cells as well as entire cell populations. Using these tools we found that calcium specifically stimulates the Gac/Rsm cascade. Different biochemical studies illustrated by a proteomics approach used to identify calcium regulated targets, which reveals a strong overlap with the previously defined RsmA-regulon, confirmed this finding. As calcium is able to override the repressed status of the Gac/Rsm cascade in growing cells suggests that its signaling mode is distinct from the density-related activation of the system. Furthermore, we found that even though calcium ions seem to be rapidly captured by *P. aeruginosa* cells, their continuous presence is a prerequisite to maximally activate the system.

Our data show that LadS, one of the Gac/Rsm-associated histidine kinases is absolutely essential for calcium-mediated stimulation of the signaling cascade. LadS belongs to the 7TMR-DISMED2 protein family and contains next to the histidine kinase domain a C-terminal extension in the form of a conserved receiver domain. We find that increasing LadS protein levels directly translate into increased activation of the signaling cascade. Along with the observation that calcium stabilizes a mutant allele harboring two additional amino acids, also if expressed in *E. coli* cells, implies that LadS is directly involved in calcium recognition. This occurs either directly or indirectly via a ubiquitous co-factor. As DISMED2 domains harbor reminiscent similarity to carbohydrate-binding modules, carbohydrates are likely co-factor candidates. In line with this idea we find that the activation of LadS by calcium ions depends on its periplasmic DISMED2 domain as well as the adjacent transmembrane helices.

While the histidine kinase activity is crucial, the receiver domain is negligible for calcium-sensing and might play a role in modulating LadS activity. Based on the observation that calcium induces a decrease in LadS receiver domain phosphorylation we postulate that the receiver domain serves as a phosphate repository in a low-calcium environment. Upon calcium-stimulation the receiver domain donates its phosphate back to the histidine, which then in turn mediates downstream signaling events.

We envision three different scenarios: i) LadS engages in phosphotransfer to an unknown response regulator; ii) LadS phosphorylates the receiver domain of GacS; or iii) phosphorylated LadS modulates protein-protein interactions *e.g.* of RetS and GacS, which would eventually result in increased GacS phosphorylation and induction of the acute-to-chronic switch.

We show that calcium-sensing does not represent a general feature of *Pseudomonas* species, but is rather an adaptation to the lifestyle of *P. aeruginosa*. Interestingly, a dysregulated calcium homeostasis and thus elevated calcium levels in different body fluids was found to be intimately linked to the CF pathology. Together with our finding that most clinical isolates from CF airways remain calcium-responsive we postulate that LadS-mediated stimulation drives the acute-to-chronic switch during *P. aeruginosa* infections of CF airways. As cells with an active Gac/Rsm cascade tend to exit more slowly from stationary phase and have an increased tolerance to antibiotics, calcium-mediated activation of the signaling pathway might also substantially contribute to the persistence of *P. aeruginosa.*

In addition we show that the Gac/Rsm cascade is only active in a subpopulation of the cells, indicating that this might be a prerequisite to ensure survival and fitness in rapidly changing environments.

Overall, we could show that calcium, as the first defined input signal, specifically activates the global Gac/Rsm cascade in *P. aeruginosa* leading to the induction of the acute-to-chronic lifestyle switch. We postulate that this mechanism contributes to chronic infections of CF airways, as the CF pathology is linked to a dysregulated calcium homeostasis. Moreover, our data also add to the growing body of evidence demonstrating that calcium signaling plays an important role not only in eukaryotic but also in prokaryotic cells.

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LIST OF ABBREVIATIONS

aa	Amino acids
AUC	Area under the curve
bp	Base pairs
CA	Catalytic and ATP-binding domain
CaSR	Calcium-sensing receptor
CBM	Carbohydrate-binding module
c-di-GMP	Bis-(3',5')-cyclic dimeric guanosine monophosphate
CF	Cystic fibrosis
CFTR	Cystic fibrosis transmembrane conductance regulator
cfu	Colony forming unit
Csr	Carbon storage regulator
DAG	Diacylglycerol
DHp	Dimerization and histidine phosphorylation domain
DISM	Diverse intracellular signaling modules
ER	Endoplasmic reticulum
Gac	Global activator
GPCR	G-protein coupled receptor
HK	Histidine kinase
Hpt	Histidine phosphotransfer
IP_3(R)	Inositol-1,4,5-trisphosphate (receptor)
LadS	Lost adherence sensor
nt	Nucleotides
PIP_2	Phosphatidylinositol-4,5-bisphosphate
PLC	Phospholipase C
PTH	Parathyroid hormone
QS	Quorum sensing
RBS	Ribosomal binding site
RetS	Regulator of exopolysaccharides and T3SS
Roc	Regulator of cup fimbriae
ROS	Reactive oxygen species
RR	Response regulator
Rsm	Regulator of secondary metabolite
RyaR	Ryanodine receptor
SCV	Small colony variant
SD	Shine-Dalgarno
sRNA	small regulatory RNA
T3SS	Type III secretion system
T4P	Type IV pili
T6SS	Type IV secretion system
TCS	Two-component system
Tn	Transposon
Vfr	Virulence factor regulator

1 INTRODUCTION

1.1 *Pseudomonas aeruginosa* – a jack of all trades

Pseudomonas aeruginosa is a ubiquitous gram-negative γ-proteobacterium. As an extremely versatile organism it can adapt to a variety of ecological niches, thriving on different nutrient sources and infect a wide range of hosts such as plants (*Arabidopsis thaliana*), insects (*Drosophila melanogaster*, *Galleria mellonella*), nematodes (*Caenorhabditis elegans)* and mammals [1]–[4]. This versatile lifestyle is orchestrated by an intricate signaling network with different regulatory modules that account for almost 10% of all encoded proteins in *P. aeruginosa* [5].

P. aeruginosa is an opportunistic pathogen and responsible for 10-20% of all nosocomial infections in immunocompromised patients [6]. Moreover, it ranks among the leading causes of morbidity and mortality in people suffering from cystic fibrosis (CF). Early stages of disease usually carry the signature of acute infections. Disease progression is associated with conversion to the chronic lifestyle accompanied by major changes in overall cell physiology. Typically, acute infections are characterized by the expression of motility organelles like flagellum and type IV pili (T4P) and virulence factors such as type 3 secretion system (T3SS), type 2 secretion system (xcp), exotoxin A and lipase [7]. In contrast, chronic infections are associated with surface-attached multicellular communities, also referred to as biofilms, and extracellular virulence factors such as pyocyanin, hydrogen cyanide and elastase, as well as with antibiotic tolerance and persistence (reviewed in [8]). The biofilm matrix consists of exopolysaccharides, DNA and proteins and serves as a protective barrier against the immune system and antibiotics (reviewed in [9]). *P. aeruginosa* strain PA01 encodes for three major exopolysaccharides: pel, psl and alginate and is widely used as a model organism for biofilm formation [10]. The adaptation process underlying the acute-to-chronic lifestyle switch is governed by multiple regulatory modules like *e.g.* quorum sensing (QS) systems. *P. aeruginosa* encodes for a total of four different QS autoinducers, two N-acyl-homoserine lactones: rhl and las as well as two 2-alkyl-4-quinolones: PQS and HHQ (reviewed in [11]). Another global signaling molecule associated with the lifestyle switch from motility to sessility is the second messenger bis-(3',5')-cyclic dimeric guanosine monophosphate (c-di-GMP). While high levels of c-di-GMP promote the formation of biofilms, low levels are associated with motility and the expression of virulence factors (reviewed in [12]). Last but not least, the Gac/Rsm signaling cascade is one of the main players during the acute-to-chronic lifestyle transition in *P. aeruginosa* and the main subject of this PhD thesis.

1.2 Cystic fibrosis

1.2.1 Disease manifestation

Cystic fibrosis (CF) is an autosomal recessive disorder caused by mutations of the CF transmembrane conductance regulator (CFTR) with an incidence rate of one out of 4'000 births in Europe [13]. More than 1'500 different possible CFTR mutations are reported, however the most prevalent one (~67%) is the deletion of a phenylalanine at position 508 (ΔF508) caused by an in-frame deletion of three base

pairs [14]. The aberrant protein is trapped in the endoplasmic reticulum (ER) and subsequently projected to proteasomal degradation [15]. The CFTR encodes for a cAMP-dependent chloride channel, which regulates the fluid transport in the respiratory and gastrointestinal tract. Loss of CFTR reduces the fluid transport and leads to mucus accumulation in the lungs and subsequently airway obstruction. Moreover, as the mucus impairs ciliary clearance, chronic bacterial colonization is inevitable and by the age of 20 years about 60-70% of the patients are infected with *P. aeruginosa* [16]–[18]. Chronic infections are typically preceded by recurrent, intermittent colonization. In about 25% of the cases re-colonization occurs with the same clone, due to persistent bacterial reservoirs in the paranasal sinuses [19], [20]. Chronic infections of the lower airways are dominated by a neutrophil-mediated inflammatory response accompanied by the production of reactive oxygen and nitrogen species (reviewed in [21]). The persistent inflammation and the associated lung deterioration are the primary cause of lethality in CF patients [22].

ER retention of misfolded proteins depends on different calcium-dependent chaperones. Interestingly, *in vitro* studies have demonstrated that CFTR ΔF508 would be functional if it would be allowed to reach the cell surface. Low temperature or calcium depletion by thapsigargin treatment increases the fraction of surface-exposed receptors, which leads to disease amelioration and provides a promising therapeutic approach [23].

1.2.2 Adaptation of *Pseudomonas aeruginosa* to the CF lung environment

Environmental strains are the primary source of *P. aeruginosa* infections. This is best illustrated by the fact that most clinical isolates are genotypically indistinguishable from environmental isolates, suggesting that the pre-existing virulence determinants might also be beneficial in the natural reservoir [11]. The shift from the natural environment to the lung of CF patients is accompanied by drastic physicochemical and nutritional changes. Together with the constant exposure to antibiotics and the lung immune system, these changes are the main driver of adaptation in *P. aeruginosa.*

Typical phenotypes associated with chronic infections are mucoidy caused by the overproduction of alginate, increased antibiotics resistance and reduced expression of virulence factors. Loss of virulence-associated traits such as QS, motility, T3SS and O-antigen components of the LPS are thought to be a consequence of the selective pressure imposed by the host immune system [24]. Sequencing of longitudinally collected *P. aeruginosa* isolates from CF patients delineated some of the molecular mechanism underlying the phenotypic changes. Genes which were repeatedly found to be mutated are listed non-exhaustively below [24], [25]:

- *mucA* (anti-σ-factor, responsible for mucoid conversion)
- *lasR* (part of the QS system, though the related rhl-system is generally not mutated)
- *mutS* (causes increased mutation frequencies)
- *mexZ* (repressor of *mexXY-oprM* operon involved in antibiotics resistance)
- *esxA* (master regulator of T3SS)
- *fleQ* (master regulator of flagellar gene expression)
- *vfr* (cAMP-responsive virulence factor regulator implicated in regulation of *e.g.* T3SS)

- *wspF* (methylesterase, causing elevated c-di-GMP levels via the constitutive activation of WspR)
- *rpoN* (alternative σ-factor σ^{54})

Interestingly, both studies state that the repertoire of mutated genes detected in clinical isolates is surprisingly large. However, most mutations represent unique patient-specific events, illustrating that a huge variety of fitness trajectories exist during adaptation to the CF lung environment.

1.3 Two component systems

Every organism is in continuous interaction and exchange with its environment. Therefore, constant monitoring followed by rapid adaptation in response to changing conditions is absolutely crucial for fitness and survival. One of the most common mechanisms of bacteria to sense and respond to environmental changes are so called two-component systems (TCSs) (reviewed in [26]–[28]). Each component of this modular system is built from at least two different domains. Typically, dimers of membrane-bound sensor histidine kinases (HK) sense extracellular stimuli via their sensor domains. This induces a conformational change and leads to autophosphorylation and activation of the intracellular transmitter domain. In more detail, ATP bound to the catalytic and ATP-binding (CA) domain of one HK donates its γ-phosphate to the conserved histidine residue located in the dimerization and histidine phosphorylation (DHp) domain of the other HK subunit. From there the signal is transduced via phosphotransfer to a conserved aspartic acid residue in the N-terminal receiver domain of its cognate response regulator (RR) (Fig 1). The final cellular response is mediated by the highly variable C-terminal output domain, which engages in protein-DNA or protein-protein interactions. Dephosphorylation of the RR, either by intrinsic autophosphatase activity, through phosphatase-activity of its cognate sensor or by an unrelated phosphatase resets the system, on alert to start another round of signal transduction. Divalent metal cations such as Mg^{2+} or Mn^{2+} are required for all three phosphotransfer reactions [29].

The genes for HKs and its cognate RRs are often organized in an operon or clustered on the chromosome. Orphan HKs and RRs render the identification of cognate partners more challenging. However, with the identification of the 'molecular interaction code' the prediction of cognate HK and RR pairs is facilitated. The decoding relies on a computational approach exploiting known interacting protein pairs [30].

HKs can be classified according to different characteristics such as domain architecture or the mode of signal perception. Three categories can be formed according to the spatial localization of their sensing unit. The largest group consists of membrane-integral HKs where the input domain is exposed to the extracellular/periplasmic space. The sensor unit consists of a loop of 50 - 300 amino acids (aa) framed by two trans-membrane helices. Based on the different nature of input signals it is not surprising that the individual loops share little homology. The second largest group consists of HKs, which sense intracellular cues. A well-studied example is the redox-sensing PAS domain, which has been implicated in oxygen and light sensing [31]. Finally, intramembrane sensors harbor 2 to 20 membrane-spanning helices, which are typically linked by loops that are shorter than 25 aa. As the

name implies, the transmembrane helices are directly involved in sensing, however accessory proteins are frequently required for efficient signal perception. The signals are usually related to membrane perturbation, transport processes or electrochemical gradients [26].

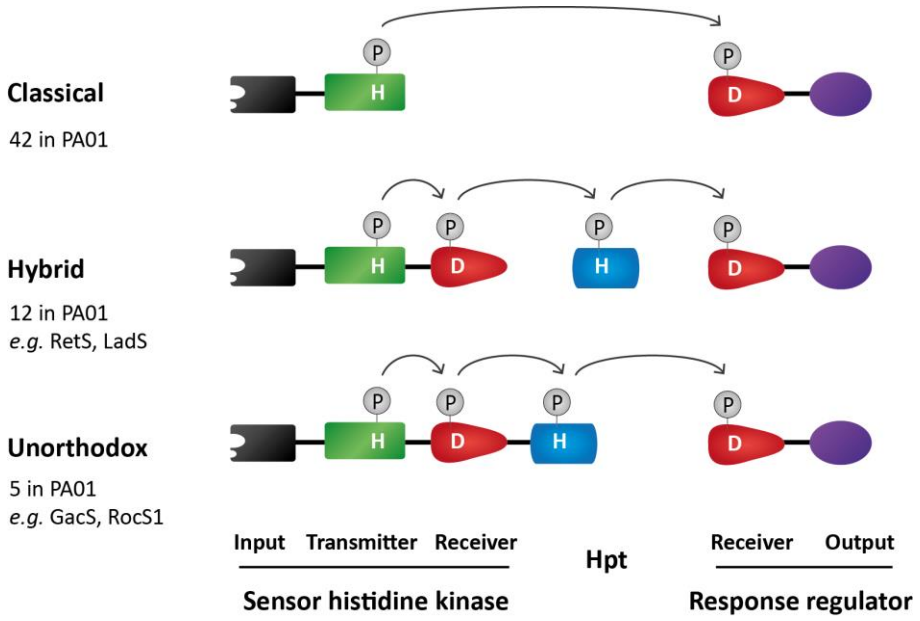

Fig 1. Schematic representation of domain architectures of two-component systems detected in *P. aeruginosa*

Based on their domain architecture three different groups of histidine kinases (HK) can be distinguished. HKs of classical two-component systems (42 systems found in *P. aeruginosa*) perceive signals via their N-terminal input domain (black), which induces autophosphorylation (P) of the transmitter domain (green) at a conserved histidine (H) residue. The phosphate is then transferred to an aspartic acid residue (D) in the receiver domain (red) of the cognate response regulator (RR). This activates the output domain (purple), which mediates the final cellular response. Hybrid and unorthodox kinases (12 and 5 systems found in *P. aeruginosa*, respectively) harbor a C-terminal extension in the form of a receiver domain. They rely on a histidine-phosphotransfer (Hpt) module for successful signal transduction to their cognate RR. In the case of unorthodox HKs the Hpt domain (blue) is an integral part of the HK. Hybrid kinases depend on one of the three Hpt proteins in *P. aeruginosa* (HptA, HptB or HptC). Adapted from [33].

Another mode of classification is based on domain architecture (Fig 1). Classical TCSs are formed by HKs, which consist of an input (black) and a transmitter domain (green). However, also more complex domain organizations exist. Unorthodox kinases as well as hybrid kinases harbor C-terminal extensions in the form of a receiver domain (red). As phosphotransfer strictly alternates between histidine and aspartic acid residues both types of HKs rely on a histidine phosphotransfer protein (Hpt) in order to activate their cognate RR. Hybrid kinases depend on external Hpt modules (blue), whereas in unorthodox kinases the Hpt domain is an integral part of the protein.

Signal transduction can also occur in a branched manner illustrated by the ‘many-to-one’ concept where different HKs affect the activity of a single RR (Fig 2A). An opposing signaling structure is described by ‘one-to-many’ where one HK can talk to several RRs (Fig 2B).

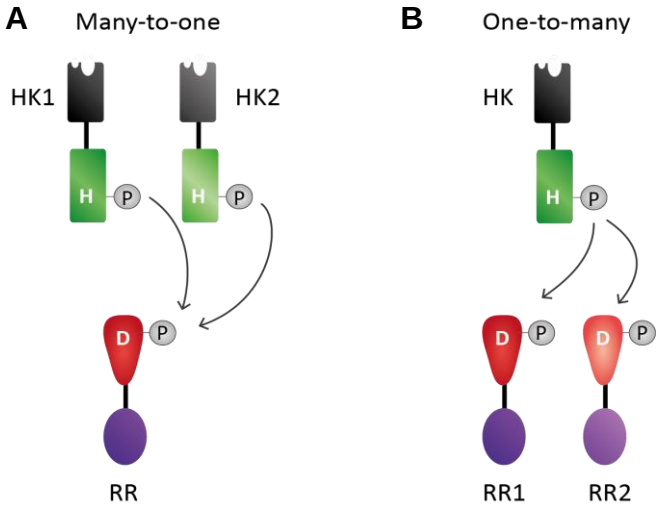

Fig 2. Schematic representation of branched signaling pathways
(A) Many-to-one: several HKs converge one a single RR. (B) One-to-many: one HK phosphorylates several RRs, *e.g.* RocS1/RocA1/RocR. (Adapted from [37]).

The first genome of *P. aeruginosa* was sequenced in 2001 and led to the identification of about 127 TCS members in strain PA01 [5]. Among the 64 HKs a surprisingly high number of non-classical HKs were identified (12 hybrid HKs and 5 unorthodox HKs) [32]. The RRs can be divided into different classes based on sequence homology (numbers reflect RRs in each group): OmpR-like (24), NarL-like (11), NtrC-like (8) and CheY-like (5). *P. aeruginosa* encodes for only three Hpt modules (HptA (PA0991), HptB (PA3345) and HptC (PA0033)) suggesting that these engage in several signal-transduction pathways, considering that there is a total of 12 hybrid HKs. The genome of PA01 contains 14 orphan HKs and 8 orphan RRs. Strikingly, 12 of the orphan HKs encode non-classical kinases [32]. Among them are some of the best studied TCSs in *Pseudomonas,* which play important roles for virulence and antibiotic resistance (reviewed in [27], [33]–[36]). This implicates that even though outnumbered by classical TCS, the hybrid and unorthodox HKs are key for successful adaptation of *P. aeruginosa* to changing environments.

As a result of the high structural similarities of the TCS modules bacteria have evolved different strategies to prevent unwanted cross-talk such as spatial and temporal control elements, the existence of the previously mentioned molecular interaction code, as well as by adjusting the molecular ratio of RR to cognate HK [37].

One of the biggest challenges in deciphering the role of phosphorylation pathways is the identification of the stimuli sensed by individual HKs. Even though a variety of different input signals such as light, temperature, pH, osmolarity, oxygen pressure, ions, redox state and QS molecules could be identified [38]–[40], for most TCSs knowledge about the corresponding input signals is still missing.

1.3.1 The Gac/Rsm signaling cascade

One of the best-studied TCSs in *Pseudomonas* is the so-called Gac/Rsm cascade (global activator / regulator of secondary metabolite), which is homologous to the Csr (carbon storage regulator) system in *E. coli* and seems to be a specialty of γ-proteobacteria. The first chapter focuses on the core components comprising the TCS GacS/GacA (PA0928/PA2586) and its downstream targets the small regulatory RNAs (sRNAs) RsmY (PA0527.1) and RsmZ (PA3621.1) which modulate the activity of a translational repressor called RsmA (PA0905). The following chapters aim to summarize the other factors directly associated with the central cascade and to provide an overview of the complex regulatory network the Gac/Rsm cascade is embedded in. A schematic summary is shown on page 9.

1.3.1.1 Core components

The HK GacS (101 kDa) and the RR GacA (23.6 kDa) were first described in 1992. Bacteria harboring deletions in *gacS* or *gacA* displayed reduced virulence and ecological fitness [41], [42]. Since both components are orphans it was recognized only over time that they form a cognate TCS. Genetic evidence was provided in 1994 [43] and direct phosphotransfer was later demonstrated in the homologous Csr system in *E. coli* [44]. Furthermore, the minimal units required for interaction were elucidated. The entire GacA molecule is required for homodimer formation as well as for interaction with GacS. GacS homodimerization relies on the cytosolic HAMP domain and the interaction between GacS and GacA is established via the transmitter (DHp and CA) and receiver domain [45]. While all three conserved phospho-sites of the unorthodox GacS sensor kinase are essential for signal transduction, the periplasmic loop is negligible. This stands in line with the finding that the loop region in general is only poorly conserved. In contrast, deletion of the linker HAMP-domain involved in dimerization renders the protein constitutively active [46].

RsmA is a small (7 kDa) sequence-specific RNA-binding protein. It forms homodimers [47] and acts as a translational repressor by binding to conserved GGA repeats (recognition motif: (AU)CAxGGAxG(AU)) in the 5' UTR of mRNAs. One of the binding sites is typically overlapping with the Shine-Dalgarno (SD) site thereby blocking the access of the 30S ribosomal subunit [48]. Typically, translational arrest leads to increased mRNA decay [49]. However, CsrA-mediated mRNA stabilization has also been demonstrated [50]. RsmA and GacA/GacS were found to be functionally linked as they regulate similar cellular processes [51], [52]. The regulatory link between GacA and RsmA is established via sRNAs that interfere with RsmA activity.

Two different classes of sRNAs influence translation in bacteria. The first group stimulates or represses translation by directly base-pairing with the mRNAs. Gram-negative bacteria rely on the RNA chaperone Hfq to mediate such interactions [53], [54]. The second class interferes with translational repression imposed by members of the Csr/Rsm family. Chromatin immunoprecipitation (ChIP) analysis demonstrated that GacA controls the expression of only two genes coding for sRNAs belonging to the latter class [55]. Both sRNAs, termed RsmY and RsmZ, contain multiple unpaired GGA motifs that are required for binding to RsmA, which frees the mRNA for ribosome access. In general, RsmA-sequestering sRNAs are typically about 100 to 400 nucleotides long and they only share little sequence identity (reviewed in [48]). Apart from the shared transcriptional control by GacA, the sRNAs are subject to several independent regulatory mechanisms, which will be discussed in

chapter 1.3.1.4 [55]–[58]. Together with the fact that RsmY shows higher expression levels than RsmZ under laboratory conditions [59], [60] and that additive as well as redundant effects of the two sRNAs on downstream targets have been demonstrated [61] suggests that the two sRNAs have distinct functionalities depending on the context. This illustrates an additional mechanism to fine-tune the Gac/Rsm signaling cascade in response to different environmental stimuli.

Several reports describe stimuli that activate the Gac/Rsm signaling cascade. However, the exact nature of the input signal as well as the corresponding sensing unit remains unclear for most of them. Haas and coworkers had found that supplementing the growth medium with signal extracts prepared from stationary phase cultures stimulated the Gac/Rsm activity. The signal is unrelated to any known QS molecules and it seems to be rather ubiquitous as not only signal extracts of other *Pseudomonas* strains but also of more distantly related species such as *Vibrio* showed stimulatory activity [62]. A more recent report describes an alternative input signal associated with kin cell lysis. PARA (*Pseudomonas* response to antagonism) is a program triggered upon cell lysis in interspecies co-cultures. A diffusible signal activates the Gac/Rsm cascade causing increased fitness in the remainder population due to upregulation of the T6SS [63]. Moreover, a phenolic plant compound, identified as a T3SS inhibitor, was shown to impact the activity of the Gac/Rsm cascade [64].

The downstream targets of RsmA were identified by several microarray studies, which demonstrated that about 9% of all encoded genes are controlled by the Gac/Rsm cascade [65]. Moreover, Brencic and Lory found that one third of all targets are positively controlled by RsmA, which most likely occurs in an indirect manner [60]. Among them are many virulence-associated components such as the T3SS, T4P, T2SS (xcp) and rhamnolipids [60], [65], [66]. Targets and processes directly repressed by RsmA include: T6SS, *lasI* and *rhlI* QS, hydrogen cyanide production, *pel* and *psl* exopolysaccharides*, mexEF-oprN* efflux pumps*,* enzymes involved in c-di-GMP synthesis as well as genes involved in iron homeostasis [60], [65], [67]–[69]. The upregulation of *mex* genes correlates with increased tolerance of *rsmA* mutants towards amikacin, nalidixic acid, trimethoprim and ceftazidime [70]. In line with this, a *gacS* mutant was found to be hypersusceptible to gentamycin, amikacin and chloramphenicol [71].

RsmA was also shown to negatively control its own translation [67], [72]. This is consistent with the observation that RsmA levels are rising with increasing cell density [59] [60]. RsmY and RsmZ also negatively autoregulate their expression, however the underlying mechanism is unknown [59]. Recently, two groups identified RsmN as a homologue of RsmA. However, even though RsmA and RsmN share ~30% identity they do not seem to act redundantly. This is illustrated by the fact that *rsmA*, but not *rsmN*, is able to complement a *csrA* deletion in *E. coli*. The observation that RsmA directly represses the translation of *rsmN,* together with the fact that deleting *rsmN* in *P. aeruginosa* has no phenotype, suggests that RsmN is more important in conditions where RsmA is absent [73], [74].

A properly balanced Gac/Rsm cascade is crucial for successful colonization and survival in different hosts. This is illustrated by the fact that mutants locking the system in a fully active state (Δ*rsmA*) or in a fully repressed state (Δ*gacS* or Δ*gacA*) were similarly attenuated in different infection models (reviewed in [11], [70]). Considering the sensitivity of the system it is not surprising that many

additional sensors and regulatory modules were found to affect the cascade. This allows the integration of various environmental stimuli in order to neatly fine-tune the system, which is key for the success of *P. aeruginosa* as a jack-of-all-trades.

1.3.1.2 RetS and LadS – inverse regulation by two hybrid sensor kinases

LadS (PA3974) and RetS (PA4856) are acronyms for "lost adherence sensor" and "regulator of exopolysaccharides and T3SS", respectively. They inversely regulate the Gac/Rsm cascade with LadS acting as an activator and RetS as a repressor. Both belong to the family of 7TMR-DISMED2 proteins and code for hybrid HKs (Fig 3). RetS is the only hybrid HK in *P. aeruginosa* featuring two conserved receiver domains. One well-studied example for such a domain organization is the response regulator PleD of *C. crescentus,* which plays an important role during the transition from swarmer-to-stalked cells. However, only one of the two aspartic acid residues is conserved in PleD and the tandem receiver domain has been shown serve as dimerization stem during PleD activation [75].

The molecular details of RetS are far better understood than of LadS and will be summarized first. RetS (103.8 kDa) was simultaneously identified by three research groups as a global regulator of biofilm formation and virulence. Strains lacking *retS* displayed decreased cytotoxicity due to the down-regulation of the T3SS and T2SS (xcp). On the other hand, *retS* mutants were associated with increased biofilm formation and the appearance of small colony variants (SCV), which are characteristic for high c-di-GMP levels [76]–[78]. Apart from phenotypic similarities to the Gac/Rsm system, RetS was genetically linked to the signaling cascade as *gacA, gacS* and *rsmZ* were identified in a transposon (Tn) screen for RetS downstream targets [77].

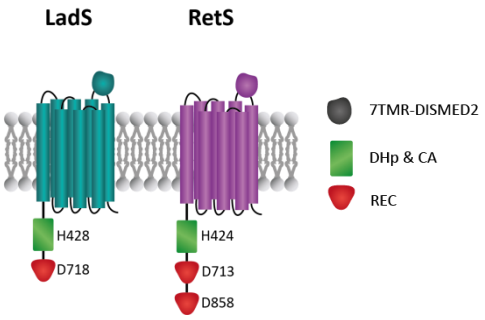

Fig 3. Schematic structure of RetS and LadS
LadS and RetS are both hybrid HKs and 7TMR-DISMED2 family members. Conserved phosphorelay residues are indicated next to the domains.

Conflicting reports exist about the functional requirements of RetS. While Laskowski and coworkers found that the conserved aspartic acid residue D858 of the second receiver domain is crucial for *in vitro* and *in vivo* functionality [79], Goodman and coworkers demonstrated that RetS fulfills its function via direct interaction with GacS independent of any of the three conserved phospho-acceptor residues [80]. Protein-protein interaction studies indicated that GacS interacts with RetS as strongly as it interacts with itself. Moreover, GacS also weakly interacted with LadS, while no interaction was shown between RetS and LadS or between the two hybrid HKs and GacA [45]. This unconventional mode of action stands in line with reports that RetS lacks intrinsic autophosphorylation activity [81]. RetS was shown to impact GacS functionality at three different levels, i) inhibition of GacS autophosphorylation [80], ii) phosphatase activity against the GacS receiver domain and iii) phosphotransfer from GacS to the second receiver domain of RetS (work presented at the *ASM Conference on Pseudomonas* 2015 by Porter and coworkers). The latter finding might explain the discrepancies observed regarding the functional requirements for D858, as the robustness of this mode of action may depend on the exact

experimental conditions. *retS* expression has been shown to be negatively regulated by Mg^{2+}-limiting conditions via the PhoP-PhoQ TCS that senses divalent cations (see chapter 1.3.2.1) [82].

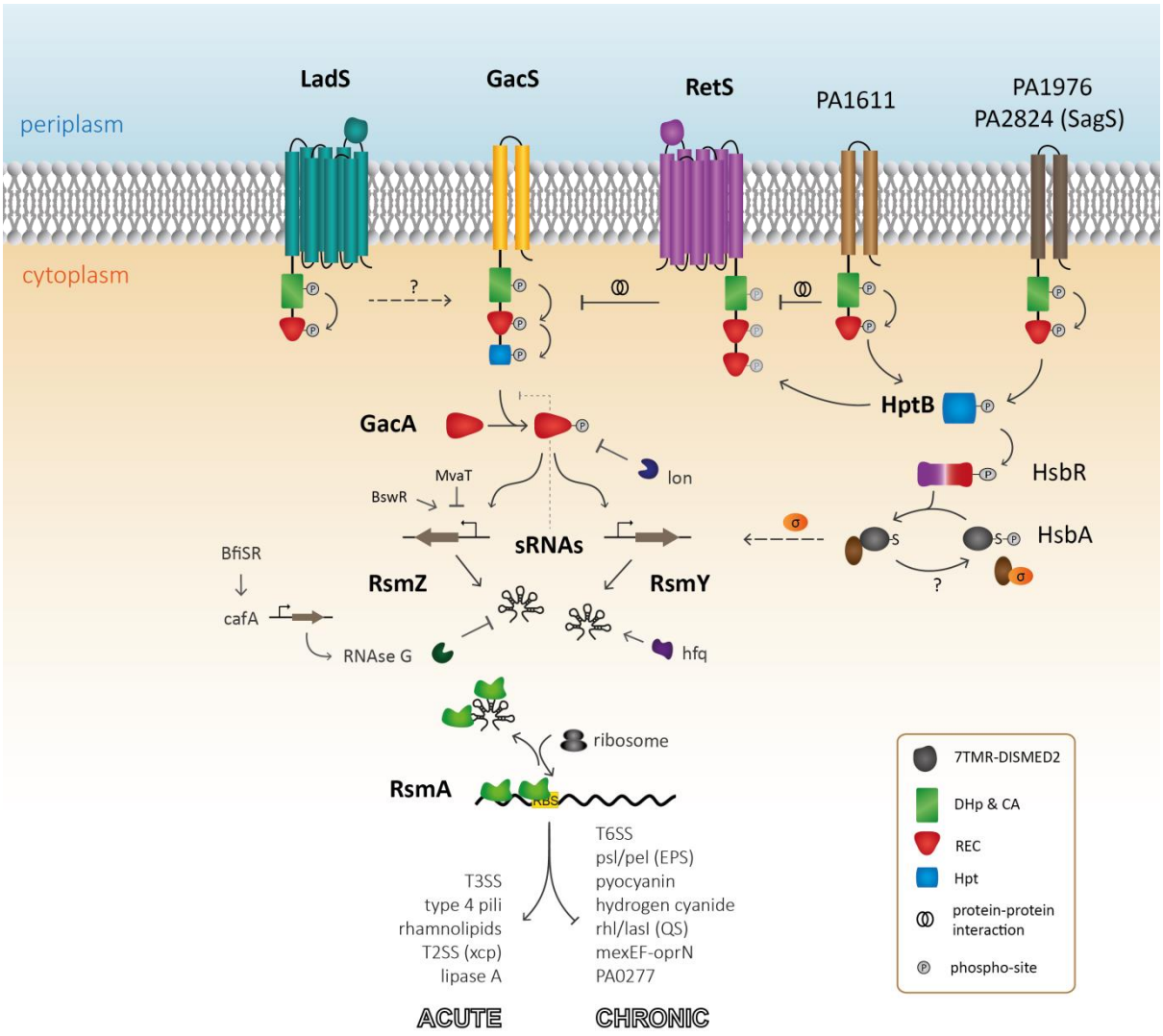

Fig 4. Schematic overview of the Gac/Rsm signaling cascade and its associated regulatory modules. Activation of the Gac/Rsm signaling cascade is key for the acute-to-chronic regulatory switch. The central TCS GacS/GacA is activated upon stimuli associated with high cell density leading to the expression of two sRNAs (RsmY and RsmZ), which bind to and thereby relieve the translational repression imposed by RsmA. Two hybrid HKs inversely affect the cascade: RetS via direct interaction with GacS negatively and LadS by unknown means positively. PA1611 was also shown to directly interact with RetS [94]. Moreover, the hybrid HKs PA1611, PA1976 and PA2824 are involved in phosphotransfer to HptB which in turn activates the RR HsbR whose Ser/Thr phosphatase activity leads to dephosphorylation of HsbA, a putative anti-anti-σ factor. In contrast to the RetS-pathway, only the expression of RsmY is affected by HptB. Additionally, also reverse phosphotransfer from HptB to RetS was observed [81]. Transcription factors affecting the expression of *rsmZ* include the H-NS family member MvaT (repressor) [55] and BswR (activator) [56]. The stability of RsmY is positively affected by the RNA chaperone protein Hfq [57], whereas RsmZ is degraded by the action of RNAse G which is induced by the TCS BfiSR [58]. Moreover, the protease Lon has been shown to degrade GacA [96].

LadS (88.2 kDa) was identified in a screen for altered biofilm formation of a *pilA* mutant. The absence of LadS resulted in flat and unstructured biofilms. Based on its sequence conservation and overlapping functionality with RetS, LadS could also be linked to the Gac/Rsm cascade. Based on epistasis experiments, LadS was placed upstream of RetS. However, no mechanistic details are known for how LadS and RetS might physically or functionally interact [83]. RetS and LadS are highly conserved among *Pseudomonas* species [51]. However, in some species like *P. syringae* LadS lacks the C-terminal receiver domain, indicating that this domain is most likely dispensable for functionality [84]. The PA14 reference strain, which is a highly virulent clinical isolate and represents the most common clonal group worldwide [85] harbors a *ladS* mutation [86]. A duplication of 49 bp induces a frameshift, which produces a protein with intact transmembrane helices but aberrant cytoplasmic domains. The observation that all other environmental isolates harbor a wild-type copy of *ladS* and that PA14 is the only sequenced strains that also lacks the *psl* exopolysaccharide cluster [87], suggests that PA14 represents a rather atypical clinical isolate.

Both RetS and LadS harbor a N-terminal 7TMR-DISMED2 domain. The family of 7TMR-DISMED2 containing proteins (DISM = diverse intracellular signaling modules) was discovered via an *in silico* approach to identify bacterial multipass-membrane receptors analogous to eukaryotic G-protein-coupled receptors (GPCRs). In *P. aeruginosa* a total of four proteins were identified, which harbor a periplasmic DISMED2 domain of roughly 150 aa in length, followed by seven membrane-spanning helices [88]. Strikingly, three out of four proteins are hybrid HKs (LadS, RetS, PA3462) and evidence exists that next to LadS and RetS, also PA3462 is involved in regulating the Gac/Rsm cascade. PA3462 is thought to interfere with GacS functionality in a similar manner as RetS via direct protein-protein interaction (work presented at the *Pseudomonas Conference 2013* by Porter and coworkers). The fourth member of this family, NicD, harbors a cytoplasmic GGDEF-domain implicated in c-di-GMP synthesis. NicD has been shown to be involved in glutamate-induced biofilm dispersal, which depends on an intact periplasmic DISMED2 sensing domain as well as on a functional GGDEF-domain [89].

Mutational analysis of RetS suggested that the periplasmic DISMED2 domain fulfills an inhibitory role during *in vivo* infections as bacteria lacking the periplasmic domain were recovered in higher numbers from the infection site compared to wild-type bacteria. In contrast, the transmembrane helices are crucial for proper signal transduction as a deletion spanning six out of seven helices and the DISMED2 domain caused severe virulence attenuation [79]. The crystal structure of the DISMED2 domain of RetS was recently solved by two groups and is shown in Fig 5A [90], [91]. The domain adopts a conserved jelly-roll fold formed by two opposing antiparallel β-sheets (β1-β3-β8-β5-β6 and β2-β9-β4-β7) which are flanked by two α-helices. This fold is characteristic for carbohydrate binding modules, which are an integral part of carbohydrate-active enzymes, such as glycoside hydrolases [92]. However, helix α1 seems to be unique to the DISMED2 domain. This is especially interesting since the subjacent β-sheets usually form the cavity for carbohydrate binding and helix α1 might interfere with ligand access as it resides where the carbohydrate would be normally placed (indicated by a star) [90]. This suggests that potentially ligands other than carbohydrates are recognized by this domain. RetS was implicated as sensor in PARA responding to the diffusible signal released by lysed kin cells. Deletion of *retS* as well as a single amino acid substitution of a tryptophan residue similarly

abrogated PARA. The tryptophan residue (W90) tightly links the α1-helix to the protein core and is situated in the putative binding pocket (Fig 5B) [63], [91].

Homology modeling of the DISMED2 domain of LadS which shares 35% sequence identity with the DISMED2 domain of RetS reveals that the overall fold is conserved including the putative binding site formed by aromatic residues creating a hydrophobic patch (Fig 5B). Vincent and coworkers also identified a second putative binding site for RetS situated at the dimer interface. However, this site is not conserved in LadS [91].

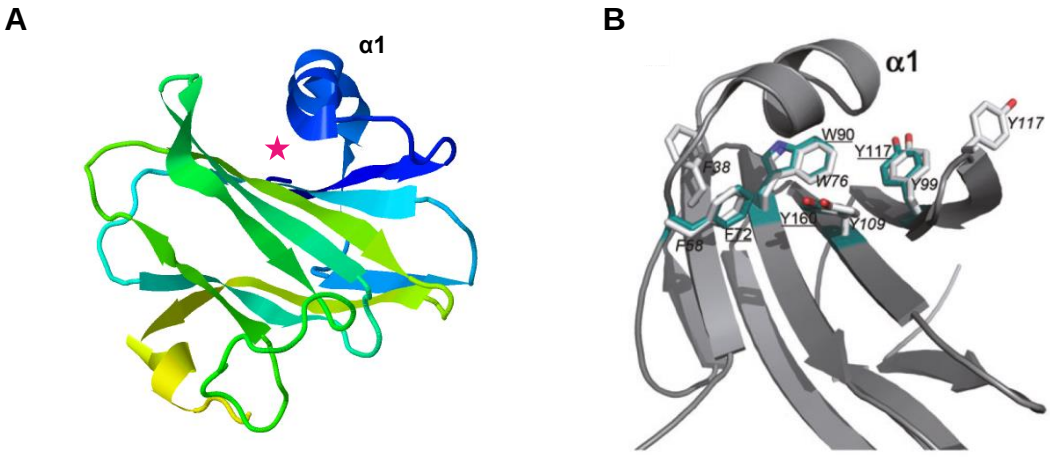

Fig 5. Structure of $RetS_{DISMED2}$ domain and comparison to $LadS_{DISMED2}$
(A) Ribbon cartoon structure showing the nine-stranded β-sandwich fold. N-terminus (blue), C-terminus (yellow). Helices α1 and α2 are not part of the conserved carbohydrate binding module. A pink star indicates the putative ligand binding site. (pdb 3JYB) [90] (B) Close-up view of putative binding site lined by hydrophobic amino acids. Residues from the $LadS_{DISMED2}$ homology model are shown in white with labels in italic. The residues of $RetS_{DISMED2}$ are shown in teal and labels are underlined. Illustration taken from [91].

1.3.1.3 HptB pathway

The HptB-dependent signaling cascade is distinct of the RetS pathway as it only affects the expression of *rsmY* [61]. *In vitro* studies have demonstrated that three different hybrid HKs are able to phosphorylate HptB: PA1611, PA1976 and SagS (PA2824). Reverse phosphotransfer from HptB to RetS was also observed, however the biological significance is unclear. Direct interaction of all four HKs with HptB was demonstrated by two-hybrid analysis [81].

HptB specifically phosphorylates the downstream located RR HsbR (PA3346) activating its atypical Ser/Thr phosphatase output domain. HsbR dephosphorylates HsbA (PA3347) which in turn potentially sequesters anti-σ factors [81]. HsbA interacts with the anti-σ^{28} factor FlgM, which is involved in regulation of swarming motility. However, the signaling mechanism causing increased *rsmY* expression is unknown [93].

Even though phosphotransfer between PA1611 and HptB has been observed, HptB is negligible for the function of PA1611. By directly interacting with RetS, PA1611 represses the activity of the Gac/Rsm cascade. This does not rely on any conserved phophoresidues. PA1775 coding for a conserved cytoplasmic membrane protein was found to negatively regulate the expression of PA1611 [94].

1.3.1.4 Other Gac/Rsm associated regulatory factors

Apart from the hybrid HKs RetS and LadS and the HptB-regulatory network several other factors were identified, which contribute to the increasing complexity of the Gac/Rsm regulon. A non-comprehensive list is provided below, which is also schematically illustrated in Fig 4.

- ANR (homologous to FNR of *E. coli*) is induced under anaerobic conditions, activating the NarXL TCS. NarL directly represses the transcription of *rsmY* and *rsmZ* [95].
- MvaT belonging to the family of H-NS transcriptional regulators directly represses the expression of *rsmZ* [55].
- BswR (PA2780) binds to the *rsmZ* promoter region and counteracts the repressing activity of MvaT [56].
- RsmY can be stabilized by the RNA chaperone protein Hfq [57].
- The TCS BfiSR activates the expression of *cafA* coding for RNAse G (homologous to RNAse E of *E. coli*), which then specifically degrades RsmZ [58].
- GacA protein stability is negatively affected by the protease Ion in *P. protegens* [96]. Our own results in *P. aeruginosa* support this finding.
- The σ-factor RpoN negatively affects GacA expression [97].
- The σ-factor AlgT activates the TCS AlgZR, which by an unknown mechanism affects the levels of RsmY, RsmZ and RsmA finally resulting in a net reduction of free RsmA [98].

Even though the Csr/Rsm system is highly conserved among different γ-proteobacteria, one interesting regulator identified in *E.coli* is absent in *P. aeruginosa*. The c-di-GMP effector CsrD is thought to directly bind to the sRNAs, which induces their degradation by potentially altering the availability for RNAse E [99].

1.3.2 Metal-sensing two-component systems

TCSs, which directly sense or are induced by metal-ions play important roles for the regulation of virulence traits upon host cell contact or adaptation processes in response to harmful environmental conditions.

1.3.2.1 PhoP/PhoQ

Most of the knowledge concerning the PhoPQ TCS, consisting out of the HK PhoQ and the RR PhoP, stems from studies in *Salmonella typhimurium* [100]–[102]. In general the system seems to be conserved and specific differences between *Salmonella* and *Pseudomonas* will be pointed out. PhoQ is activated by low periplasmic concentrations of Mg^{2+} and Ca^{2+} ions, which causes the upregulation of genes important for magnesium homeostasis such as specific transport systems. Surprisingly, none of the PhoP/PhoQ regulated genes seems to be involved in calcium homeostasis [102], [103]. Moreover, the PhoPQ system controls its own transcription [104]. Direct cation binding as well as distinct binding sites for Ca^{2+} and Mg^{2+} have been demonstrated [103], though the calcium binding pocket is unrelated to any known calcium binding motif [105].

Cation-rich environments repress the TCS by inducing PhoQ transmitter phosphatase activity towards phosphorylated PhoP. In contrast to *Salmonella* where PhoQ also acts as a kinase, in *P. aeruginosa* PhoQ only seems to harbor phosphatase activity. Phosphorylation of PhoP is most likely achieved by an alternative kinase [106].

One crucial difference between *Salmonella* and *Pseudomonas* is their mode of lifestyle. While *Pseudomonas* resides extracellularly, *Salmonella* is an intracellular pathogen. In agreement with this, PhoPQ is upregulated upon contact with epithelial cells in *P. aeruginosa* [107]. The two contrasting habitats also result in different requirements for the PhoPQ system. Both *phoP* and *phoQ* mutants show decreased virulence and increased susceptibility to polymyxin B in *Salmonella*. On the other hand only *phoQ* is essential for *P. aeruginosa* virulence. Moreover, deletion of *phoQ* renders *P. aeruginosa* more resistant to polymyxin B due to upregulation of *arnBCADTEF-pmrE* operon, which is involved in addition of aminoarabinose to the lipid A moiety of LPS [108] [109].

1.3.2.2 PmrA/PmrB

Similarly as PhoPQ, also PmrAB responds to limiting Mg^{2+} concentrations. Even though the two systems induce a very similar cellular response including induction of cationic antimicrobial peptide and polymyxin B resistance, only very few genes seem to be co-regulated. PmrAB, like PhoPQ, regulates its own transcription. However, unlike in *Salmonella*, no cross-regulation between the two systems was observed in *P. aeruginosa* [110].

1.3.2.3 BqsS/BqsR

There are conflicting reports about the input signal for this TCS. Patrauchan and coworkers show that the TCS is induced by calcium, leading to the induction of PA0320, which is involved in regulation of swarming motility and pyocyanin production. PA0320 contains a bacterial oligonucleotide/ oligosaccharide-binding domain and is thought to localize to the periplasm ([66] and poster at the *ASM Conference on Pseudomonas* 2015). Moreover, a recent report suggests homology to the stress-related protein YgiW of *E.coli* [112]. Kreamer and coworkers on the other hand found that the system senses iron, especially Fe^{2+} and does not respond to Fe^{3+} or calcium. Interestingly, PA0320 also ranks among the highest upregulated proteins in their hands [113]. Another study implicates the TCS in biofilm decay as *bqsS* and *bqsR* mutants showed increased biofilm formation [114]. Interestingly, the TCS has also been involved in intrinsic tobramycin resistance [115]. Discrepancies among the studies could arise from different cation exposure times. While Patrauchan and coworker directly supplemented the growth medium with calcium, Kramer and coworkers exposed the cells only for 30 minutes to the different ions.

Moreover, several heavy-metal sensing TCS were identified in *P. aeruginosa*, which generally induce tolerance to the sensed metals.

- CzcR/CzcS: Zn^{2+}-responsive TCS. Confers resistance to zinc, cadmium, cobalt and cross-resistance to imipenem. However, imipenem-resistance does not confer zinc tolerance [116].

- CopR/CopS: Transcriptionally induced Cu^{2+}-responsive TCS [117].
- ColR/ColS: Responds to excess of zinc, iron, cadmium and manganese [118].

1.3.3 Other two-component systems

The last section is dedicated to TCSs, which either play an important role during chronic infections or show interesting signaling architectures.

1.3.3.1 RocS1/RocR/RocA1

This regulatory network illustrates the signaling concept of 'one-to-many' mentioned previously. It comprises a central unorthodox HK and two RRs, RocR and RocA1. As the acronym implies, roc = regulator of cup, the signaling cascade is mainly involved in regulation of cup fimbriae. This cellular appendage is important for initial attachment of *P. aeruginosa* cells. However, under laboratory conditions they are not expressed. Cup fimbriae consist out of three components: a major fimbrial subunit, a chaperone and an usher. Presynthesized fimbriae are transported to the periplasm via the Sec pathway where they are bound to the chaperone before they are exported via the pore-forming usher. RocR and RocA1 act antagonistically and inversely regulate the expression of *cupC* genes (RocR positively and RocA1 negatively) and genes associated with the T3SS (RocR negatively and RocA1 positively). How this antagonism is established and the role of the c-di-GMP degrading EAL-output domain of RocR is still unclear [119], [120]. Overall, crosstalk between a single HK and two RRs with inverse signaling properties allows easy and rapid fine-tuning of the downstream targets [121]. The discovery that RocS2, another HK, is also involved in cup fimbriae regulation via the action of RocA1 rendered the network even more complex. Importantly for our work, no interaction of RocA1 or RocA2 with GacS was observed [120].

1.3.3.2 CbrA/CbrB

CbrAB is mechanistically very similar to the Gac/Rsm system. Poor carbon sources activate the TCS inducing the expression of the sRNA crcZ, which relieves the translational repression imposed by Crc. This results in upregulation of genes required for the uptake and usage of less preferred carbon sources as well as a decrease in biofilm formation and virulence gene expression [122], [123].

1.3.3.3 KinB/AlgB

Conversion to mucoidy is one of the major adaptation processes associated with chronic infections in CF lungs. Alginate expression is controlled by the global σ-factor AlgT (also AlgU or σ^{22}), which is usually sequestered by the anti-σ-factor MucA. Upon certain stimuli including envelope stress MucA is degraded by the action of AlgW and MucP, thereby freeing AlgU [124]. MucA ranks among the top mutated targets in clinical isolates [125].

The KinB/AlgB TCS is involved in induction of acute virulence traits [126]. While the phosphatase activity towards AlgB is crucial, the kinase activity of KinB is negligible for functionality. Deletion of *kinB* results in mucoidy, which is partially mediated via the upregulation of AlgU [127].

Overall, the so far studied TCSs of *P. aeruginosa* are organized in an intricate network with steadily increasing complexity. This allows *Pseudomonas* to optimally adapt to its current environment. However, one of the biggest problems is still that for most of the TCSs no defined input signal is known.

1.4 Calcium signaling

As the fifth most prevalent element in our biosphere we ubiquitously encounter calcium, mostly in its ionic form [128]. The role for this very simple messenger, which does not need to by synthesized or chemically modified, is well established in eukaryotes and has been shown to regulate almost every aspect of a cell's life including death (reviewed in [129]). Calcium signaling has also gained more and more attention in prokaryotes. The maintenance of a steep calcium gradient over the cell membrane with intracellular levels being several orders of magnitude lower than outside is an important feature common to both domains of life. The concentration gradient is a prerequisite for calcium signaling and also crucial to avoid cellular intoxication as calcium readily precipitates phosphate [130]. Since Mg^{2+} ions are unable to do so it is not surprising that intra- and extracellular Mg^{2+}-concentrations reside in a very similar range (mM), which renders Mg^{2+} unsuitable as a signaling molecule [131]. Even though Ca^{2+} ions are ubiquitously present, different calcium signatures as defined by spatial organization and temporal changes can trigger distinct responses [130].

1.4.1 Calcium signaling in eukaryotes

Ions are intrinsically difficult to see or quantitate in living organisms. Therefore, several tools were developed to visualize Ca^{2+}, such as ratiometric calcium-sensitive dyes (*e.g.* Fura-2) or aequorin, a photoprotein, which was isolated from the same organism as GFP (*Aequora victoria*) [132], [133]. The advantage of aequorin lies in the fact that it can easily be targeted to specific cellular compartments. Aequorin is usually expressed as apoprotein. Its prosthetic group coelenterazine needs to be provided externally, but due to high membrane permeability can be supplemented in the growth medium.

Using such tools the intracellular calcium concentrations were estimated to be about four orders of magnitude lower (10^{-7} M) than in the extracellular fluid (10^{-3} M) [130]. The total calcium concentration in blood was found to be about 2.5 mM, whereof ~60% is sequestered by plasma proteins or complexed with other ions [128]. Human joints and bones can harbor concentrations up to 4 mM [134]. In comparison sea water contains about 10 mM calcium [135].

Intracellularly calcium is sequestered by cytosolic proteins like parvalbumin or by organelles including the ER and mitochondria, which involves proteins such as calrecticulin and calsequestrin (reviewed in [82]). Calcium mobilization from intracellular stores occurs either through ryanodine receptors (ryaR) or inositol-1,4,5-trisphosphate receptors (IP_3R) depending on the excitability of cells. Excitable cells such as muscle cells and neurons use electrochemically-, calcium- or cAMP-gated ryaRs, whereas non-excitable cells rather rely on IP_3Rs. Calcium-regulated processes frequently depend on the adaptor protein calmodulin (calcium modulated protein), which upon calcium binding gets recruited to proteins involved in cell division, DNA synthesis or muscle contraction (reviewed in [136]).

Maintenance of calcium homeostasis strongly relies on the extracellular calcium-sensing GPCR simply termed CaSR for calcium-sensing receptor. Parathyroid glands express CaSRs on their surface. Low extracellular calcium levels lead to the release of parathyroid hormone (PTH), which results in overall increased calcium plasma levels by stimulating bone resorption, renal reabsorption and intestinal calcium absorption. The underlying signaling mechanism is depicted in Fig 6.

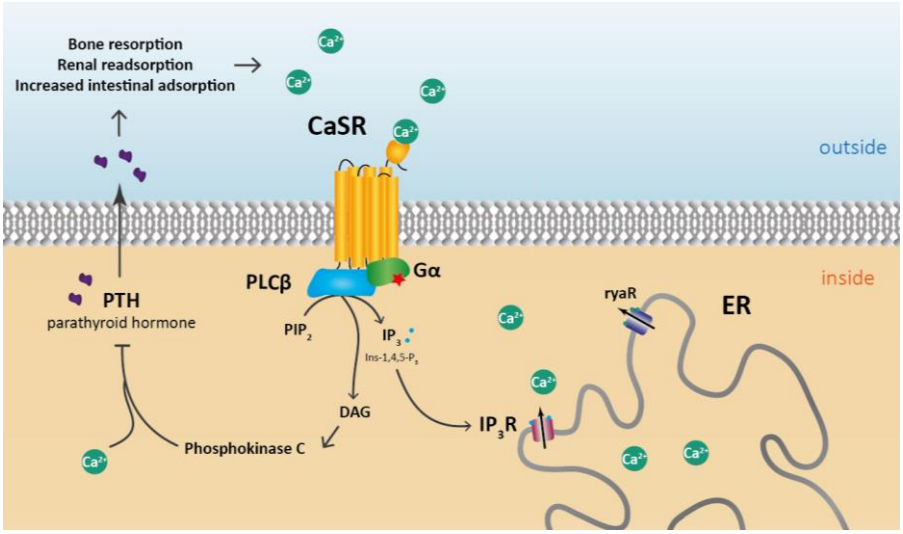

Fig 6. Schematic overview of the extracellular calcium signaling pathway in parathyroid glands
Parathyroid glands harbor calcium-sensing GPCRs (CaSRs) on their surface. Ca^{2+} directly binds to the extracellular domain of the CaSR, which then leads via the action of a G-protein to the activation of phospholipase C (PLC). PLC cleaves phosphatidylinositol-4,5-bisphosphate (PIP_2) into diacylglycerol (DAG) and IP_3. IP_3 binds to IP_3Rs in the ER leading to calcium mobilization, which in turn also activates ryaRs. Ca^{2+} as well as phosphokinase C activated by DAG directly reduce the release of PTH. Hence, low extracellular calcium levels lead to the release of parathyroid hormone (PTH), which increases bone resorption, renal reabsorption and intestinal calcium absorption resulting in overall increased calcium plasma levels [137], [138].

Ca^{2+} ions are directly recognized by the extracellular domain of the CaSR, which then leads via the action of a G-protein to the activation of phospholipase C (PLC). PLC cleaves phosphatidylinositol-4,5-bisphosphate (PIP_2) into diacylglycerol (DAG) and IP_3. IP_3 binds to the IP_3Rs in the ER stimulating the release of calcium, which in turn also activates ryaRs. Ca^{2+} as well as phosphokinase C activated by DAG directly reduce the release of PTH and lead to signal termination [137], [138].

GPCRs are characterized by an extracellular ligand-binding domain followed by seven transmembrane-helices and are usually active as dimers. In the case of CaSR the extracellular domain comprises a 612 aa bilobed Venus flytrap-like module with one calcium-binding site located at the crevice between the lobes [139]. However, the use of calcimimetics as well as proteins without extracellular domain suggest the presence of additional binding sites [137], [140].

Interestingly, an *in silico* approach aiming at the identification of analogous bacterial multipass-membrane receptors lead to the discovery of the prokaryotic 7TMR-DISMED2 protein family [88].

1.4.2 Calcium signaling in prokaryotes

The importance of calcium signaling was long underestimated in prokaryotes, however several reports demonstrated that also bacteria maintain calcium homeostasis by controlled influx and efflux

mechanisms [111], [141]–[143]. Moreover, meanwhile calcium is also appreciated as a versatile signaling molecule. However, direct calcium-binding and a causal functional relationship could be demonstrated only for a handful of proteins (summarized in several reviews [130], [131]) and in Table 1.

1.4.2.1 Calcium homeostasis

Intracellular calcium concentrations of bacteria are very similar to the ones found in eukaryotes and lie in the range of 100 – 300 nM [146], [147], with the fraction of bound Ca^{2+} largely exceeding the fraction of free Ca^{2+} [141]. Several factors contribute to the maintenance of the steep gradient across the cell membrane including intrinsic low permeability of the cell envelope, strictly controlled influx and efflux mechanisms alongside with a high cytosolic buffering capacity. Intact calcium homeostasis was found to be essential for *Streptococcus pneumoniae* colonization and survival as deletion of a Ca^{2+}-exporter lead to strong attenuation in a mouse model [148]. Calcium influx is generally mediated by channels, whereas efflux relies on P- and F-type ATPases and electrochemical gradient driven systems. However, most of the transporters are not dedicated calcium transporters. The periplasm in gram-negative bacteria accounts for 8-16% of the total cell volume [149] and has been shown to harbor similar calcium concentrations as the extracellular space. Moreover, it has been demonstrated that under some circumstances the periplasm can even concentrate calcium ions in respect to the extracellular milieu. This is attributable to membrane-derived oligosaccharides and anionic proteins which act as Ca^{2+} chelators. The expression of the former is reduced in a high osmolarity environment [146].

A study addressing calcium homeostasis in *P. aeruginosa* estimated the basal intracellular calcium concentration to 140 $\pm$ 50 nM, which upon stimulation with external [mM] calcium rapidly increased 13-fold, followed by a steady decline. If cells were continuously exposed to 5 mM Ca^{2+} the basal intracellular calcium concentration was found to be 57% higher than before (220 $\pm$ 40 nM). Homology search identified 18 putative calcium transporters and at least 13 were found to be involved in maintaining calcium homeostasis indicating large functional redundancy [147].

1.4.2.2 Global cellular processes affected by calcium

Calcium has been shown to impact diverse aspects of bacterial lifestyles including heterocyst differentiation [150], spore formation [151], [152], fruiting body formation [153] and chemotaxis [154]. Reports in *E. coli* have demonstrated that calcium controls the switch between tumbling and swimming. While repellents lead to increased calcium levels accompanied with higher tumbling frequencies, attractants reduce the intracellular calcium concentration [155], [156]. Another well studied example of a calcium-induced adaptation process is the low-calcium response in *Yersinia,* which involves a set of plasmid-borne virulence genes (such as the T3SS) that are transcriptionally induced under low-calcium conditions [157]. Calcium-induced swarming has been demonstrated in *Vibrio parahaemolyticus* as well as *P. aeruginosa* [147] [158]. Moreover, a study from the 70's suggests that Ca^{2+} as well as Mg^{2+} ions lead to an increased tolerance of *P. aeruginosa* towards different classes of antibiotics including tetracycline, gentamycin, polymyxin B and carbenicillin [159]. Other calcium-induced phenotypes in *P. aeruginosa* include elevated protease IV production [160],

increased biofilm formation in the mucoid isolate FRD1 accompanied with higher production of extracellular proteases AprA, LasB and PrpL [161] and activation of the T3SS in mucoid strains by alginate-mediated calcium-chelation [162].

Butane 2,3-diol, a byproduct of glucose fermentation in the gut, was found to act as a physiological activator of calcium channels in *E.coli* especially during stationary phase. In line with this, stationary phase cells were found to have higher intracellular calcium levels compared to exponential growing ones. [163]. Interestingly, transferring stationary phase *Salmonella* cells into fresh medium led to a steep increase in intracellular calcium levels peaking 4 min after the shift. The authors speculate that this calcium spike might serve a regulatory function during the adaptation to the new environment [164].

Even though only a fraction of all existing reports about calcium-induced cellular changes were discussed here, it is evident that calcium affects a very wide range of processes. Moreover, what is also evident is that most of the studies are descriptive in nature and do not provide any underlying mechanistic details. The next chapter will focus on examples where direct calcium binding has been demonstrated including some examples where also the underlying functional importance was elucidated.

1.4.2.3 Calcium-binding proteins

Calcium-binding proteins can be assigned to two classes. Either they are directly involved in calcium homeostasis in the form of transporters or act as calcium effectors and mediate downstream signaling events. *In silico* tools are very potent in mining large proteomes in search of specific calcium-binding motifs. However, for most proteins identified the biological significance is missing. An unbiased approach to identify calcium-binding proteins is $^{45}CaCl_2$ autoradiography, which led to the discovery of several proteins with unclassified binding motifs in *B. subtilis* [165].

The largest and best characterized group of calcium-binding motifs detected across all kingdoms of life is the EF-hand motif, which has been first described in 1973 [166]. The calcium-binding loop typically contains several aspartic acid residues organized in the following consensus motif: DxDxDG and is flanked by two α-helices, resulting in an overall helix-loop-helix structure. Usually EF-hand motifs are found in adjacent pairs [167]. Another common motif found in prokaryotes is the so called β-roll motif of repeat in toxin (RTX) proteins, which consists of tandemly-repeating GGxGxDxUx nonamers. A third group comprises the greek key motifs of βγ-crystallins formed by four adjacent antiparallel β-strands. A unique feature of this class is that calcium coordination is mediated via serine/threonine and not like otherwise common via aspartic acid residues. Furthermore, Big-domains (bacterial proteins with immunoglobulin-like domains) have also been implicated in calcium-binding. (Reviewed in [111]).

For several *P. aeruginosa* proteins direct calcium binding has been demonstrated. An overview including some examples from other bacteria is provided in Table 1.

Table 1 Overview of different calcium-binding proteins

Protein	Organism	Ca^{2+}-binding motif	Function	Importance of calcium-binding	Ref
EfhP (PA4107)	*P. aeruginosa*	EF-hand	Important for maintenance of calcium homeostasis, biofilm formation and resistance to oxidative stress	Effector function	[168]
PilY1 (PA4554)	*P. aeruginosa*	EF-hand like	Involved in twitching and swimming motility as well as in host cell adherence	Crucial for integrin binding	[147]/ [148]
AprA (PA1249)	*P. aeruginosa*	RTX	Alkaline protease	Essential for folding	[171]
Tse3 (PA3484)	*P. aeruginosa*	Annexin-like	HSI-I T6SS effector with muramidase activity, secreted into the periplasm of its prey. Immunity protein Tsi3 prevents self-intoxication.	Crucial for membrane association and enzymatic function	[172]
PlcR (PA0843)	*P. aeruginosa*	EF-hand	Chaperone which is required for release of PclH (main component of phospholipase C)	Not known	[173]
PA-IL/ PA-IIL (PA2570/PA3361)	*P. aeruginosa*	?	Evolutionary unrelated lectins, which establish host interaction by recognizing either galactose (PA-IL) or fucose moieties (PA-IIL)	Enhances carbohydrate binding	[174], [175]
LapG	*P. fluorescens*	?	LapA: main adhesin and key for initial attachment. Activity regulated by calcium-dependent protease LapG which cleaves LapA, resulting in biofilm loss.	Crucial for enzymatic activity	[176]
LapF	*P. putida*	RTX	Important for biofilm maturation	Induction of multimerization	[177]
CabA (AprA homologue)	*Vibrio vulnificus*	RTX	cabABC operon is induced by c-di-GMP, critical for proper biofilm formation.	Induction of multimerization; essential for function	[178]

1.4.3 Dysregulated calcium homeostasis is associated with cystic fibrosis

The earliest report describing the phenomenon of an altered calcium homeostasis in association with CF stems from 1967. The authors detected increased calcium levels in the submaxillary saliva from CF patients compared to healthy individuals [179]. This finding is supported by many other studies, which found elevated calcium levels in parotid and submaxillary saliva [180], fibroblasts [181], [182], [183] and other body fluids like tears and seminal fluid [184]. Other indicators of hypercalcemia like increased calmodulin and calcitonin levels were also associated with CF patients [185]. The underlying cause of this pathology is thought to be related to the increased calcium mobilization from the mitochondria and ER. Mitochondria of CF patients were found to accumulate 2-3x more calcium than controls. Increased calcium influx and efflux resulted in the net accumulation of calcium [183] [186], [187]. Along the same line, the activity of the Ca^{2+}-ATPase was found to be greatly reduced in erythrocytes from CF patients compared to control cells [188]. Moreover, the persistent hyper-inflammation in chronically infected patients further contributes to the elevated calcium levels [189].

Molecular evidence linking the defect in CFTR to the dysregulated calcium homeostasis is provided by a study, which demonstrates that partial restoration of the CFTR by low temperature reduces the aberrant calcium mobilization. This is most likely caused by affecting the number or the spatial organization of the membranous IP_3Rs [189].

1.4.4 *Pseudomonas aeruginosa* interferes with eukaryotic calcium signaling

Several reports demonstrate that *P. aeruginosa* infections cause increased intracellular calcium levels in host cells. This effect relies on type IV pili [190] and is thought to be mediated via pyocyanin and one of the QS autoinducers. Pyocyanin is a redox-active compound which leads to the formation of reactive oxygen species (ROS) by accepting electrons from NAD(P)H and subsequent formation of oxygen radicals via the reduction of O_2. This is thought to stimulate calcium-mobilization from the ER via the increased production of IP_3. Moreover, the effective pyocyanin concentration was found to be similar to the levels detected in sputum of CF patients (75-200 μM) providing strong support for its *in vivo* relevance [191]. Another mechanism is illustrated by an example of interkingdom signaling where the las QS molecule was shown to induce IP_3R-dependent calcium release from the ER, promoting apoptosis [190]. Moreover, also the production of proinflammatory cytokines such as IL-8 was shown to occur in a calcium-dependent manner [192].

2 AIM OF THESIS

Chronic *Pseudomonas aeruginosa* infections are among the most frequent nosocomial infections and are the leading cause of morbidity and mortality in cystic fibrosis (CF) patients. The establishment of persistent infections is usually associated with loss of motility and virulence and the emergence of biofilm formation. While the molecular details underlying the acute-to-chronic transition of *P. aeruginosa* are still poorly understood, the second messenger c-di-GMP as well as the global family of Csr/Rsm posttranscriptional regulators have been implicated in this switch. This thesis focuses on the Gac/Rsm cascade with the aim to obtain a better understanding of the underlying regulation of this global signaling cascade, its associated kinases and the corresponding input signals. This will be achieved by developing fluorescent reporter tools as proxy of the Gac/Rsm cascade activity to analyze the behavior of single cells as well as entire cell populations. Furthermore, the *in vivo* relevance of the signaling cascade will be addressed by analyzing *P. aeruginosa* isolates from CF patients.

3 DEVELOPMENT OF DUAL ACUTE-CHRONIC REPORTERS

In order to study the regulation underlying the Gac/Rsm cascade, one of the main players involved in the acute-to-chronic lifestyle switch of *P. aeruginosa,* as well as to probe the chronic potential of clinical isolates from cystic fibrosis (CF) airways, we aimed to establish a simple and versatile fluorescent-based reporter system. Promoter fusions of target genes that are positively or negatively controlled by RsmA served as proxies for the "acute" and "chronic" lifestyle (illustrated in green and red throughout the entire thesis, respectively) and were combined on a single vector backbone. As the inverse regulation of the type 3 (T3SS) and the type 6 secretion system (T6SS) was previously used to classify the lifestyle status of *P. aeruginosa* [193], we choose respective promoter regions as readouts for the first design of our dual reporter. While *popN* is implicated in the control of T3SS activity and is positively controlled by RsmA, *tssA* is a part of the HSI-I T6SS and was shown to underlie strong negative control by RsmA (detailed promoter regions are illustrated in Fig 7) [60], [65], [77].

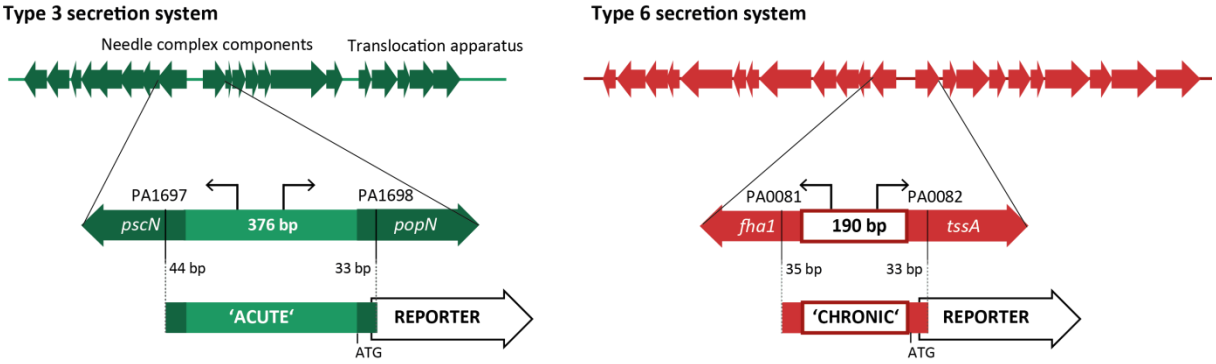

Fig 7. Schematic representation of selected promoter regions used to construct dual acute-chronic reporter tools
Genomic context of promoter regions used to create different fluorescence-based reporter tools, representing the "acute" or "chronic" lifestyle of *P. aeruginosa.* Acute (green): T3SS promoter = intergenic region upstream of *PA1698* (*popN).* Chronic (red): T6SS promoter = intergenic region upstream of *PA0082* (*tssA)*. The length of the sequence used for translational fusions is indicated. For transcriptional fusions an additional ribosomal binding site (RBS) was introduced between the promoter and the reporter gene.

The process from the first design to the final dual acute reporter constructs required several rounds of optimization (outlined in Fig 8) and will be briefly summarized. After settling on the promoter regions and on pME6032 as vector backbone we addressed the question of suitable fluorescent reporter genes, which had to meet the following requirements: no overlapping excitation/emission spectra to allow the simultaneous detection of both readouts and the fluorophores should ideally be as bright as possible. We opted to use *cerulean* and *venus,* which encode enhanced versions of CFP and YFP, respectively. The very first design of the dual reporter construct is indicated on the left where the two promoter regions face back-to-back (P_{T3SS} fused to *venus* and P_{T6SS} to *cerulean*). However, we found that both intergenic regions contain a second promoter facing in the opposite direction, which potentially leads to false positive expression profiles.

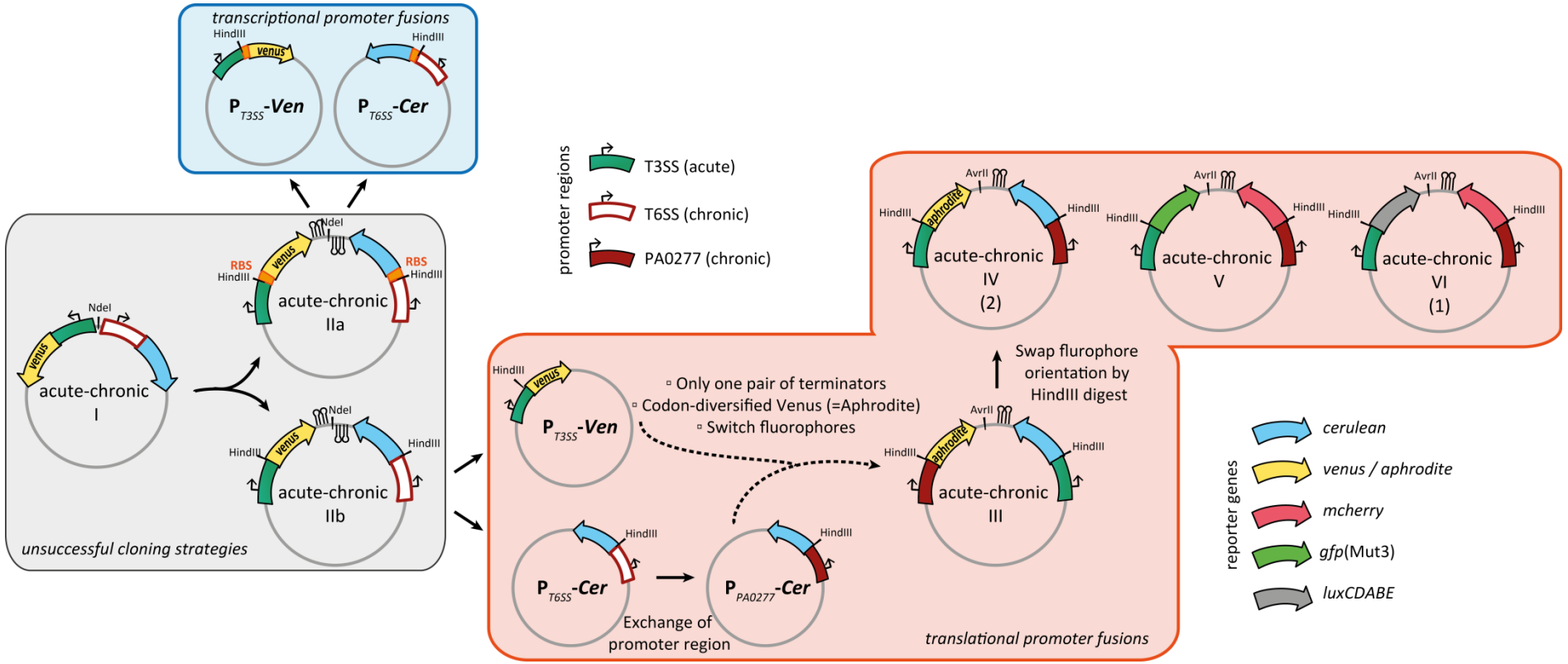

Fig 8. Development of the Gac/Rsm-responsive acute-chronic dual reporter tools

The process from the very first design on the left to the final dual reporters on the right is schematically illustrated. 'Acute-chronic I': acute (P_{T3SS}::*venus*) and chronic (P_{T6SS}::*cerulean*) back-to-back promoter fusions. Swap of promoter orientation in combination with the introduction of two terminators pairs (T_0, T_1) as spacers yielded 'acute-chronic II' (a / b = transcriptional / translational promoter fusions), which however could not be finalized. Therefore, individual translational (orange box) and transcriptional (blue box) promoter fusions were constructed (acute (P_{T3SS}::*venus*) and chronic (P_{T6SS}::*cerulean*)).

The chronic reporter was optimized by exchanging P_{T6SS} for the stronger P_{PA0277}. Moreover, to minimize sequence homology *venus* was exchanged for the codon-diversified *aphrodite* and only one terminator pair was used as spacer resulting in 'acute-chronic III' by combination of acute (P_{T3SS}::*cerulean*) and chronic (P_{PA0277}::*aphrodite*) translational promoter fusions. Based on this design several other variants suitable for analysis of single cells were constructed: acute-chronic IV (acute: P_{T3SS}::*aphrodite* and chronic: P_{PA0277}::*venus*) and acute-chronic V (acute: P_{T3SS} ::*gfp*(Mut3) and chronic: P_{PA0277}::*mcherry*) or for analysis of cell populations: acute-chronic VI (acute: P_{T3SS}::*luxCDABE* and chronic: P_{PA0277}::*mcherry*)

To solve this problem, we adapted our cloning strategy by reversing the orientation of the two fusions and introducing transcription terminators between the reporter constructs to minimize any interference. Translational and transcriptional versions are schematically illustrated (Fig 8, IIa and IIb). Unfortunately, for reasons we came to realize only later, the dual reporters IIa and IIb could not be completed. Therefore, without knowing that these reporters meets our expectation, we decided to first test individual "acute" and "chronic" reporter cassettes before making the effort to combine them.

Single translational (orange box) and transcriptional fusions (blue box) were generated successfully and transformed into wild-type, Δ*rsmYZ* or Δ*rsmA* strains to check for the respective expression patterns. Representative images are shown in Fig 9.

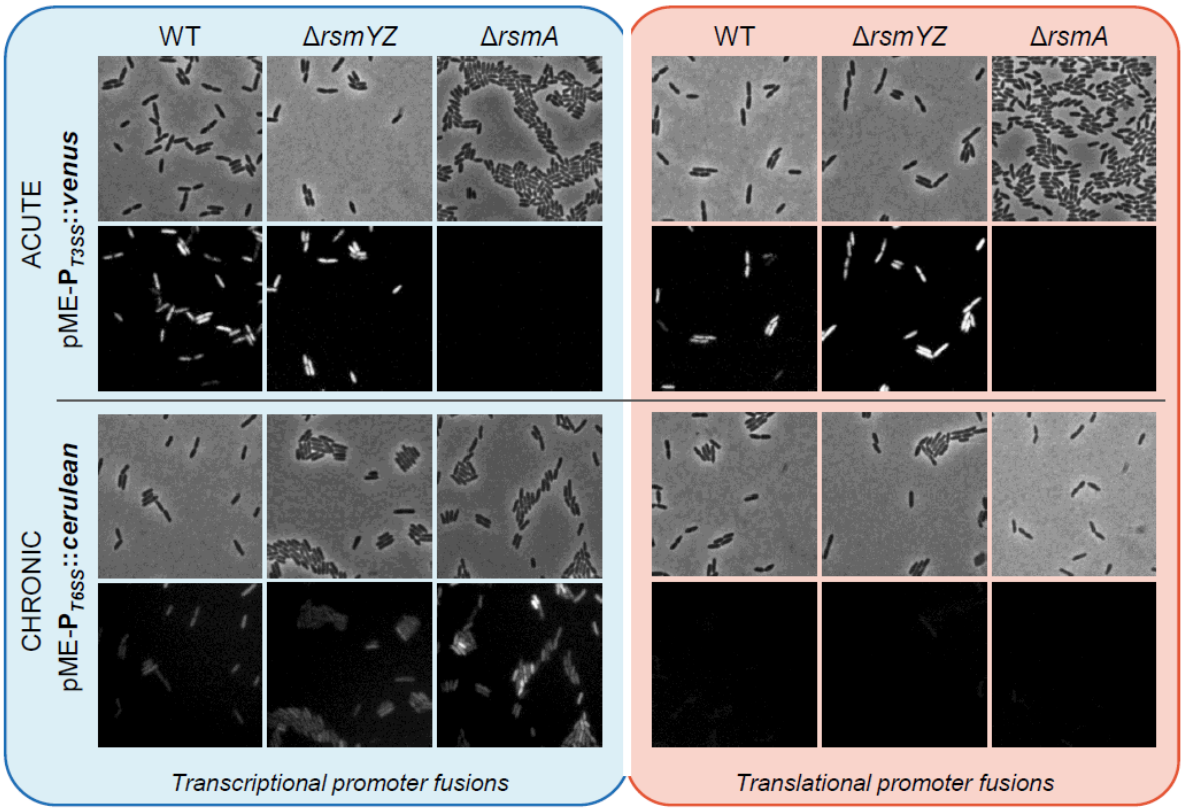

Fig 9. Single acute and chronic reporter constructs (transcriptional vs. translational fusions)
Fluorescence microscopy images of *P. aeruginosa* strains (WT, *ΔrsmYZ*, Δ*rsmA*) transformed with transcriptional (blue box) or translational (orange box) reporter constructs: acute: P_{T3SS}::*venus* and chronic: P_{T6SS}::*cerulean*. Image contrast is adjusted to the same level.

As expected, the "acute" reporter (P_{T3SS}::*venus*) is maximally expressed if the Gac/Rsm cascade is fully repressed (*ΔrsmYZ*) while no signal is detectable in the absence of RsmA. Wild-type cells show an intermediate phenotype. This pattern, observed with translational and transcriptional reporter fusions, supports the notion that the positive control of RsmA occurs indirectly. In contrast, the transcriptional "chronic" reporter fusion (P_{T6SS}::*cerulean*) exhibited similar expression levels in all three strains, making it unsuitable as proxy for the Gac/Rsm cascade activity. Unfortunately, the translational version failed to produce a signal above the detection limit.

In search for an alternative "chronic" readout we took advantage of the different microarray studies defining the RsmA regulon [60], [65], [77]. *PA0277*, coding for a hypothetical Zn-dependent protease

was among the targets with the strongest negative regulation by RsmA. Similar as before, a translational reporter cassette was constructed and tested in wild-type, Δ*rsmYZ* or Δ*rsmA* strain backgrounds (promoter region is schematically illustrated in Fig 10).

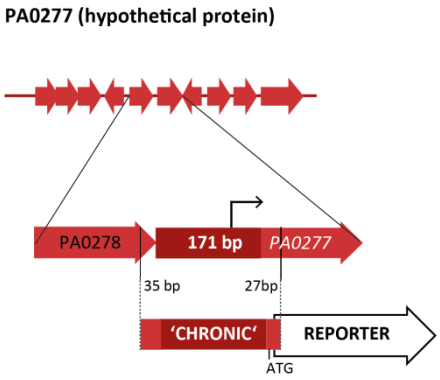

Fig 10. Schematic representation of the promoter region used as chronic readout in final reporter tools
Genomic context of *PA0277* which encodes a Zn-dependent protease and is negatively controlled by RsmA. The length of the sequence used for translational promoter fusions is indicated. For transcriptional fusions an additional RBS was introduced between the promoter and the reporter gene.

Representative images are depicted in Fig 11, which shows a largely improves signal-to-noise ration compared to the previous T6SS-based "chronic" reporter. Moreover, the reporter is expressed in an RsmA-dependent manner with only background signal in the absence of *rsmY* and *rsmZ*.

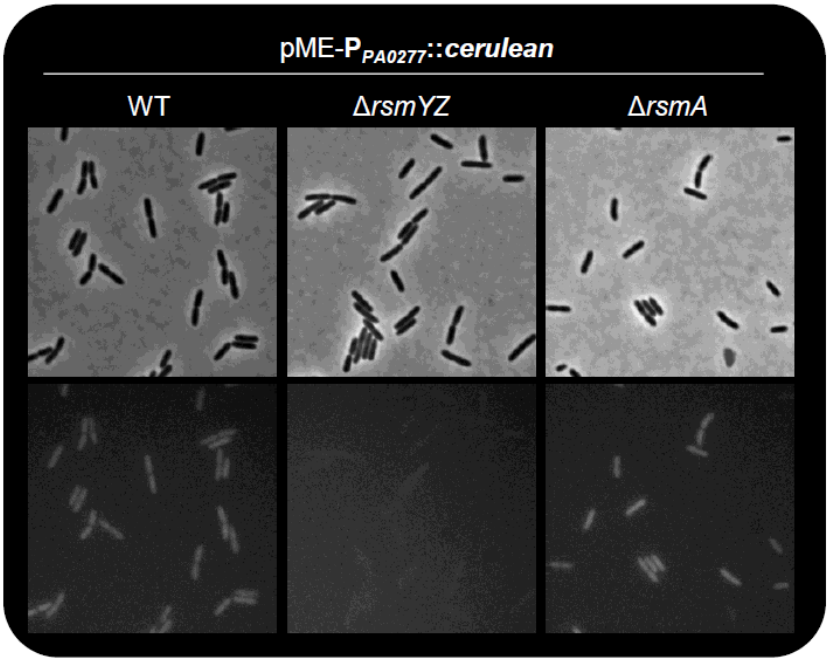

Fig 11. Chronic P_{PA0277}::*cerulean* reporter shows an RsmA-dependent expression profile with increased signal intensity
Fluorescence microscopy images of *P. aeruginosa* strains (WT, Δ*rsmYZ*, Δ*rsmA*) transformed with the translational P_{PA0277} ::*cerulean* reporter construct. Image contrast is adjusted to the same level.

Based on this promising result we pursued our initial goal and combined the acute and chronic reporter cassettes to generate reporter acute-chronice III (Fig 8). Apart from exchanging the "chronic"

promoter region, the fluorophores were swapped to fuse the stronger T3SS-promoter to the reporter with weaker signal intensity (*cerulean*) and *vice versa*. Moreover, upon identifying the highly homologous sequences of *venus* and *cerulean* as weak points during previous cloning attempts, *venus* was exchanged for *aphrodite,* a codon-diversified version. For the same reason one of the two terminator pairs (T_0, T_1) was abandoned. Using this strategy we successfully completed the first version of the dual Gac/Rsm responsive reporter tool ☺ (Fig 8) and the corresponding expression patterns completely agreed with our expectations (Fig 12). In the absence of RsmA expression of the "chronic" reporter (P_{PA0277}, red) is very high whereas the activity of the "acute" promoter is minimal (P_{T3SS}, green). The opposite holds true for a strain lacking both sRNAs. Furthermore, comparing wild-type cells grown under T3SS-inducing (LB + 5mM EGTA, Fig 12, left) and non-inducing (LB, Fig 12, right) conditions demonstrates that, in accordance with literature reports, calcium-poor environments trigger the expression of the T3SS.

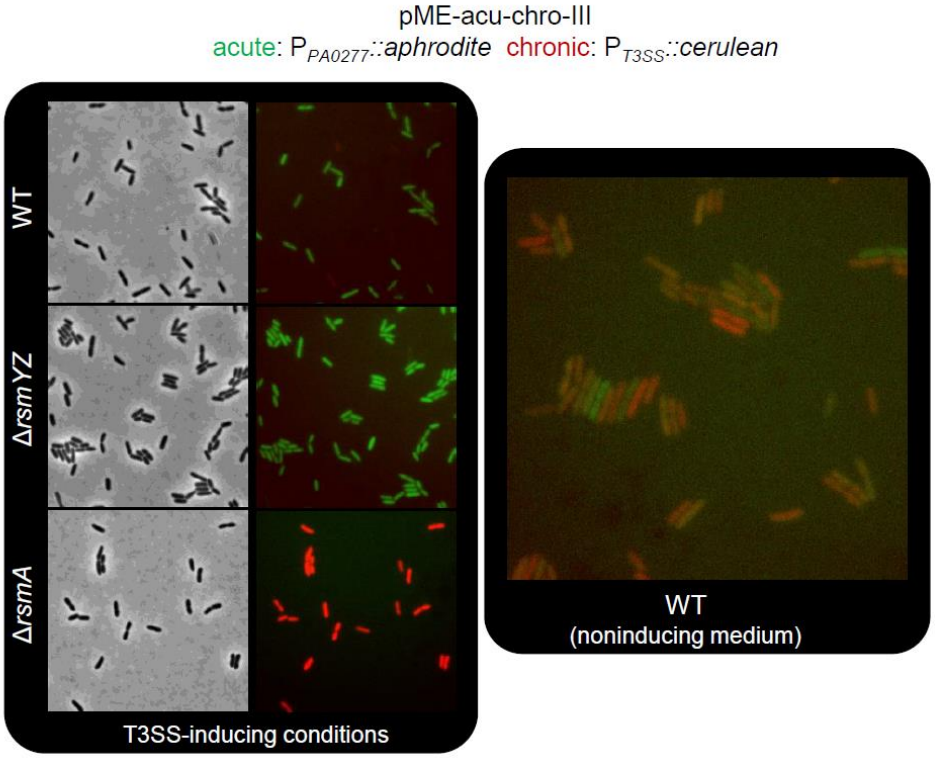

Fig 12. Expression profile of dual acute-chronic reporter III
Fluorescence microscopy images of *P. aeruginosa* strains (WT, *ΔrsmYZ*, Δ*rsmA*) transformed with acute-chronic III reporter (acute: P_{PA0277} ::*aphrodite* and chronic: P_{T3SS}::*cerulean*). Left: cells grown in LB +5 mM EGTA, right: cells grown in LB. Phase and overlay of CFP and YFP channels is displayed. Except for Δ*rsmA* where the YFP-intensity was lowered for more convenient display, all images are adjusted to the same level.

Based on the dual acute-chronic III reporter, several other variants were constructed leading to improved signal strength or increased combinatorial possibilities. Swapping the fluorophores via the inbuilt restriction sites resulted in the acute-chronic IV reporter and acute-chronic V codes for a *gfp-mcherry* version. Representative images of the latter are depicted in Fig 13, which show the same RsmA-dependent expression profile as observed for acute-chronic III. Even though full derepression of the Gac/Rsm cascade seems to cause the formation of mCherry inclusion bodies (Fig 13) no impact on cell growth or viability was observed.

These different reporters provide useful tools to study the behavior and expression profiles of single cells. However, the signal intensity of the fluorescent reporters is too weak to assess expression profiles at the population level in a plate reader. Therefore, we exchanged the *gfp*-cassette of the acute-chronic V reporter with the luciferase genes *luxCDABE* yielding dual reporter version VI. Luminescence-based readouts are more sensitive and, because *P. aeruginosa* lacks background luminescence, allow the detection of also weak signals. Moreover, automated acquisition of absorbance, fluorescence and luminescence in short intervals over the entire growth curve provides a powerful tool generating highly reproducible results.

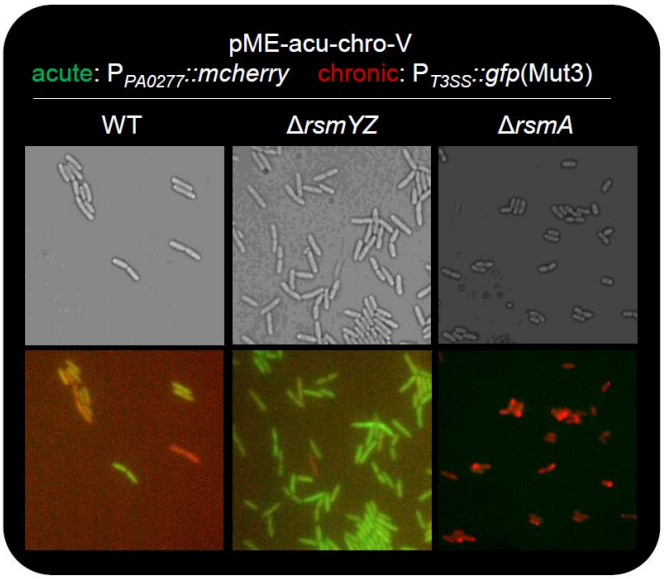

Fig 13. Expression profile of dual acute-chronic reporter V
Fluorescence microscopy images of *P. aeruginosa* strains (WT, Δ*rsmYZ*, Δ*rsmA*) transformed with acute-chronic V reporter (acute: P$_{PA0277}$ ::*mcherry* and chronic: P$_{T3SS}$::*gfp*(Mut3)). Cells were grown in T3SS-inducing conditions (LB +5 mM EGTA). Phase and overlay of GFP and mCherry channels is displayed. Except for Δ*rsmA* where the RFP-intensity was lowered for more convenient display, all images are adjusted to the same level.

Taken together, we successfully constructed dual Gac/Rsm-responsive reporter tools, which warrant expression analysis both of cell populations and of single cells. These tools were built to achieve the goal of this thesis: unraveling the regulatory modules underlying the Gac/Rsm cascade and studying the adaptation processes *in vivo* using clinical isolates (see next chapter).

4 PAPER DRAFT

A calcium-responsive kinase induces the acute-to-chronic lifestyle switch in *Pseudomonas aeruginosa*

(Manuscript in preparation)

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Keywords: *P. aeruginosa,* cystic fibrosis, calcium, Gac/Rsm cascade, LadS

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4.1 Abstract

Chronic *Pseudomonas aeruginosa* infections are the leading cause of morbidity in cystic fibrosis (CF) patients. Disease progression is typically associated with the loss of motility and virulence and the emergence of biofilm formation. This acute-to-chronic lifestyle switch is governed by the global family of Csr/Rsm posttranscriptional regulators. The activity of RsmA is regulated by an intricate network assembled around the central Gac two-component system and several associated histidine kinases. Using Gac/Rsm-responsive dual reporter systems we showed that calcium stimulates the signaling cascade via the action of the associated hybrid histidine kinase LadS. Activation of LadS by calcium ions depends on the periplasmic DISMED2 domain and on the histidine kinase activity. Moreover, calcium-sensing by LadS is not a general feature of *Pseudomonas* species but rather represents an adaptation to the lifestyle of *P. aeruginosa*. Based on this and on the observation that most clinical isolates from CF airways remain calcium-responsive we propose that elevated calcium levels, which are commonly found in CF lungs, can act as trigger to induce the acute-to-chronic virulence switch during persistent *P. aeruginosa* infections of CF airways. These results add to the growing body of evidence suggesting that calcium signaling plays an important role not only in eukaryotic but also in prokaryotic cells.

4.2 Introduction

Calcium is a well-established signaling molecule with broad implications in the regulation of eukaryotic cell physiology. The importance of calcium signaling in prokaryotes was long underestimated, however various reports over the past years suggest that calcium also interferes with diverse bacterial processes. Calcium is involved in heterocyst differentiation [1], spore formation [2], [3], fruiting body formation [4] and chemotaxis [5]. Bacteria maintain cellular calcium homeostasis and establish a steep gradient over the cell membrane with roughly four orders of magnitude lower intracellular calcium levels (100 - 300 nM) as compared to the periplasmic and extracellular fluid [6], [7]. This is very similar to eukaryotes and is achieved through strictly controlled influx and efflux mechanisms and high cytosolic buffering capacity. The latter is responsible for the fact that only a fraction of the total calcium is present as free ions [8]. As calcium readily precipitates phosphate it is crucial to be meticulously excluded from the cytosol to avoid cellular intoxication. Moreover, a concentration gradient along the membrane is a prerequisite for calcium signaling. Direct calcium-binding and a causal functional relationship with calcium signaling could be demonstrated for only a handful of proteins (summarized in several reviews [9]–[11]). Recently, an EF-hand-like protein called EfhP was identified in *P. aeruginosa,* which plays an important role in calcium homeostasis, biofilm formation and resistance to oxidative stress [12]. The EF-hand motif is the largest and best characterized group of calcium-binding motifs detected across all kingdoms of life [13]. Moreover, calcium was shown to regulate swarming motility, resistance against antibiotics and protease IV production in *P. aeruginosa* [7], [14], [15].

P. aeruginosa is the leading cause of morbidity and mortality in people suffering from cystic fibrosis (CF). CF is an autosomal recessive disorder affecting roughly one out of 4’000 newborns in Europe and is caused by mutations of the CF transmembrane conductance regulator (CFTR) [16]. Loss of

CFTR leads to mucus accumulation in the lungs, which impairs the ciliary clearance function leading to 60-70% of the patients airways being infected with *P. aeruginosa* by the age of 20 years [17]–[19]. Early stages of disease usually carry the signature of acute infections, which are associated with motile planktonic cells expressing a diverse set of virulence factors such as effectors of the type 3 secretion system (T3SS). Prolonged infection of CF lungs triggers an adaptation process towards reduced virulence and increased biofilm formation [20], [21]. Biofilm communities are a hallmark of chronic infections and serve as protective strategy against the host immune system and the constant exposure to antibiotics (reviewed in [22], [23]). While the molecular details underlying the acute-to-chronic transition of *P. aeruginosa* are still poorly understood, several regulatory components involved in this switch were identified recently. Among them is the ubiquitous second messenger bis-(3',5')-cyclic dimeric guanosine monophosphate (c-di-GMP). While high levels of c-di-GMP promote biofilm formation, low levels are associated with a motile and more virulent lifestyle (reviewed in [24], [25]). Likewise, the Gac/Rsm global signaling cascade is of central importance for the switch between acute and chronic infections [26]. GacS/GacA is a conserved two-component system (TCS) positively controlling the expression of two small RNAs (RsmY and RsmZ), which in turn bind to and inactivate the translational regulator RsmA (see overview in Figure 1A). RsmA directly represses genes involved in the community lifestyle including diguanylate cyclases [27], exopolysaccharide components (pel and psl), antibiotic factors like hydrogen cyanide, hydrolytic enzymes or type 6 secretion (T6S) as well as quorum sensing (QS) systems [26], [28]–[31]. In contrast RsmA stimulates the expression of acute virulence traits such as T3SS and type IV pili (T4P) [29], [26]. LadS and RetS, two associated histidine kinases (HKs) inversely regulate the Gac/Rsm cascade. LadS acts as an activator and RetS as a repressor of GacA activity. Both belong to the family of hybrid sensor HKs, which harbor next to the kinase domain a C-terminal extension in the form of a receiver domain harboring a conserved aspartic acid residue. As phosphotransfer strictly alternates between histidine and aspartic acid residues, hybrid HKs are thought to rely on external histidine phosphotransfer modules in order to activate their cognate response regulators (RRs) [32]. Moreover, both proteins harbor a 7TMR-DISMED2 periplasmic sensor domain that shares homology to carbohydrate-binding modules, which are an integral part of carbohydrate-active enzymes, such as glycosidase hydrolases [33], [34]. The DISMED2 domain was discovered by an *in silico* approach to identify bacterial multipass-membrane receptors analogous to eukaryotic G-protein-coupled receptors (GPCRs) [35]. RetS fulfills its regulatory function via direct interaction with GacS and independent of its conserved phosphorelay residues [36] [37]. How LadS mechanistically stimulates the Gac/Rsm cascade is so far unknown, however LadS is thought to act upstream of RetS as simultaneous deletion of both hybrid HKs phenocopies a *retS* mutant [30]. Interestingly, the increased virulence of the *P. aeruginosa* reference strain PA14 was shown to partially result from a mutation in *ladS,* which causes elevated T3SS activity, increased cytotoxicity and poor biofilm formation. Although PA14 represents the largest clonal group worldwide, *ladS* is intact in all other *P. aeruginosa* isolates tested [38], [39]. While this supports the notion that LadS downregulates the acute virulence behavior of *P. aeruginosa,* it raises questions about the specific signals recognized by LadS and the ecological niches in which LadS is operative. One of the biggest challenges in deciphering the role of phosphorylation pathways is the identification of activating input signals. The GacS network was shown to respond to a signal associated with high

cell density as supplementing the growth medium with signal extracts prepared from stationary phase cultures stimulated the Gac/Rsm signaling cascade. The exact nature of the signal remains unclear, however it is unrelated to any known QS molecules and seems to be rather ubiquitous as also signal extracts of distantly related species stimulated the Gac/Rsm cascade [40]. More recently, a signal associated with kin cell lysis was shown to activate the GacS pathway. While Rets is essential, LadS is negligible for signal perception and transmission [41].

Here we demonstrate that calcium induces the Gac/Rsm signaling cascade via the action of LadS. To our knowledge this is the first defined input signal for the Gac/Rsm cascade. Activation of LadS by calcium ions depends on the periplasmic DISMED2 domain and on an intact histidine kinase domain. Moreover, calcium-sensing by LadS is not a general feature of *Pseudomonas* species, but rather represents an adaptation to the lifestyle of *P. aeruginosa*. Based on this and on the observation that most clinical isolates from CF airways remain calcium-responsive, we propose that elevated calcium levels, which are commonly found in CF lungs [42]–[47], can act as trigger to induce the acute-to-chronic virulence switch during persistent lung infections.

4.3 Materials and Methods

Ethics statement

The clinical *Pseudomonas aeruginosa* isolates used in this study were cultured from patient samples collected for routine microbiological testing at the University Children's Hospital, Basel. Sub-culturing and analysis of bacteria was performed anonymously. No additional procedures were carried out on the patients. Cultures were sampled following regular procedures with written informed consent in agreement with the guidelines of the ''Ethikkommission beider Basel EKBB''.

Bacterial strains and plasmids

Strains used in this study are listed in Table S1. *P. aeruginosa* strains and *E. coli* DH5α were grown at 37°C, other *Pseudomonas spp.* at 30°C in either LB broth or one of the following minimal media: M9 medium supplemented with sodium-succinate (15 mM) or Ca^{2+}-free medium (0.1 M HEPES, 1.5 mM NaH_2PO_4, 1.5 mM K_2HPO_4, 18 mM NH_4Cl, 2 mM $MgSO_4$, 65 µM $FeSO_4 \cdot 7H_2O$, 6.3 µM $ZnSO_4 \cdot 7H_2O$, 8.3 µM $MnSO_4 \cdot H_2O$, 3.3 µM $CuSO_4 \cdot 5H_2O$, 0.9 µM H_3BO, 15 mM sodium-succinate, pH 6.9). If required, the latter was supplemented with 2.5 mM $CaCl_2$ (unless stated otherwise). Antibiotics were used at the following concentrations: for *E. coli* Amp 100 µg/ml, Gm 20 µg/ml, Tc 12.5 µg/ml and for *P. aeruginosa* Gm 30 µg/ml, Tc 100 µg/ml. For inducible plasmids IPTG was added to a final concentration of 0.5 – 1 mM.

Lysates of transducing phage E79tv2 [48] were used to construct *ΔgacS* and *ΔgacA* strains and were prepared and used as described previously [49]. Plasmids and oligonucleotides used in this study are listed in Table S1 and S2, respectively. Cloning was carried out in accordance with standard molecular biology techniques. Deletion constructs for *ladS*, *rsmA* and *psl* were produced by SOE-PCR using primers A-H and Ca-Cd and ligated between *Eco*RI and *Hin*dIII of pEX18-Tc or pME3087, respectively. Deletions were achieved by two-step allelic exchange. After transformation of the resulting vectors into PA01, tetracycline-resistant colonies were plated on 5%-sucrose plates to force the formation of double crossovers in case of *ladS* deletion [50]. The same protocol was followed to create Δ*retS* and Δ*pel.* To generate Δ*rsmA and* Δ*pelAΔpsl* strains counterselection was achieved by carbenicillin enrichment [51]. Briefly, individual tetracycline-resistant colonies were grown overnight in LB. In the morning cultures were diluted 1:100 into fresh medium. After 2 h tetracycline [20 µg/ml] was added to inhibit growth of cells which have lost the tetracycline-cassette. After incubation for 1 h carbenicillin [2 mg/ml] was added

to select against growing bacteria. Cells were incubated for another 4 – 6 h before being harvested by centrifugation, washed in LB and used to set-up a fresh overnight culture to start a new round of counterselection. After completing the second round, dilution series were plated onto LB agar plates. In all cases, tetracycline-sensitive colonies were tested for successful deletion by colony PCR.

The plasmid pME-acu-chro was created in three steps. First the *luxCDABE* cassette was amplified from pUC18T-mini-Tn*7*T-lux using primers Af and Ag, followed by digest with *Sac*I and *Xho*I and ligation into pME6032 yielding pME-lux. Next, P_{T3SS} was amplified from genomic DNA (gDNA) of PA01 using primer pair I/J, followed by digest with *Bam*HI and *Hin*dIII and ligation into pME-lux yielding pME-P_{T3SS}-lux. Finally, the P_{PA0277}-mcherry-T_0,T_1 fragment was amplified using primer pair R/N from pME-acu-chro-4, phosphorylated and ligated into *Xho*I-digested and blunted pME-P_{T3SS}-lux, resulting in the final pME-acu-chro vector. Plasmids pME-acu-chro-3 and -4 were constructed by first amplifying the individual reporter cassettes via SOE-PCR. The following primers were used to create acute cassettes: P_{T3SS}-*cerulean*-T_0,T_1: I-N, P_{T3SS}-*gfp*(Mut3)-T_0,T_1: I, O-Q, M-N and chronic cassettes P_{PA0277}-*aphrodite*: R-U and P_{PA0277}-*mcherry*: R-S, V-W. The following templates were used for: the promoter regions: gDNA of PA01; *cerulean*: pCERC-2 [52]; *gfp*(Mut3): P_{cdrA}::*gfp*(Mut3) [53]; *aphrodite*: pRSET FLIPglu-600uDelta11 Ares-Aphrodite [54]; *mcherry*: pCWR336 [55] and the terminators T_0,T_1: pUC18T-mini-Tn*7*T-Gm [56]. Acute cassettes were digested with *Bam*HI and *Avr*II, chronic cassettes with *Avr*II and *Eco*RI and respective combinations were ligated into the *Eco*RI-*Bam*HI fragment of pME6032 yielding the final vectors. The plasmid pME-acu-chro-2 was created via *Hin*dIII digest of pME-acu-chro-3, religation and testing by PCR for swapped fluorophore orientation. The plasmids pME-P_{T3SS}-Ven and pME-P_{T3SS}-RBS-Ven were produced by SOE PCR using the following primers pairs (and templates) to amplify the individual components for translational or transcriptional fusions, respectively: P_{T3SS}: I/J or I/Bq (gDNA of PA01), *venus:* K/Bs or Br/Bs (pxVENN-2), T_0,T_1: M/Bt for both fusions (pUC18T-mini-Tn*7*T-Gm). Outside primers I/Bt were used to create final reporter fusions. The PCR products were phosphorylated and cloned into *Eco*RI-*Bam*HI-digested and blunted pME6032. The plasmids pME-P_{PA0277}-Ven and pME-P_{PA0277}-RBS-Ven were created by amplifying the promoter region using primer pair R/S from gDNA of PA01. The PCR product was phosphorylated, digested with *Hin*dIII and cloned into the *Bam*HI-digested, blunted and *Hin*dIII-digested fragment of pME-P_{T3SS}-Ven and pME-P_{T3SS}-RBS-Ven vectors, respectively. The plasmids P_{rsmY}::*mcherry and* P_{rsmZ}::*mcherry* were constructed by amplifying the respective promoter regions from gDNA of PA01 using primer pairs X/Y and Z/Aa and *mcherry* was amplified from pCWR336 using primers Ab/Ac. The promoter regions were digested with *Xba*I and *Sph*I and *mcherry* with *Sph*I and *Hin*dIII, followed by triple ligation with the *Xba*I-*Hin*dIII fragment of P_{cdrA}::*gfp*(Mut3). The plasmid P_{rsmY}::*mcherry*-Tc was created by exchanging the *aac1* cassette for the tetracycline cassette from pME6032 using λ-Red-mediated mutagenesis [57]. Briefly, the PCR product obtained by primers Ad/Ae was transformed into P_{rsmY}::*mcherry*-harboring AB330-competent cells and homologous recombination was induced by thermal shock. A version of P_{rsmY}::*mcherry* for chromosomal integration was produced by releasing the complete reporter cassette via *Not*I from P_{rsmY}::*mcherry,* blunting and ligation into SmaI-digested pUC18T-mini-Tn*7*T-Gm yielding the final pTn*7*-P_{rsmY}::*mcherry*. pTn*7*-*ladS*-FLAG was constructed by SOE-PCR using primers Ah-Aj followed by digest with *Bam*HI and *Hin*dIII and ligation into pUC18T-mini-Tn*7*T-Gm. Release of *ladS*-FLAG by digest with *Sac*I and *Kpn*I and ligation into pME6032 yielded pME-*ladS*-FLAG. Different *ladS* mutations were introduced by SOE-PCR with Ak/Al as outside primers and pME-*ladS*-FLAG as template. The following internal primers were used: H428A: Am/An; D718N: Ao/Ap; D718E: Aq/Ar; ΔDISM2: As/At; $_{DISM2}$QL: Au/Av; RG_{DISM2}: Aw/A. pME-*ladS*-RG_{DISM2}-D80A-FLAG was created using the same outside primers, pME-*ladS*-RG_{DISM2}-FLAG as template and internal primers Ay/Az. Generally, after SOE-PCR the inserts were *Sac*I and *Kpn*I digested and ligated into pME6032. pTn*7*T-*ladS*-ΔREC-FLAG was constructed using primer Ak in combination with Ba / Aj followed by digest with *Bam*HI and *Hin*dIII and ligation into pUC18T-mini-Tn*7*T-Gm. Release of *ladS*-ΔREC-FLAG by digest with *Sac*I and *Kpn*I followed by ligation into pME6032 yielded pME-*ladS*-ΔREC-FLAG. pME-$ladS_{cyt}$-FLAG was constructed using primers Bb/Al and ligation via *Sac*I and *Kpn*I into pME6032. pME6032-FLAG was constructed

by amplifying 3x-FLAG from pME-*ladS*-FLAG using Bc/Bd and ligation via *Kpn*I and *Xho*I into pME6032. pME-*ladS*$_{Pseudomonas\ spp.}$-FLAG were constructed by amplifying *ladS* from their respective gDNA using primer pairs: Be/Bf (Pf-5), Bu/Bv (F113), Bw/Bx (Pf-01) and cloning via *Sac*I and *Kpn*I into pME6032-FLAG. *ladS*-SBW25 was constructed using primers By/Bz and cloning via *Bam*HI and *Kpn*I into pME6032. Different *ladS*-PA01/Pf-5 hybrids (HB1-HB5) were constructed as followed using either pME-*ladS*-FLAG (♦) or pME-*ladS*-Pf-5-FLAG (◊) as template. Primers to create final fusion are underlined. HB1: Be/Bh (◊) and Bg/Al (♦); HB2: Ak/Bj (♦) and Bi/Bf (◊); HB3: Ak/Bo (♦) and Bp/Bf (◊); HB4: Be/Bk (◊) and Bl/Al (HB2); HB5: Be/Bm (◊), Bn/Bo (♦) and Bp/Bf (◊). All fusions were digested with *Sac*I and *Kpn*I and ligated into pME6032-FLAG except for HB1 which was ligated into pME6032.

Growth curves and population-based promoter activity measurements

Liquid cultures were grown in LB supplemented with the appropriate antibiotics. Clinical isolates were streaked on LB plates containing tetracycline (100 µg/ml). Resulting colonies were resuspended in LB and optical density of all strains was adjusted to 0.05. 96 flat bottom clear plates (Falcon) (or in case of luminescent reporters: 96-well clear bottom black side plates (Costar)) pre-filled with 190 µl of the desired medium were inoculated with 10 µl of the individual cultures in triplicates (final OD_{600} 0.0025) and fluorescence (mCherry: 580 nm / 620 nm; YFP: 513 nm / 559 nm), luminescence and OD_{600} were recorded every 30 min for 20 h using a Synergy 2 (or H4) plate reader (Biotek). The data were analyzed using GraphPad Prism by integrating the area under the recorded curves and subsequent normalization for growth. All assays were repeated at least twice.

Timelapse microscopy

Microscopy analysis of cells carrying the pME-acu-chro-2 reporter plasmid was performed using a Olympus IX71 microscope equipped with an UPlanSApo 100×/1.40 oil objective (Olympus) and a coolSNAP HQ2 (Photometrics) charge-coupled device camera in a temperature-controlled chamber set to 30°C. Cells were cultured in either calcium-rich (LB with 10 mM $CaCl_2$) or calcium-poor medium (LB with 5 mM EGTA / 20 mM $MgCl_2$) until mid-log phase and imaged on 1.2% agar pads containing appropriate supplements (low calcium pads with 5 mM EGTA / 20 mM $MgCl_2$ and high calcium pads with 10 mM $CaCl_2$). Images were acquired every 20 min and processed with softWoRx version 3.3.6 and ImageJ 1.47h.

Immunoblot analysis

Protein levels were generally analyzed directly after completion of plate reader measurements. Cells were resuspended in SDS sample buffer at OD 1 and separated on SDS-PAGE gels (7.5%). Proteins were blotted onto polyvinylidene difluoride (PVDF) membranes (Millipore) using a Trans-Blot® SD Semi-Dry Transfer Cell (BioRad) at constant voltage (25V) for 30 min. Membranes were blocked for 1 h with 5%-milk-PBS-Tween at room temperature followed by overnight incubation with primary antibodies (α-FLAG (1:5'000; Sigma) and α-RNA-Pol β (1:7'000; BioLegend) as loading control) at 4°C. Primary antibodies were detected with HRP-coupled α-mouse antibody (1:10'000; Dako). Chemiluminescence was detected by ECL reagent (LumiGlo or LumiGlo Reserve, KPL), prepared according to manufacturer's instruction and imaged using a ImageQuant LAS-4000 system (Fujitsu Life Sciences).

Quantification of LadS ~P and GacS ~P levels in vivo

Strains were grown until early exponential phase in either calcium-poor (LB with 5 mM EGTA / 20 mM $MgCl_2$) or calcium-rich medium (LB with 10 mM $CaCl_2$). Volumes corresponding to 1 - 2 ml à OD 0.25 were collected and washed once with PBS. The cell pellets were resuspended in 60 µl lysis buffer (10 mM Tris-HCl, one tablet Phos-STOP (Roche), one spatula tip DNAse (Roche), pH 7.5). 40 µl 10% SDS was added followed by incubation for 6 min at room temperature. Lysates were diluted into SDS sample buffer and analyzed by SDS–PAGE gels (7.5%)

supplemented with 50 mM Phos-tag acrylamide (Wako) and 100 mM $MnCl_2$. Gels were run at 4°C at 100V for 5 h. Before immunoblotting the gels were incubated for 20 min in transfer buffer (1x Tris-Glycine, 20% ethanol, 0.03% SDS) containing 5 mM EDTA and for another 20 min in transfer buffer without EDTA. Proteins were blotted onto PVDF membranes in a wet-tank system at constant voltage (100V) for 1 h. The following steps are similar as outlined above.

Sample preparation for proteomics analysis

Strains of interest (Δ*ladS* overexpressing either *ladS* or mutant *ladS*-H428A) were grown in either Ca^{2+}-free medium or Ca^{2+}-free medium supplemented with 2.5 mM $CaCl_2$ until late logarithmic/early exponential growth phase. $2x10^9$ cells were lysed in 50 µl lysis buffer (2% sodium deoxycholate (SOC), 0.1 M ammoniumbicarbonate (ABC)) and disrupted by two cycles of sonication for 20s (Hielscher Ultrasonicator). Protein concentration was determined by BCA assay (Thermo Fisher Scientific). Proteins were reduced with 5 mM TCEP for 10 min at 95°C, alkylated with 10 mM iodoacetamide for 30 min in the dark at room temperature. Samples were diluted with 0.1 M ABC solution to a final concentration of 1% SOC before digestion with trypsin (Promega) at 37°C overnight (protein to trypsin ratio: 50:1). After digestion, the samples were supplemented with TFA and HCl to a final concentration of 0.5% and 50 mM, respectively. Precipitated SOC was removed by centrifugation (15 min, 4°C at 14'000 rpm). Peptides were desalted on C18 reversed phase spin columns according to manufacturer's instruction (Macrospin, Harvard Apparatus), dried under vacuum and stored at -80°C until further processing.

LC-MS analysis and label-free quantification

1 µg of peptides of each sample was subjected to LC–MS analysis using a dual pressure LTQ-Orbitrap Elite mass spectrometer connected to an electrospray ion source (both Thermo Fisher Scientific) as described recently [58] with a few modifications. Briefly, peptide separation was carried out using an EASY nLC-1000 system (Thermo Fisher Scientific) equipped with a RP-HPLC column (75 µm × 45 cm) packed in-house with C18 resin (ReproSil-Pur C18–AQ, 1.9 µm resin; Dr. Maisch GmbH, Ammerbuch-Entringen, Germany) using a linear gradient from 95% solvent A (0.15% formic acid, 2% acetonitrile) and 5% solvent B (98% acetonitrile, 0.15% formic acid) to 28% solvent B over 60 min at a flow rate of 0.2 µl/min. The data acquisition mode was set to obtain one high resolution MS scan in the FT part of the mass spectrometer at a resolution of 240'000 full width at half-maximum (at m/z 400) followed by MS/MS scans in the linear ion trap of the 20 most intense ions using rapid scan speed. The charged state screening modus was enabled to exclude unassigned and singly charged ions and the dynamic exclusion duration was set to 30 s. The ion accumulation time was set to 300 ms (MS) and 25 ms (MS/MS).

For label-free quantification, the generated raw files were imported into the Progenesis LC-MS software (Nonlinear Dynamics, Version 4.0) and analyzed using the default parameter settings. MS/MS-data were exported directly from Progenesis LC-MS in mgf format and searched against a decoy database the forward and reverse sequences of the predicted proteome from *Pseudomonas aeruginosa* (NCBI, download date: 06/09/2011, total of 23,970 entries) using MASCOT (version 2.4.0). The search criteria were set as follows: full tryptic specificity was required (cleavage after lysine or arginine residues); 3 missed cleavages were allowed; carbamidomethylation (C) was set as fixed modification; oxidation (M) as variable modification. The mass tolerance was set to 10 ppm for precursor ions and 0.6 Da for fragment ions. Results from the database search were imported into Progenesis and the final peptide feature list and the protein list containing the summed peak areas of all identified peptides for each protein, respectively, were exported from Progenesis LC-MS. Both lists were further statically analyzed using an in-house developed R script (SafeQuant) and the peptide and protein false discovery rate (FDR) was set to 1% using the number of reverse hits in the dataset [58].

Drug susceptibility testing

Strains were grown overnight in LB. Cells were harvested by centrifugation and resuspended in 3 ml Ca^{2+}-free medium with 100 µM $CaCl_2$ at an OD_{600} of 0.12. Aliquots of dilutions (10^{-6}/10^{-7}) were plated for cfu counting (t = 0 h). Tobramycin (Teva Pharmaceuticals) was added to a final concentration of 20 µg/ml and cells were incubated shaking at 37°C. After 1 h, 3 h, 5 h and 7 h of drug challenge number of surviving cells were determined by cfu plating.

4.4 Results

4.4.1 Dual Gac/Rsm-responsive reporters as a tool to study the acute-to-chronic switch in *P. aeruginosa*

The Gac/Rsm signaling cascade is one of the key regulatory pathways involved in the acute-to-chronic switch in *P. aeruginosa* (Figure 1A). To study this transition both at the population and at the single cell level we used fluorescence/luminescence based promoter fusions of RsmA-regulated genes as a proxy for Gac/Rsm activity. Translational fusions to *popN* and *PA0277* were used as readouts for acute (green) and chronic (red) infections, respectively. While *popN* is implicated in the regulation of T3SS activity, which is positively controlled by RsmA, *PA0277* is a putative Zn-dependent protease, and underlies strong negative control by RsmA [26], [29], [31]. Reporter fusions were combined on the same plasmid with promoters pointing in opposite directions to avoid interference. Reporter functionality was validated using different Gac/Rsm-cascade mutants (Figure 1B). Mutations causing constitutive repression of the pathway such as deletions of the small RNAs *rsmY* and *rsmZ* or of *gacA* coding for the central response regulator, strongly induced expression of the "acute" marker and lead to downregulation of the "chronic" reporter. In contrast, deleting *rsmA* strongly induced the expression of the "chronic" marker, while repressing the activity of the "acute" reporter. From this we concluded that our reporter fusions serve as accurate readouts for the activity of the Gac/Rsm cascade.

4.4.2 Calcium inversely regulates expression of the acute-chronic reporter

Chelation of calcium ions is commonly used *in vitro* to induce the T3SS [59]. In line with these reports, we found that supplementing M9 medium with EGTA strongly enhanced the "acute" reporter activity. However, unexpectedly we also observed a significant decrease in "chronic" marker expression (Figure 1C, left). Even though EGTA potentially chelates a variety of different cations, only calcium was able to restore the expression profile recorded in the absence of EGTA. Moreover, the EGTA-imposed expression profile was mirrored using a Ca^{2+}-free minimal medium supplemented with $CaCl_2$ (Figure 1C, right).

The calcium-induced switch in gene expression is also observable at the single cell level. Bacteria were grown in liquid broth under high or low calcium conditions until mid-log phase and subsequently imaged on low or high calcium agar pads, respectively (Figure 1D, green = "acute", red = "chronic"; Movies S1 and S2). The switch occurs rapidly and is established after 2 – 3 hours. Of note, "chronic" marker expression was homogeneous throughout the population, whereas only a subpopulation of the cells showed T3SS promoter activity. Similar observations were made previously [60].

Next we aimed at investigating how calcium is sensed by the Gac/Rsm system and at identifying essential components for the calcium-induced lifestyle switch. First, deletions of core Gac/Rsm components were tested for their ability to respond to calcium. Irrespective of the calcium levels, strains lacking *gacA* or the small RNAs were unable to activate the cascade in response to calcium and induce the expression of the "chronic" marker (Figure 1E). In contrast, the "acute" marker retains its ability to respond to calcium even in the absence of *gacA* and *rsmYZ,* demonstrating that an assembled T3SS machinery autonomously responds to low-calcium conditions via the secretion-induced partner-switching mechanism [61]. However, RsmA is a prerequisite for a functional T3SS.

Consequently, none of the reporters shows a calcium-dependent expression profile in a *rsmA* mutant (Figure 1E). Overall, this demonstrates that all core components of the Gac/Rsm cascade are essential for the calcium-induced lifestyle switch.

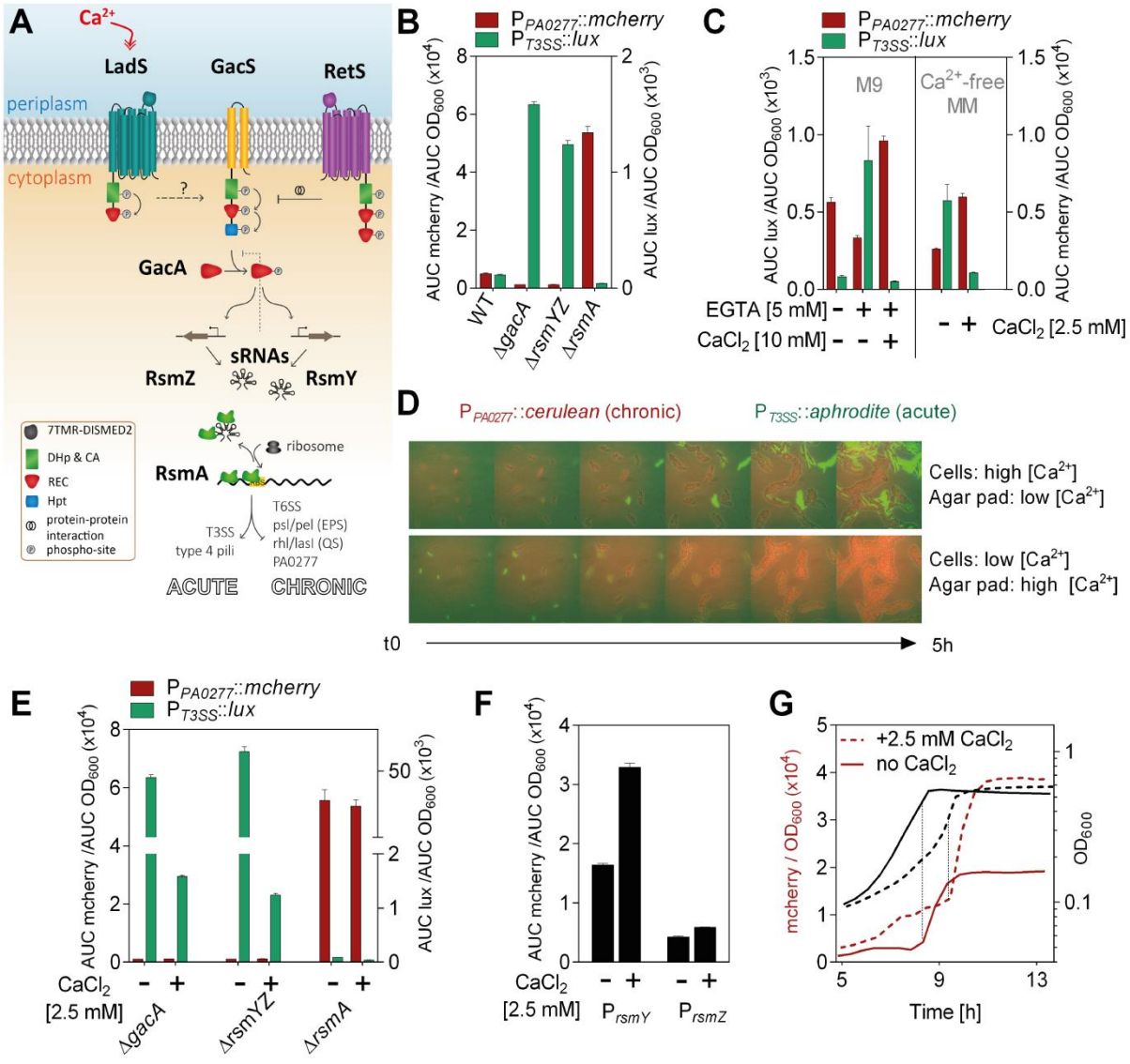

Figure 1. Calcium stimulates the activity of the Gac/Rsm cascade
(A) Schematic representation of the Gac/Rsm signaling cascade. (B) Expression profiles of the pME-acu-chro reporter (acute: P_{T3SS}::*lux* and chronic: P_{PA0277}::*mcherry*) in strains carrying deletions in Gac/Rsm associated genes. (C) Expression profiles of the pME-acu-chro reporter in *P. aeruginosa* cells grown in M9 medium supplemented with EGTA (5mM) and $CaCl_2$ (20mM) (left) or in Ca^{2+}-free minimal medium (MM) supplemented with 2.5 mM $CaCl_2$ (right). (D) Fluorescence time-lapse microscopy of logarithmically growing *P. aeruginosa* cells harboring plasmid pME-acu-chro-2 (acute: P_{T3SS}::*aphrodite* and chronic: P_{PA0277}::*cerulean*). Cells were either cultured in calcium-rich medium and imaged on low-calcium agar pads (top) or vice versa (bottom). One frame per hour is depicted. (E) Expression profiles of the pME-acu-chro reporter (acute: P_{T3SS}::*lux* and chronic: P_{PA0277}::*mcherry*) in strains carrying deletions in Gac/Rsm associated genes under low and high calcium conditions. (F) Activity of *rsmY* and *rsmZ* small RNA promoter fusions under low and high calcium conditions. (G) P_{rsmY}::*mcherry* expression curves normalized for growth (red) and corresponding absorbance readings (black) of wild-type cells grown under low and high calcium conditions over time. (B-G) Strains were incubated overnight in a microplate reader at 37°C either in M9 medium (B, C (left)) or in Ca^{2+}-free MM (C (right), E-G). Absorbance, fluorescence and luminescence was measured every 30 min. Bars represent the area under the curve (AUC) normalized for growth. Standard deviations are indicated.

4.4.3 Calcium stimulates the activity of the Gac/Rsm cascade

In order to strengthen the emerging premise that calcium stimulates the activity of the Gac/Rsm cascade, we tested the effect of calcium on the promoter activity of the two small RNAs, *rsmY* and *rsmZ*. In line with our hypothesis we found that calcium significantly increases *rsmY* transcription and, although to a much lesser extent, *rsmZ* transcription (Figure 1F). Moreover, in agreement with RsmA being a translational repressor, transcription of the "chronic" marker *PA0277* was not affected by the presence of calcium (Figure S1). Closer examination of the *rsmY* promoter activity profile suggested that calcium-sensing is not restricted to a specific growth phase (Figure 1G). In the absence of calcium, the promoter activity of *rsmY* remains low in logarithmically growing cells and increases only when cells approach stationary phase. In contrast, when calcium is present in the medium *rsmY* expression shows a significant increase already during early logarithmic growth, which is retained in the stationary phase. Interestingly, cells grown in the presence of calcium show a prolonged lag phase compared to cells cultured in calcium-poor medium (Figure 1G). The transition from exponential to stationary growth is accompanied by a strong activation of the signaling cascade likely caused by the production of input signals associated with high cell density. Thus, calcium can override the repressed status of the Gac/Rsm cascade in growing cells, suggesting that its signaling mode is distinct from the density-related activation of the system.

4.4.4 LadS is essential for calcium-induced stimulation of the Gac/Rsm cascade

To uncover the calcium-sensing unit we expanded our analysis to the Gac/Rsm-associated kinases LadS, RetS and PA1611 and to the recently identified EfhP calcium-binding protein [12], [62]. Intriguingly, disruption of *ladS* rendered cells calcium-blind, as indicated by the acute-chronic dual reporter that directly probes RsmA activity (Figure 2A) and the *rsmY* promoter strength (Figure 2B). Similar to cells lacking *gacA* (Figure 1E) calcium is still able to repress the T3SS promoter activity to some extent, likely due to its specific autonomous control by calcium. Complementation with *ladS* under the control of its native promoter (Δ*ladS* att::*ladS*) fully restored calcium-sensitivity (Figure 2B). Moreover, gradually increasing LadS levels with an inducible copy of *ladS* stimulated *rsmY* promoter activity in calcium-rich medium but not in a medium lacking calcium (Figure 2B). Thus, LadS overexpression boosts the calcium response, strengthening the view that LadS itself is responsible for calcium-sensing. In agreement with this we found that GacS phosphorylation levels are strongly enhanced by calcium in a LadS-dependent manner (Figure 2C). Finally, Gac/Rsm stimulation was specific for calcium as none of the other divalent cations tested (Mg^{2+}, Mn^{2+}, Fe^{2+}, Zn^{2+}, Cu^{2+}) were able to elicit a similar concentration-dependent induction of the *rsmY* promoter activity (Figure 2D).

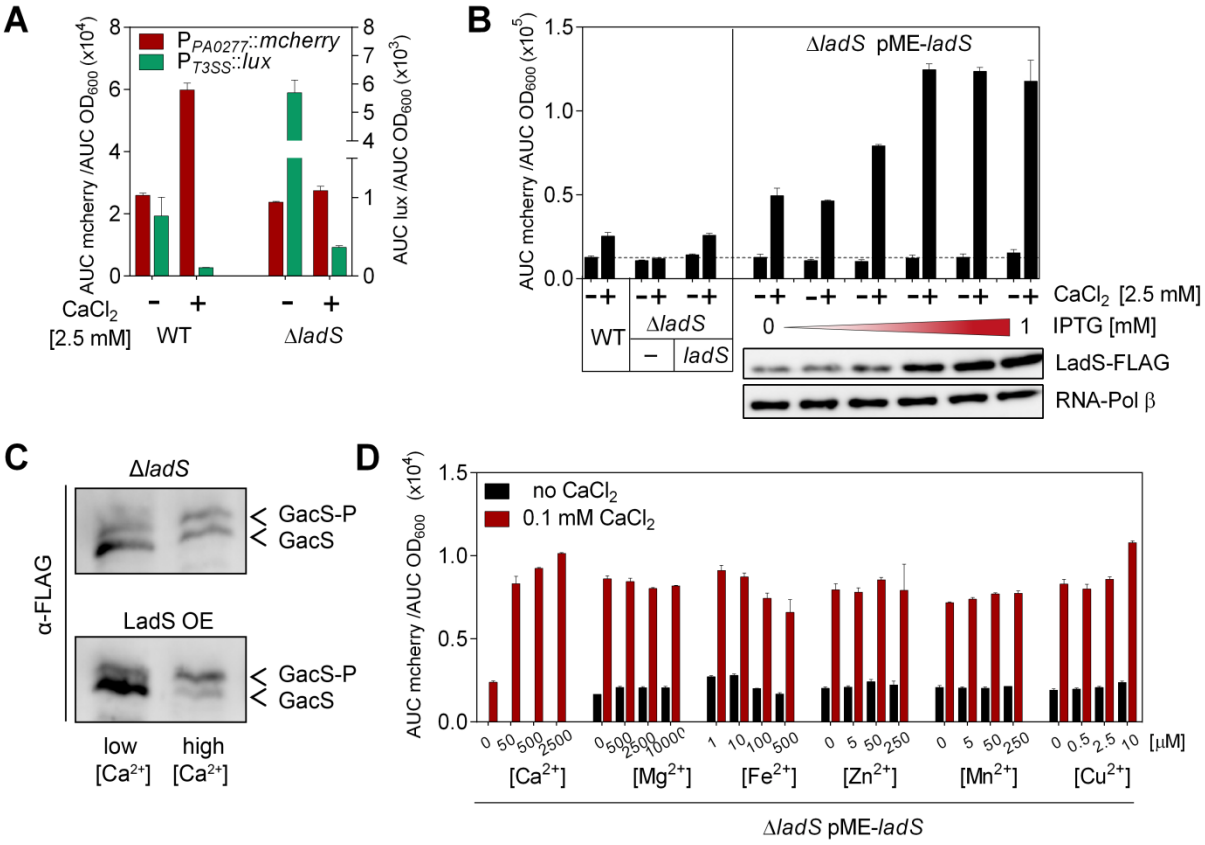

Figure 2. LadS is essential for calcium-mediated stimulation of the Gac/Rsm signaling cascade
(A) Expression profiles of the pME-acu-chro reporter (acute: P_{T3SS}::*lux* and chronic: P_{PA0277}::*mcherry*) in *P. aeruginosa* wild-type and Δ*ladS* strains grown under low and high calcium conditions. (B) Left: Activity of *rsmY* promoter in wild-type, Δ*ladS* and Δ*ladS att*::*ladS* strains at low or high calcium conditions. Right: Effect of calcium on *rsmY* promoter activity in strains expressing *ladS*-FLAG from an IPTG-inducible promoter. Increasing IPTG levels are indicated and immunoblot analysis with α-FLAG antibodies is shown below. RNA-Polymerase subunit β served as loading control. (C) Phos-Tag™ SDS-PAGE followed by immunoblot analysis (α-FLAG) showing GacS-FLAG phosphorylation under low and high calcium conditions in strains lacking (Δ*ladS*) or overexpressing *ladS*. Phosphorylated and non-phosphorylated GacS is indicated. (D) Effect of different cations on *rsmY* promoter activity. Δ*ladS* harboring a chromosomal P_{rsmY}::*mcherry* fusion and an IPTG-inducible plasmid-borne *ladS* copy was grown with increasing concentrations of the indicated cations in the presence (red bars) or absence of calcium (black bars). Strains were grown overnight in a microplate reader. Absorbance, fluorescence and luminescence was measured every 30 min. Bars represent the area under the curve (AUC) normalized for growth. Standard deviations are indicated.

4.4.5 Calcium-induced signal transduction requires the periplasmic DISMED2 domain and histidine kinase activity of LadS

LadS is a hybrid histidine kinase with conserved phosphorelay residues (H428 and D718) and harbors a 7TMR-DISMED2 periplasmic sensor domain, which is shared by only three additional *P. aeruginosa* proteins, RetS, PA3462 and NicD (PA4929). To identify essential features of LadS for calcium-sensing *rsmY* promoter activity was assessed in strains expressing different *ladS* mutant variants under low and high calcium conditions (Figure 3A). Deletion of the periplasmic DISMED2 domain as well as mutation of the conserved histidine residue (H428A) renders the cells calcium-blind. However, while the H428A substitution completely abolished *rsmY* expression, disruption of the DISMED2 domain led to constitutive *rsmY* promoter activity even in the absence of calcium, indicating that the domain usually restrains LadS function. Point mutations in the receiver domain D718N and D718E led to a

slight decrease in the overall transcriptional activity, but the receiver domain *per se* is negligible for calcium-sensing. The importance of the DISMED2 domain and the transmembrane helices is emphasized by a soluble form of LadS ($LadS_{cyt}$), which renders the protein instable. Evidence that calcium impacts LadS either directly or indirectly stems from mutations, which introduce two additional amino acids flanking either side of the DISMED2 domain ($_{DISM2}$QL and RG_{DISM2}) and strongly destabilize the protein in a Ca^{2+}-free medium (Figure 3A,B). Strikingly, in calcium-rich medium LadS-

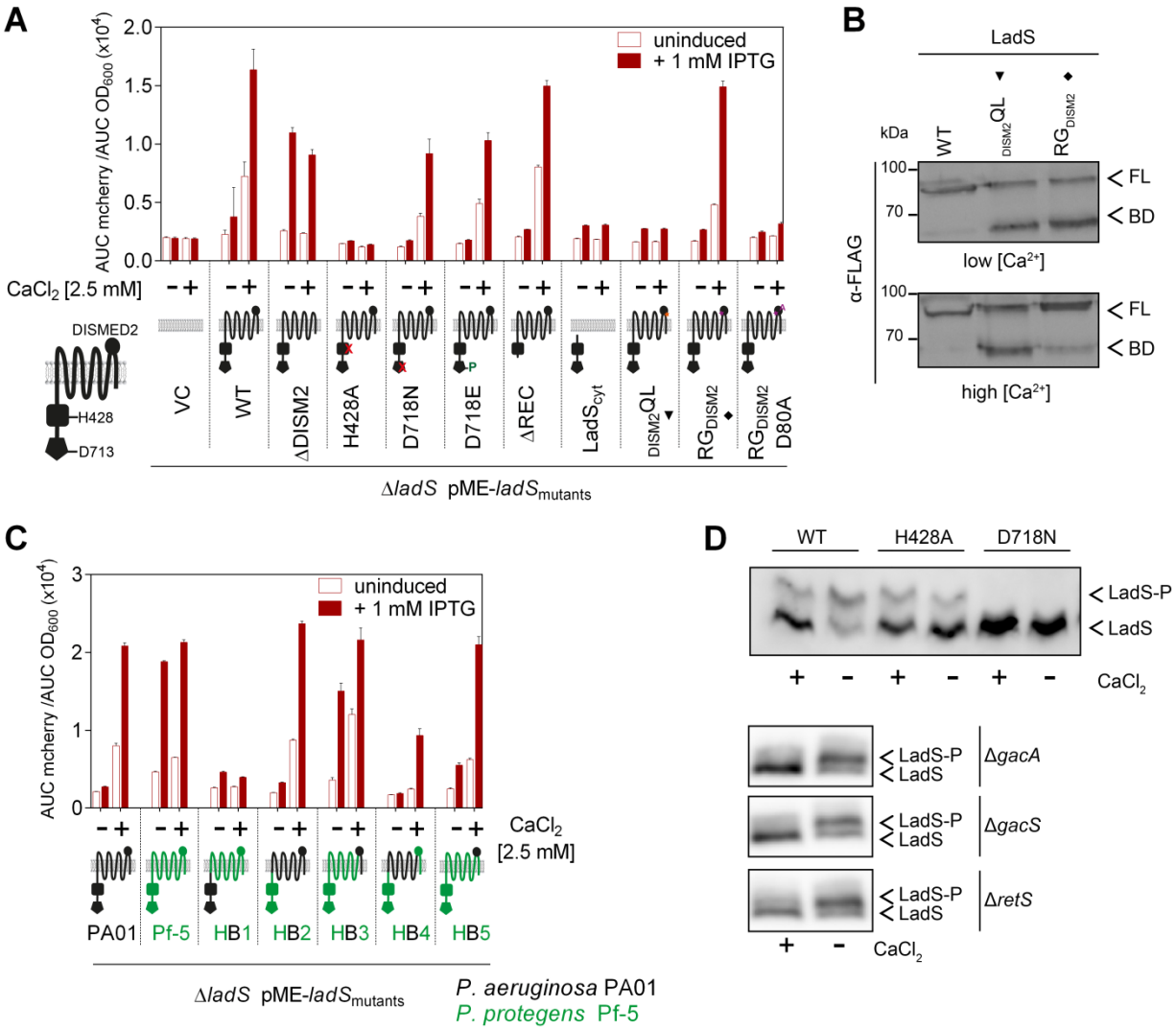

Figure 3. LadS calcium-sensing requires the periplasmic DISMED2 domain and histidine kinase activity
(A) Activity of the *rsmY* promoter in strains expressing different *ladS* mutants under low and high calcium conditions. All *ladS* alleles were plasmid-expressed in a Δ*ladS* mutant with the empty vector serving as control (VC). Mutant variants are outlined schematically below the graph. (B) Immunoblot analysis with α-FLAG antibodies of strains expressing wild-type and mutant *ladS*-FLAG ($_{DISM2}$QL and RG_{DISM2}) under low and high calcium conditions. Arrows mark full-length LadS (FL) and a breakdown product (BD). (C) Calcium-dependent activity of the *rsmY* promoter in strains expressing different *ladS* hybrid proteins. Hybrid proteins are indicated schematically below the graph. Black = *P. aeruginosa* PA01; green = *P. protegens* Pf-5. Strains were grown overnight in a microplate reader. Absorbance and fluorescence was measured every 30 min. Bars represent the area under the curve (AUC) normalized for growth. Standard deviations are indicated. (D) Phos-Tag™ SDS-PAGE followed by immunoblot analysis (α-FLAG) showing wild-type and mutant LadS-FLAG phosphorylation under low and high calcium conditions in different strain backgrounds. Phosphorylated and non-phosphorylated LadS is indicated.

RG_{DISM2} is stabilized and this effect coincides with calcium-induced expression of *rsmY* (Figure 3A). In contrast, calcium is unable to stabilize the LadS-$_{DISM2}$QL mutant form and a strain expressing this protein fails to respond to calcium. The same pattern is visible for constructs expressed in *E. coli* cells, indicating that the calcium-mediated stabilization is independent from *P. aeruginosa*-specific factors (Figure S4).

As illustrated by the EF-hand motif (DxDxDG), which is the most common calcium-binding motif, aspartic acid residues are typically implicated in calcium-binding [13]. Therefore, several aspartic acid residues in the periplasmic domain of LadS-RG_{DISM2} were replaced by alanine to identify a potential calcium binding site. One such mutation (D80A) completely abrogated both calcium-mediated induction of *rsmY* expression as well as protein stabilization (Figures 3A, S2). Altogether, these data define the LadS transmembrane helices and periplasmic DISMED2 domain as essential components of calcium-mediated signal transduction. Moreover, the data imply that while histidine kinase activity of LadS is crucial for calcium-sensing, its C-terminal receiver domain is not required for this process.

4.4.6 LadS-mediated calcium-sensing is specific for *P. aeruginosa*

The Gac/Rsm cascade with its associated kinases is highly conserved among different *Pseudomonas* species [63]. In order to address the question if calcium-sensing is a common feature we cross-complemented *P. aeruginosa* Δ*ladS* with *ladS* from *P. fluorescens* strains SBW25, Pf-01 and F113 and from *P. protegens* strain Pf-5 and scored for calcium-dependent *rsmY* promoter activity. Irrespective of the calcium levels, LadS from *P. protegens* strongly induced *rsmY* expression, which is dependent on its receiver domain and the catalytic histidine residue (Figure 3C, S3A). However, none of the *ladS* homologues restored calcium-sensitivity in *P. aeruginosa*. Likewise, overexpressing of the cognate *ladS* alleles in the individual *Pseudomonas* strains failed to promote calcium-dependent *rsmY* expression (data not shown). In contrast, expressing *ladS* of *P. aeruginosa* in *P. fluorescens* F113 restored calcium sensitivity of the Gac/Rsm pathway in this organism (Figure S3B). This strongly indicated that LadS itself is able to sense calcium and that this ability is specific to LadS from *P. aeruginosa.*

Next we made use of the above finding to further dissect the requirements of LadS to sense calcium. Hybrids were generated between LadS from *P. aeruginosa* PA01 (black) and *P. protegens* (Pf-5) (Figure 3C). Combining the transmembrane part of Pf-5 with the cytosolic domains of PA01 strongly reduced the constitutive expression of *rsmY* as found in Pf-5 wild-type, but failed to restore calcium-sensitivity (HB1). In contrast, a hybrid containing membrane and periplasmic portions of the PA01 protein and cytosolic domains from Pf-5 (HB2), responded to calcium like wild-type LadS from PA01. Moreover, grafting the DISMED2 domain of PA01 onto an otherwise wild-type LadS from Pf-5 is sufficient to completely restore calcium-sensing (HB5). Finally, intermediate phenotypes displayed by hybrids HB3 and HB4 suggested that the signaling capacity is not determined by the DISMED2 domain alone but may result from the interplay of this domain with residues in the neighboring transmembrane helices. Together, these data provide strong evidence for a central role of the periplasmic DISMED2 domain of LadS in calcium sensing and indicate that the ability to respond to calcium might be specific for *P. aeruginosa*.

4.4.7 Calcium affects LadS phosphorylation

The observation that the catalytic histidine H428 in the DHp domain of the histidine kinase, but not the D718 phosphoryl acceptor of the C-terminal receiver domain of LadS is required for calcium-mediated signal transduction led to the following hypothesis. LadS activation by calcium induces autophosphorylation on the conserved histidine residue however, signal transduction must occur in a receiver-domain independent manner. To test this we assessed LadS phosphorylation by Phos-Tag SDS-PAGE of cells grown under low and high calcium conditions. Contrary to our expectations, we found that cells grown in calcium-rich medium showed a smaller fraction of phosphorylated LadS as compared to cells grown at low calcium (Figure 3D, top). LadS-D718N showed no phosphorylation at all, which indicates that only aspartate phosphorylation is stable enough to be detected. LadS-H428A did not show any differential phosphorylation pattern, which is in line with the previously observed calcium-blind phenotype. Reduced phosphorylation of D718 under high calcium conditions (Figure 3D) would thus be an indicator for active LadS. Deletion of potential downstream components like *gacA, gacS* or *retS* did not influence the phosphorylation pattern (Figure 3D, bottom).

4.4.8 The LadS-mediated calcium regulon strongly overlaps with known RsmA-regulated targets

The global effect of calcium-induced LadS-dependent signaling was dissected by total proteome profiling comparing bacteria expressing wild-type *ladS* and the calcium-insensitive mutant *ladS*-H428A under high or low calcium conditions. Overall about 49% of the proteins encoded by *P. aeruginosa* were detected by this method. Calcium-induced changes for LadS and LadS-H428A are indicated by volcano plots in Figures 4A and B. The RsmA regulon has been defined by several transcriptomics studies [26], [29], [31] and we used these data sets to group the proteins regulated by calcium according to their underlying regulation by RsmA (green and red for positive and negative regulation, respectively) (Figure 4A,B). Strikingly, a strong overlap between these studies and our data was observed, illustrated by the fact that the majority of targets which are directly repressed by RsmA (red) are upregulated in the presence of calcium, while proteins that are under positive control by RsmA (green) are downregulated in the presence of calcium (Figure 4A). This includes components of the T3SS and T6SS as well as PA0277. In accordance with our previous findings LadS-H428A showed a substantially smaller fraction of calcium-regulated proteins as compared to wild-type LadS with most RsmA-repressed targets clustering around the y-axis. Direct comparison of the two data sets allows differentiating between LadS-independent and LadS-dependent calcium-regulated targets. For example, many components of the T3SS are downregulated in the presence of calcium independent from LadS. This is in agreement with the previously described secretion-induced partner-switching mechanism, which activates the T3SS expression in calcium-deprived environments [61]. One prominent class of proteins found to be strongly upregulated in both strains encodes for proteins involved in iron acquisition. This includes the *pvd* operon encoding the siderophore pyoverdine as well as different factors involved in heme degradation and uptake (*hemO, phu* system). This is most likely attributed to regulation by the calcium-binding protein EfhP, which has been shown to strongly induce the production of pyoverdine [12].

We also correlated our data to a previously published microarray study addressing calcium-induced changes in the context of T3SS regulation [64]. The data is summarized in Figure 4C in a qualitative heatmap for easier visualization. We considered only significantly regulated targets (net fold difference >1.4x) and compared them to components that showed the same net effect in terms of up- or downregulation under calcium-limiting conditions (EGTA and Ni-NTA) in the published data set. This resulted in the identification of 96 targets, 33 of which were down-regulated and 63 were up-regulated by calcium in our hands. Similarly, Wolfgang *et al.* had found upregulation of the same 63 genes and had identified 24 out of the 33 genes with decreased expression. 74 of the 96 genes were also detected in at least one of the transcriptomics studies targeting the RsmA regulon, with only 4 outliers showing inverse regulation as compared to our proteomics data (Figure 4C). The observed differences are likely attributed to posttranscriptional regulation, which remains undetected in microarray studies. Overall, these findings are in strong agreement with the notion that calcium via the action of LadS affects mainly RsmA-regulated targets.

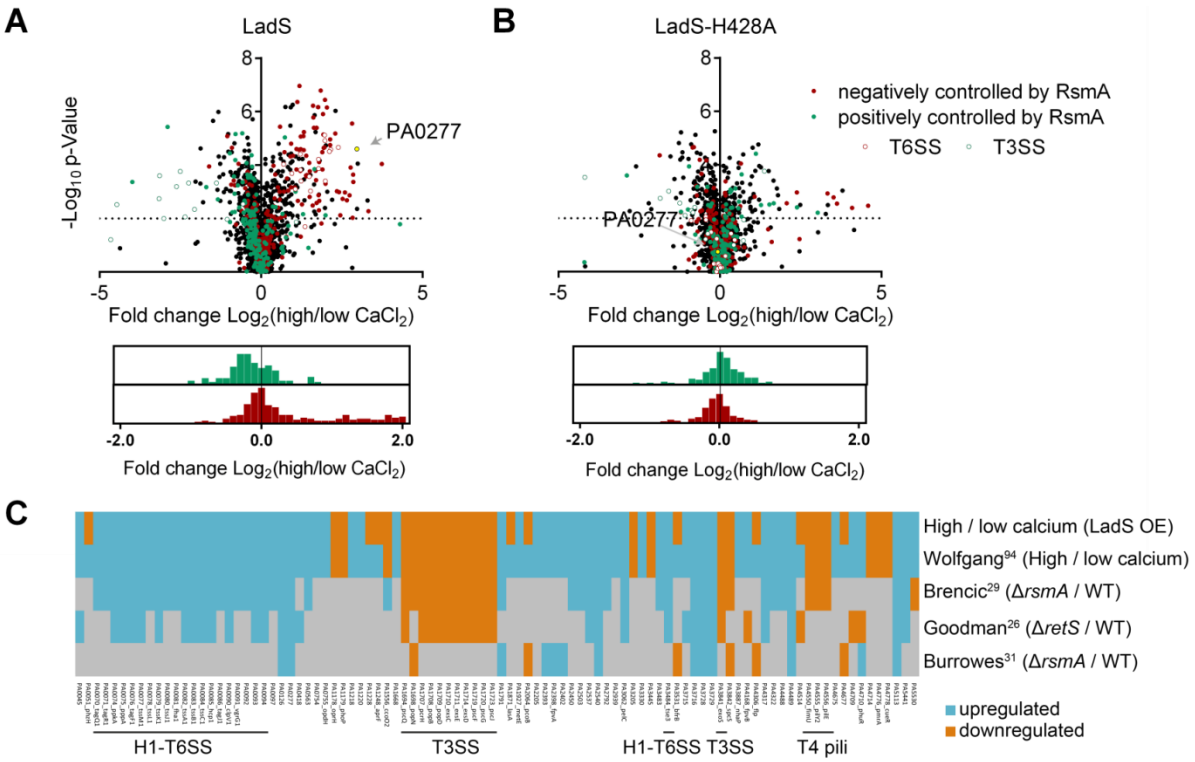

Figure 4. LadS-mediated calcium regulon overlaps with known RsmA-regulated targets
Volcano plot diagrams of differentially regulated proteins of strains expressing wild-type *ladS* (A) or *ladS*-H428A (B) grown to early stationary phase (OD ~ 0.9) in Ca^{2+}-free MM or Ca^{2+}-free MM supplemented with 2.5 mM $CaCl_2$. Total proteome was analyzed using LC-MS/MS. Experiments were performed in triplicates. Known RsmA-regulated targets identified by Burrowes *et al.* [31], Goodman *et al.* [26] and Brencic *et al.* [29] are indicated either in green (positive regulation by RsmA) or red (negative regulation by RsmA). T3SS and T6SS clusters are indicated by empty circles. The chronic marker PA0277 is indicated by an arrow. The dotted line indicates significance level p<0.01. (C) A qualitative heatmap comparing our data to previously published microarray data. Calcium-regulated genes assessed in two different media (TSB +5 mM EGTA vs. TSB+5 mM $CaCl_2$ and LB+10 mM NTA vs. LB+10 mM NTA+20 mM $CaCl_2$) in the context of T3SS regulation by Wolfgang *et al.* [97]. The RsmA regulon was defined in [31], [26], [29]. Data displayed were subjected to the following restrictions: net fold change >1.4x, p<0.05 (proteomics data) and identical trends in both media [98].

4.4.9 Activation of the Gac/Rsm cascade leads to reduced growth and increased drug tolerance

We observed that strains with a highly active Gac/Rsm cascade such as *retS* and *rsmA* mutants have a significantly longer lag phase and slower growth compared to wild-type bacteria. In contrast, strains harboring deletions in *gacS* or *rsmYZ* tend to grow slightly better (Figure 5A). Different survival rates were ruled out as the underlying cause for this observation since viable cell numbers, as determined by colony forming units (cfus), were similar in all cultures (data not shown). Overexpression of *rsmY* bypasses the effect of a *gacS* mutation and the growth profile of this strain resembles the one of an *rsmA* mutant (Figure 5A). As mentioned previously and in agreement with the above findings that calcium induces the Gac/Rsm cascade through LadS, we found that calcium induced a similar growth defect. Moreover, this is dependent on LadS and GacA (Figure 5B,C). Using calcium-mediated induction we could also show that the degree of the growth limitation correlates with the activity level of the Gac/Rsm cascade (Figure 3A). While calcium induced a strong growth defect in strains harboring wild-type LadS, this effect was completely abolished in the LadS-H428A mutant strain. In agreement with the reduced *rsmY* promoter activity compared to wild-type LadS, strains with point mutations in the LadS receiver domain (D718E, D718N) showed intermediate growth phenotypes (Figure 5C). Because an activated Gac/Rsm cascade is associated with the formation of strongly adherent cells, we wanted to exclude that cell clumping is responsible for this apparent growth limitation phenomenon. However, growth rates of mutants lacking both major exopolysaccharide systems, pel and psl, showed a similar calcium-induced growth defect (Figure 5B).

The molecular basis of Gac/Rsm induced growth variation is unclear. However, conditions reducing growth rates can be beneficial as they might enhance bacterial survival in response to environmental challenges like antibiotic treatment and by that may contribute to persistent chronic infections (reviewed in [65]). In order to test if activation of the Gac/Rsm cascade causes increased drug tolerance we performed a time-kill experiment. Stationary cells were diluted into fresh medium and at the same time challenged with 10 μg/ml tobramycin for increasing amounts of time. In line with our hypothesis we found that overexpression of *rsmY* in Δ*ladS* or Δ*pel*Δ*psl* strains leads to significantly increased survival rates as compared to control strains harboring empty vectors (Figure 5B). Elevated levels of LadS also caused increased survival, although to a lesser extent than the overexpression of *rsmY.* This is likely due to the fact that *rsmY* overexpression causes a stronger derepression of the signaling cascade compared to the upstream residing *ladS.* Ongoing experiments aim to address this point in more detail.

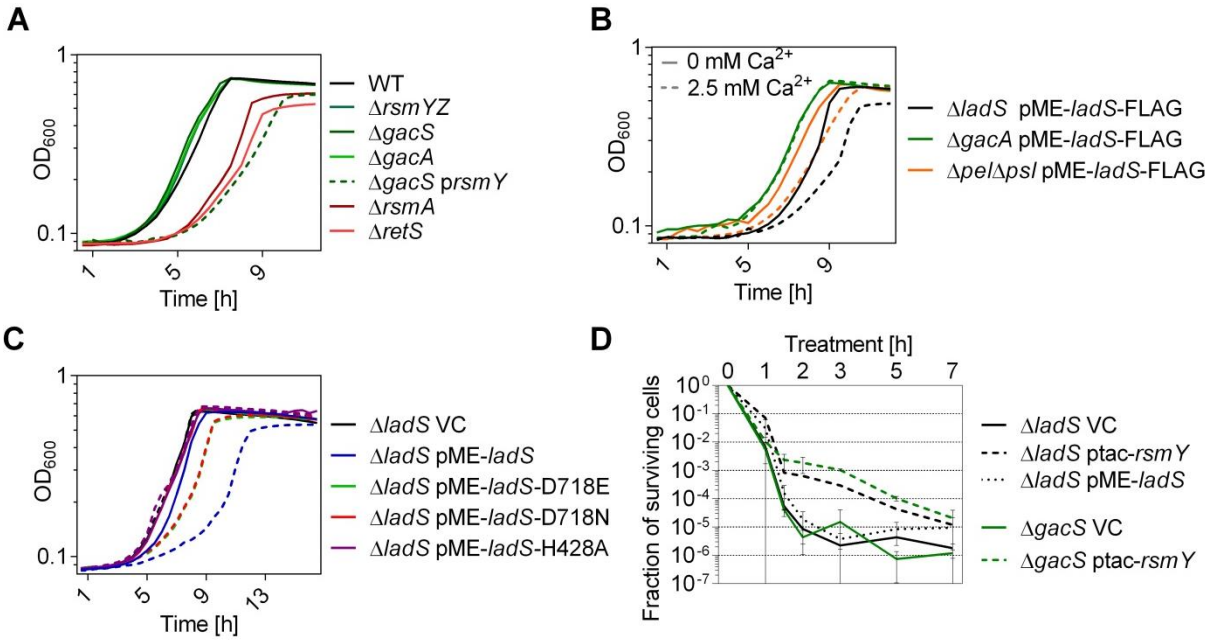

Figure 5. Activation of the Gac/Rsm cascade leads to reduced growth and increased drug tolerance of *P. aeruginosa*

(A-C) Growth curves of different strains in Ca^{2+}-free MM (continuous lines) or Ca^{2+}-free MM supplemented with 2.5 mM $CaCl_2$ (dotted lines). (B) All strains harbor plasmid pME-*ladS*-FLAG. (C) Δ*ladS* is transformed with different plasmids carrying *ladS* point mutant alleles as indicated. (D) Time kill experiment with 10 µg/ml Tobramycin using cultures diluted to OD 0.12. Samples were removed at indicated time points and plated for cfu counting. Graph shows fraction of surviving cells.

4.4.10 Calcium-sensing is retained in *P. aeruginosa* isolates from CF airways

The pathology of cystic fibrosis (CF) has been linked to a deregulated calcium homeostasis, leading to elevated calcium levels in different body fluids [43], [44], [46]. This observation, together with our findings that the Gac/Rsm pathway responds to calcium, suggested that LadS-mediated stimulation might contribute to chronic *P. aeruginosa* infections of CF airways. To gather evidence for this idea, we used the dual acute-chronic and the *rsmY* reporter to assay the effect of calcium on Gac/Rsm activity in twenty *P. aeruginosa* lung isolates from CF patients. Genome sequence analysis indicated that in the majority of analyzed strains the core components of the Gac/Rsm cascade are conserved and likely functional. In line with previous reports we find that expression of the T3SS is abolished in most clinical strains. Interestingly, calcium-sensitivity is retained in 17 out of 20 strains tested. Moreover, in almost all isolates “chronic” marker (*PA0277*) expression (Figure 6A) correlates with *rsmY* promoter activity (Figure 6B) indicating that the Gac/Rsm cascade and its sensitivity towards calcium is retained in the human host. A few outliers presented a different behavior. In response to calcium strain 147 induced the expression of *rsmY*, while the “chronic” marker remained at background levels. This might be due to mutations specifically affecting *PA0277* transcription. Furthermore, strains 129, 368 and 388 are insensitive to calcium. Strain 388 was shown to harbor a single nucleotide deletion in *ladS* (position 342) leading to a frameshift and disruption of the

cytoplasmic domains. For strains 129 and 368 no obvious candidate mutations were identified provoking this calcium-blind phenotype. Overall our findings suggest that there is selective pressure in the human host for *P. aeruginosa* to retain a functional Gac/Rsm regulatory system and that elevated calcium levels in lungs of CF patients might facilitate chronic behavior of *P. aeruginosa* through the activation of the LadS sensor histidine kinase.

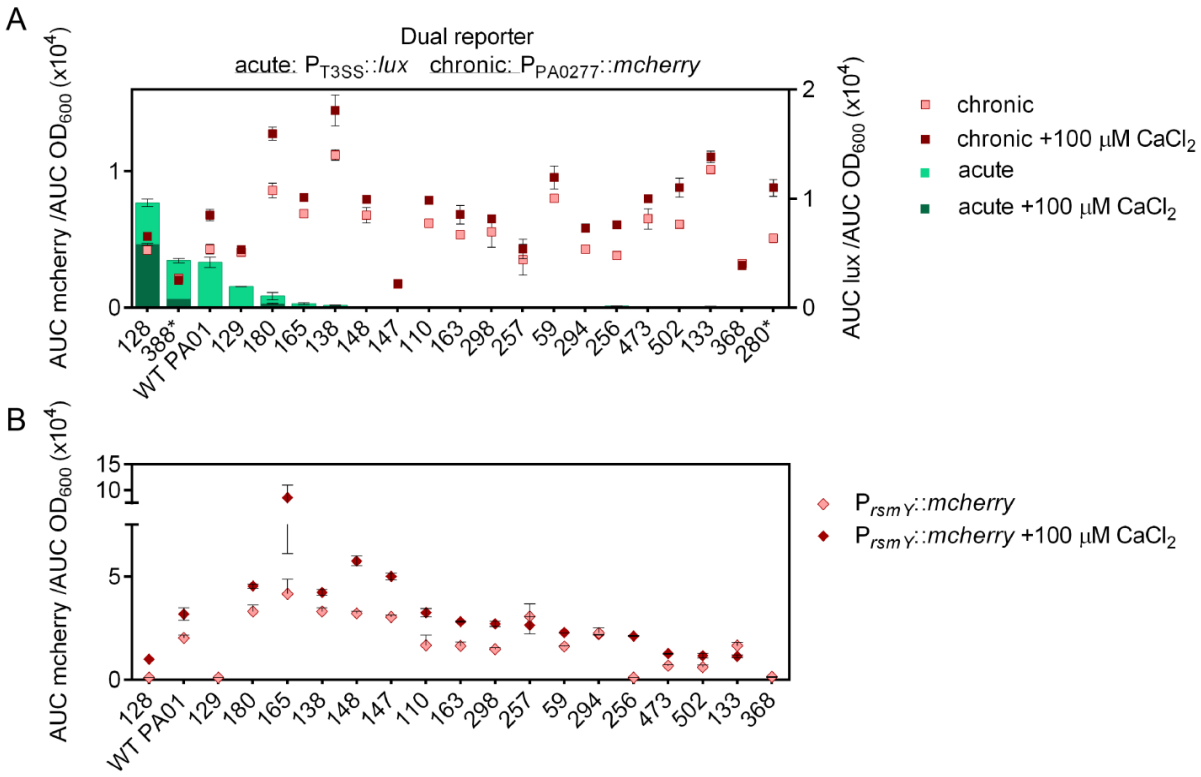

Figure 6. Calcium-sensing is retained in most clinical isolates from chronically infected CF patients

Reporter expression profiles of different clinical *P. aeruginosa* isolates from CF patients harboring either (A) pME-acu-chro plasmid (acute: P$_{T3SS}$::*lux* and chronic: P$_{PA0277}$::*mcherry*) or (B) P$_{rsmY}$::*mcherry*. Cells were cultured in Ca^{2+}-free medium (light colors) or Ca^{2+}-free medium supplemented with 100 µM $CaCl_2$ (dark colors) overnight in a microplate reader. Absorbance, fluorescence and luminescence was measured every 30 min. Bars represent the area under the curve (AUC) normalized for growth. Standard deviations are indicated. Asterisks indicate strains, which for technical reasons could only be transformed with pME-acu-chro.

4.5 Discussion

The global Gac/Rsm signaling cascade is one of the key factors regulating the transition from initial acute to long-term persistent *P. aeruginosa* infections of CF airways [30], [66]–[68]. Using Gac/Rsm responsive dual reporter tools we found that calcium acts as an environmental trigger activating the signaling cascade. Calcium-mediated stimulation resulted in reduced expression of genes involved in acute virulence such as the T3SS and increased expression of genes that are normally repressed by RsmA and contribute to chronic behavior. In line with a direct influence on the Gac network we found that calcium increased the levels of phosphorylated GacS and induced the expression of the small RNA *rsmY* in a GacA dependent manner. Only a mild stimulatory effect of calcium was observed for *rsmZ*, which is most likely due to a technical problem [69]. The observation that strains lacking either *rsmY* or *rsmZ* behave very similar in terms of calcium-dependent dual acute-chronic reporter expression, *i.e.* activation of the "chronic" and repression of the "acute" marker, confirms that calcium acts upstream of the GacA response regulator (Figure S5). To our knowledge this is the first defined input signal for the Gac/Rsm cascade. This finding raises several questions regarding the role of calcium in *P. aeruginosa* host persistence, how calcium is exactly sensed and how the intracellular signal transmission is achieved.

Calcium is the fifth most prevalent element in our biosphere [70] and a well-established signaling molecule. In *P. aeruginosa* calcium-binding proteins are involved in diverse processes including maintenance of calcium homeostasis [12] and the establishment of host cell contacts [71], [72]. We found that calcium-sensing does not represent a general feature of *Pseudomonas* species, but is rather represents an adaptation to the lifestyle of *P. aeruginosa*. Interestingly, a dysregulated calcium homeostasis is intimately linked to the pathology of CF and various body fluids show elevated calcium levels compared to healthy individuals [43], [44], [46]. Together with our findings that most *P. aeruginosa* isolates from CF airways remained calcium-responsive even after decade-long colonization of CF lungs, we postulate that calcium is used as a specific signal for *P. aeruginosa* in CF patients driving the acute-to-chronic behavioral transition. In line with this, we find that cells with an active Gac/Rsm cascade tend to exit more slowly from stationary phase and have an increased tolerance to antibiotics, both traits that are beneficial to persist in the host [65].

LadS, a Gac/Rsm associated hybrid histidine kinases with a 7TMR-DISMED2 sensory domain is essential for calcium-mediated signal transduction. Increased levels of LadS directly translate into increased activity of the signaling cascade in the presence, but not in the absence of calcium. This implies that LadS is directly involved in calcium recognition. Because LadS does not harbor any of the known calcium-binding domains or motifs, the mode of signal recognition remains unclear. The periplasmic residing DISMED2 domain adopts a jelly-roll fold, which is reminiscent of carbohydrate-binding modules [73], [33] and is shared by three other *P. aeruginosa* proteins RetS, NicD and PA3462. One fundamental difference is that at the position where the carbohydrate would be normally placed in classical carbohydrate-binding modules the DISMED2 domain harbors an additional helix. This suggests that signals other than carbohydrates might be recognized. This additional helix is found in all four 7TMR-DISMED2 proteins [74]. For two family members in *P. aeruginosa* cognate input signals were discovered, which are thought to act via their periplasmic DISMED2 domains. NicD

mediates biofilm dispersal in response to glutamate [75] and RetS senses signals related to kin cell lysis, however the physico-chemical nature of the latter cue is unknown [41].

The following experimental observations suggest that the DISMED2 domain of LadS is also of central importance for calcium-sensing. i) The presence of calcium stabilizes LadS-RG_{DISM2} harboring two additional amino acids flanking the DISMED2 domain, which coincides with calcium-induced stimulation of the Gac/Rsm cascade; ii) Mutation of a single aspartic acid residue (D80A) renders LadS-RG_{DISM2} calcium-blind; iii) Deletion of the DISMED2 domain renders LadS calcium-blind and constitutively active. Interestingly, the last finding suggests that this domain is important to restrain the activity of LadS. The observation that closely related LadS homologues lack the ability to sense calcium suggests that calcium-sensing is not a general feature of DISMED2 containing proteins but that the DISMED2 domain of LadS in *P. aeruginosa* has diversified during evolution and adopted novel ligand binding capabilities. Alternatively, calcium-sensing is a more ancient property that was selectively lost in other *Pseudomonas* species.

Intriguingly, calcium ions have frequently been found to be associated with carbohydrate binding proteins [71], [76]–[78]. Therefore, it is tempting to speculate that the DISMED2 domain of LadS has lost the sugar-binding capacity, but has retained the ability to recognize calcium ions. It is important to note that we have no evidence that calcium directly binds to LadS and at this stage it cannot be excluded that LadS interacts with calcium indirectly. Although the existence of a *P. aeruginosa* specific proteinaceous co-factor is unlikely as calcium is able to stabilize the LadS-RG_{DISM2} mutant protein, even when expressed in *E.coli.* Based on the homology of the DISMED2 domain with carbohydrate-binding modules and the observation that calcium can enhance the binding of carbohydrates [33], [72], we tested the effect of a variety of carbohydrates on calcium-dependent activation of the Gac/Rsm cascade. However, none of the supplements showed a significant effect on LadS activity.

After signal perception, the second prominent question relates to the intracellular signal transduction mechanisms employed by LadS. While the receiver domain is negligible, all other domains of LadS are essential for signaling. This suggests that the receiver domain might be important to modulate LadS activity, possibly by acting as intramolecular phosphoryl sink or storage. We propose that signal transduction either occurs via classical phosphorelay or via modulation of protein-protein interaction. Using Phos-Tag SDS-PAGE we assessed LadS phosphorylation of cells grown under low and high calcium conditions. However, only aspartate phosphorylation was stable enough to be detected. Interestingly, we observed phosphorylation of the receiver domain aspartate in a LadS mutant lacking the primary histidine phosphoryl acceptor. It is thus possible that an alternative HK can target the LadS receiver domain. Alternatively, unspecific phosphorylation via acetyl phosphate could be responsible for this phenomenon [79], [80]. Based on the observation that calcium reduces the phosphorylation of the receiver domain we propose the following signaling mechanism. Under low-calcium conditions LadS retains some basal activity with the receiver domain serving as a phosphate repository, which then in calcium-rich conditions donates its phosphate back to the histidine in the DHp domain and from there to another acceptor protein. Such phosphate sink systems where two RRs compete for the phosphoryl group from a single HK have been shown to accelerate signal termination and present a way to neatly fine-tune the signaling output [81]. Potential downstream targets of LadS are response regulators of unknown nature. Moreover, LadS might engage in phosphotransfer with GacS, even

though we are unaware of any reports showing cross-phosphorylation between non-homologous histidine kinases. However, the observation that RetS interferes with GacS activation through protein-protein interaction [66] opens up the possibility for novel unconventional signaling modes. Alternatively, LadS might act by modulating protein-protein interactions, for example the interaction of RetS and GacS. Clearly, more experiments are required to disentangle the specific requirements for LadS-mediated calcium signaling.

Taken together, our findings contribute to the better understanding of the environmental factors triggering the acute-to-chronic switch in chronically infected CF airways, which will hopefully help to discover new therapeutic approaches.

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4.7 References

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4.8 Supplementary Information

4.8.1 Supplementary Figures

In general, strains were grown in Ca^{2+}-free MM with or without 2.5 mM $CaCl_2$ (as indicated) overnight in a microplate reader. Absorbance, fluorescence and luminescence was measured every 30 min. Bars represent the area under the curve (AUC) normalized for growth. Standard deviations are indicated.

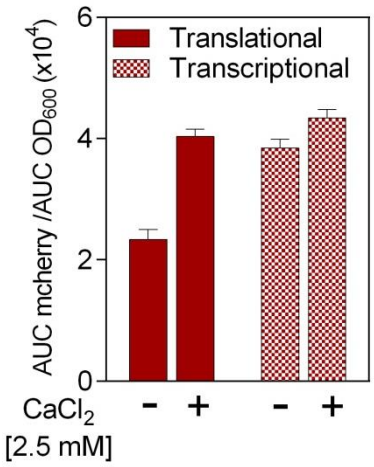

Figure S1. Transcription of the chronic marker *PA0277* is not affected by calcium
Expression profiles of transcriptional and translational *PA0277* promoter fusions under low and high calcium.

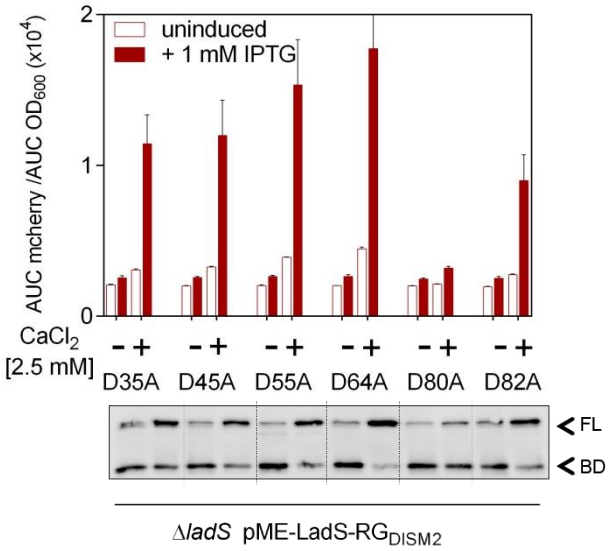

Figure S2. Mutational analysis of aspartic acid residue in the periplasmic DISMED2 domain of LadS-RG$_{DISM2}$
Calcium-dependent activity of the *rsmY* promoter in strains expressing *ladS*-RG$_{DISM2}$ harboring different D-to-A mutations. Immunoblot analysis with α-FLAG antibodies of the corresponding strains is shown below. Arrows mark full-length LadS (FL) and a breakdown product (BD).

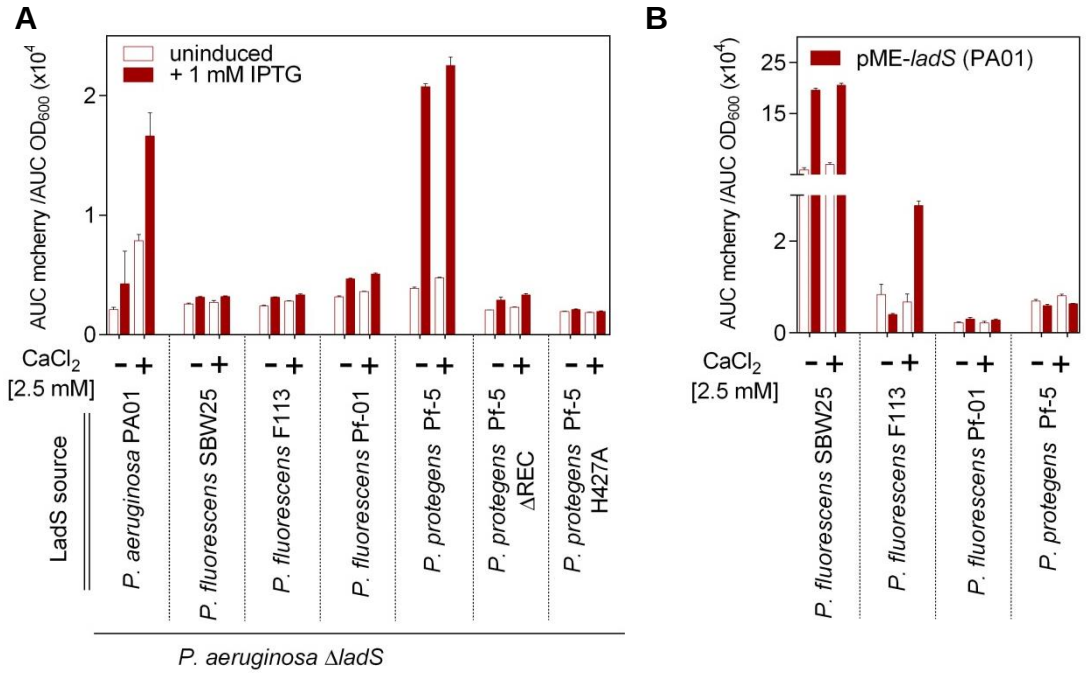

Figure S3. LadS cross-complementation
(A) Calcium-dependent activity of the *rsmY* promoter in strains with *ladS* expression from different *Pseudomonas spp.* in *P. aeruginosa ΔladS* under low and high calcium. (B) Calcium-dependent activity of the *rsmY* promoter in different *Pseudomonas* strains (light) or upon expressing *ladS* of *P. aeruginosa* (dark).

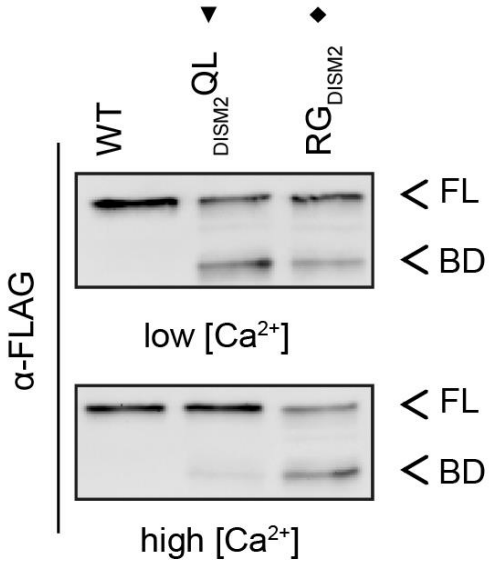

Figure S4. Calcium-mediated stabilization of LadS mutant versions (RG_{DISM2} and $_{DISM2}QL$) in *E. coli* DH5α
Immunoblot analysis with α-FLAG antibodies of *E. coli* DH5α strains expressing wild-type and mutant *ladS* ($_{DISM2}QL$ and RG_{DISM2}) under low and high calcium conditions. Full-length LadS (FL) and breakdown product (BD) are marked by arrows.

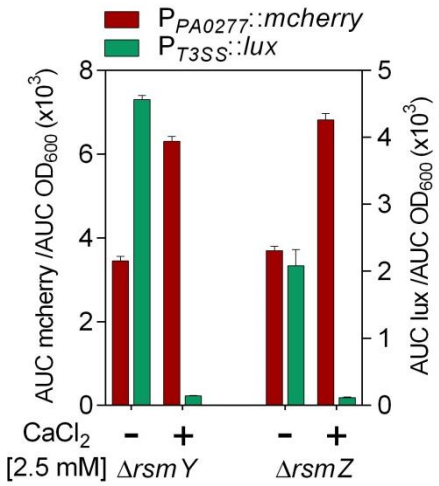

Figure S5. RsmY and RsmZ are redundant for calcium-induced activation of the Gac/Rsm cascade

Expression profiles of pME-acu-chro reporter (acute: P_{T3SS}::*lux* and chronic: P_{PA0277}::*mcherry*) in *ΔrsmY* and *ΔrsmZ* strains grown under low and high calcium conditions.

4.8.2 Supplementary Tables

4.8.2.1 Table S1 Bacterial strains and plasmids used in this study.

Strains	Description	Reference
PA01	WT *P. aeruginosa*	[82]
Δ*ladS*	Clean deletion of *ladS* in PA01	This study
Δ*retS*	Clean deletion of *retS* in PA01	This study
Δ*gacA*	Transduction with E79tv2 of *gacA*:: Ω Sp^R/Sm^R from PAO6281 [83] into PA01	This study
Δ*gacS*	Transduction with E79tv2 of *gacS*:: Ω Sp^R/Sm^R from PAO6327 [84] into PA01	This study
Δ*ladS*Δ*gacS*	Transduction with E79tv2 of *gacA*:: Ω Sp^R/Sm^R from PAO6281 [83] into Δ*ladS*	This study
Δ*rsmA*	Clean deletion of *rsmA* in PA01	This study
Δ*rsmYZ*	PAO6421, clean deletion of *rsmY* and *rsmZ*	[85]
Δ*pel*Δ*psl*	Clean deletion of *pel* and *psl* expolysaccharides in PA01	This study
SBW25	*P. fluorescens* SBW25	[86]
F113	*P. fluorescens* F113	[87]
Pf-01	*P. fluorescens* Pf-01	[88]
Pf-5	*P. protegens* Pf-5	[89]
DH5α	General *E.coli* strain used for cloning	[90]
AB330	λ cI857 Δ(cro-bioA), gal+, lac+	A. Böhm

Plasmids	Description	Reference
pME6032	P_K, 9.8 kb pVS1 derived shuttle vector, Tc^R	[91]
pME6032-FLAG	3xFLAG inserted via into *Kpn*I and *Xho*I into pME6032, Tc^R	This study
pUC18T-mini-Tn*7*T-Gm	Tn*7* insertion vector, Amp^R, Gm^R	[56]
pUC18T-mini-Tn*7*T-Gm-*lux*	Template for *luxCDABE*, Tn*7* insertion vector, Gm^R, Amp^R	[92]
pCERC-2	Template for *cerulean*, Kan^R	[52]
pXVENN-2	Template for *venus*, Kan^R	[52]
pRSET FLIPglu-600uDelta11 Ares-Aphrodite	Template for codon-diversified form of *venus* (=*aphrodite*), Amp^R	[54]
pCWR336	Template for codon-optimized *mcherry*; Kan^R	[55]
pTNS2	helper plasmid for Tn*7* integration events, Amp^R	[56]
pFLP2	FRT cassette excision vector, Amp^R	[93]
pEX18-Tc	*oriT*+, *sacB*+, gene replacement vector, Tc^R	[50]
pME3087	suicide vector for allelic replacement; *ColE1*-replicon, I*ncP-1*, *Mob,* Tc^R	[51]

Reporter tools		
pME-acu-chro	pME6032 carrying dual translational promoter fusion (acute: P_{T3SS}::*lux* and chronic: P_{PA0277}::*mcherry*), Tc^R	This study
pME-acu-chro-2	pME6032 carrying dual translational promoter fusion (acute: P_{T3SS}::*aphrodite* and chronic: P_{PA0277}::*cerulean*), Tc^R	This study
pME-acu-chro-3	pME6032 carrying dual translational promoter fusion (acute: P_{T3SS}::*cerulean* and chronic: P_{PA0277}::*aphrodite*), Tc^R	This study
pME-acu-chro-4	pME6032 carrying dual translational promoter fusion (acute: P_{T3SS}::*gfp(*Mut3) and chronic: P_{PA0277}::*mcherry*), Tc^R	This study
pME-P_{PA0277}-RBS-*Ven*	pME6032 carrying transcriptional *PA0277* promoter fusion to *venus*, Tc^R	This study
pME-P_{PA0277}-*Ven*	pME6032 carrying translational *PA0277* promoter fusion to *venus*, Tc^R	This study
pME-P_{T3SS}-RBS-*Ven*	pME6032 carrying transcriptional T3SS promoter fusion to *venus*, Tc^R	This study
pME-P_{T3SS}-*Ven*	pME6032 carrying translational T3SS promoter fusion to *venus*, Tc^R	This study
P_{cdrA}::*gfp*(Mut3)	pUCP22-*Not*I based cyclic di-GMP level reporter, Gm^R	[53]
P_{rsmY}::*mcherry*	pUCP22-*Not*I carrying transcriptional *rsmY* promoter fusion, Gm^R	This study
P_{rsmZ}::*mcherry*	pUCP22-*Not*I carrying transcriptional *rsmZ* promoter fusion, Gm^R	This study
P_{rsmY}::*mcherry*-Tc	pUCP22-*Not*I carrying transcriptional *rsmY* promoter fusion, Tc^R	This study
pTn*7*T-P_{rsmY}::*mcherry*	Tn*7*-integratable transcriptional *rsmY* promoter fusion, Amp^R, Gm^R	This study
Expression constructs		
pTn*7*T-*ladS*-FLAG	Tn*7*-integratable vector carrying *ladS* from *P. aeruginosa* PA01 under control of its native promoter, 3xFLAG, Gm^R	This study
pME-*ladS*-FLAG	pME6032 carrying *ladS* of *P. aeruginosa* PA01, 3xFLAG, Tc^R	This study
pME-*ladS*-H428A-FLAG	pME6032 carrying *ladS* with kinase-inactivating H428A point mutation, 3xFLAG, Tc^R	This study
pME-*ladS*-D718N-FLAG	pME6032 carrying *ladS* with inactivating D718N point mutation in REC domain, 3xFLAG, Tc^R	This study
pME-*ladS*-D718E-FLAG	pME6032 carrying *ladS* with phospho-mimetic D718E point mutation in REC domain, 3xFLAG, Tc^R	This study
pME-*ladS*-ΔREC-FLAG	pME6032 carrying *ladS* with a deletion of the receiver domain, 3xFLAG, Tc^R	This study
pME-*ladS*-ΔDISM2-FLAG	pME6032 carrying *ladS* with a deletion of the periplasmic 7TMR-DISMED2 domain, 3xFLAG, Tc^R	This study
pME-$ladS_{cyt}$-FLAG	pME6032 carrying soluble *ladS*, 3xFLAG, Tc^R	This study
pME-*ladS*-$_{DISM2}$QL-FLAG	pME6032 carrying *ladS* with an insertion of 'QL' C-terminally flanking 7TMR-DISMED2 domain, 3xFLAG, Tc^R	This study
pME-*ladS*-RG_{DISM2}-FLAG	pME6032 carrying *ladS* with an insertion of 'RG' N-terminally flanking 7TMR-DISMED2 domain, 3xFLAG, Tc^R	This study
pME-*ladS*-RG_{DISM2}-D80A-FLAG	pME6032 carrying *ladS*-RG_{DISM2} with D80A point mutation, 3xFLAG, Tc^R	This study
pME-*ladS*-SBW25	pME6032 carrying *ladS* from *P. fluorescens* SBW25, Tc^R	This study
pME-*ladS*-F113-FLAG	pME6032 carrying *ladS* from *P. fluorescens* F113, 3xFLAG, Tc^R	This study
pME-*ladS*-Pf-01-FLAG	pME6032 carrying *ladS* from *P. fluorescens* Pf-01, 3xFLAG, Tc^R	This study
pME-*ladS*-Pf-5-FLAG	pME6032 carrying *ladS* from *P. protegens* Pf-5, 3xFLAG, Tc^R	This study

pME-*ladS*-HB1 to HB5	pME6032 carrying different *ladS* 'PA01 - Pf-5' hybrids, 3xFLAG, Tc^R	This study
ptac-*rsmY*	pME6918, Tc^R	[94]
Deletion constructs		
pMPELA	pEX19-Ap carrying *pelA* deletion cassette, Amp^R	[95]
pME3087-Δ*psl*	pME3087 carrying *psl* deletion cassette as *Eco*RI-*Hin*dIII fragment, Tc^R	This study
pME3087-Δ*rsmA*	pME3087 carrying *rsmA* deletion cassette as *Eco*RI-*Hin*dIII fragment, Tc^R	This study
pEX18-Tc-Δ*ladS*	pEX18-Tc carrying *ladS* deletion cassette as *Eco*RI-*Hin*dIII fragment, Tc^R	This study
pEXG2-Δ*retS*	*retS* deletion construct, Tc^R	[96]

4.8.2.2 Table S2 Primers used in this study.

Primer	Description	Sequence
A	ΔladS up F	GATCGAATTCCAGGTTCGGCGTGGCGATGG
B	ΔladS up R	GGTGACGATCGGCTGGCACACAAGAAGAAAGAGAATCAGC
C	ΔladS down F	GCTGATTCTCTTTCTTCTTGTGTGCCAGCCGATCGTCACC
D	ΔladS down R	GATCAAGCTTCACCAGAGCCGTACTCACC
E	ΔrsmA up F	GATCAAGCTTGATGATGAAGAGGAATCCATGGAACAGC
F	ΔrsmA up R	GATAAAAATTAATGGTTTGCATTCCTTTCTCCTCACGCGAATATTTCAGG
G	ΔrsmA down F	CGTGAGGAGAAAGGAATGCAAACCATTAATTTTTATCTAATTTTCC
H	ΔrsmA down R	GATCGAATTCGTGAAACTGCTTTACCGCCTTCCAGG
I	P_{T3SS} F	GATCGGATCCCGCATCCGGACGATGAGAGG
J	P_{T3SS}.Cer R	TCCTCGCCCTTGCTCACCATAAGCTTCGCGGCGGAGGAACTCTGGA
K	Ven/Cer F	ATGGTGAGCAAGGGCGAGGA
L	Cer R	CGATACCGTCGACCTCGAACGGGCTGCAGCTAGCTTACTTGTAC
M	T_0,T_1 F	GTTCGAGGTCGACGGTATCG
N	T_0,T_1 R	GATCCCTAGGCCGGGCCGCAAGCTCCTAGC
O	P_{T3SS}.gfp R	AGTTCTTCTCCTTTACGCATAAGCTTCGCGGCGGAGGAACTCTGGA
P	gfp F	ATGCGTAAAGGAGAAGAACT
Q	gfp R	CGATACCGTCGACCTCGAACTTATTTGTATAGTTCATCCA
R	P_{PA0277} F	GACTGAATTCTCGGCGCCGACCTCGCCTGG
S	P_{PA0277} R	AAGCTTGGCGGAAAGGGAAAGACGGA
T	Aphr F	TTTCCCTTTCCGCCAAGCTTATGGAGTTGTTTACGGGCGTC
U	Aphr R	GATCCCTAGGCTATATGCCCGCCGCCGTGA
V	mCherry F	TTTCCCTTTCCGCCAAGCTTGTGTCGAAGGGTGAAGAAGA
W	mCherry R	GATCCCTAGGTTACTTGTAGAGCTCATCCA
X	P_{rsmY} F	GATCTCTAGAGCTGGGAAGGCTCGCGATGATGAGG
Y	P_{rsmY} R	GATCGCATGCGGTTTGAAGATTACGCATCTCTGC
Z	P_{rsmZ} F	GATCTCTAGACGGAAAACCTTAGACCCACTGAAGACC
Aa	P_{rsmZ} R	GATCGCATGCCAGGAGTGATATTAGCGATTCC
Ab	RBS-mcherry F	GATCGCATGCATTAAAGAGGAGAAATTAAGCGTGTCGAAGGGTGAAGAAGATAATATGG
Ac	mCherry R	GATCAAGCTTTTACTTGTAGAGCTCATCCATGCCGCCGGTCG
Ad	Tet F	GCGCGTTACGCCGTGGGTCGATGTTTGATGTTATGGAGCAGCAACGTCAATCGTCACCCTTTCTCGG
Ae	Tet R	CGAACAACTCCGCGGCCGGGAAGCCGATCTCGGCTTGAACGAATTGTCAGCGATCGGCTCGTTGCCCTGC
Af	Lux F	GATCGAGCTCAAGCTTATGACTAAAAAAATTTCATT
Ag	Lux R	GATCCTCGAGTCAACTATCAAACGCTTCGG
Ah	LadS F-*Bam*HI	GATCGGATCCCATGGCGCGTGAGCTTACC
Ai	LadS-3xFLAG R	ATATCATGATCTTTATAATCACCGTCATGGTCTTTGTAGTCGAAGGCGGACTTGGTGACGATCG
Aj	3x-FLAG R	GATCAAGCTTTTACTATTTATCGTCGTCATCTTTGTAGTCGATATCATGATCTTTATAATC
Ak	LadS F-*Sac*I	GATCGAGCTCCATGGCGCGTGAGCTTACC
Al	3xFLAG-R-*Kpn*I	GATCGGTACCTTACTATTTATCGTCGTCATC
Am	LadS H428A F	CGCCACCGTCACCGCCGAACTGCGCACC
An	LadS H428A R	GGTGCGCAGTTCGGCGGTGACGGTGGCG
Ao	LadS D718N F	CGGCGTACTGCTCAACTGCCAGATGCCG
Ap	LadS D718N R	CGGCATCTGGCAGTTGAGCAGTACGCCG
Aq	LadS D718E F	GGCGTACTGCTCGAGTGCCAGATGCCGG
Ar	LadS D718E R	CCGGCATCTGGCACTCGAGCAGTACGCC
As	LadS ΔDISM2 R	GATGCCGGCGCCGTTCACCGACACCGCACCCGCCAGGCACG
At	LadS ΔDISM2 F	CGTGCCTGGCGGGTGCGGTGTCGGTGAACGGCGCCGGCATC
Au	DISM2-QL R	CCTTCAGCTGGGGCGACCACAGGGTCAG
Av	DISM2-QL F	CTGACCCTGTGGTCGCCCCAGCTGAAGGCCTACCTGGAGG
Aw	RG-DISM2 R	GACTGCCCCAGCGGCAGGCGGCCGCGCTCGACCTGCTCGTTGAAG
Ax	RG-DISM2 F	CGAGCGCGGCCGCCTGCCGCTGGGGCAGTC
Ay	LadS-D80A F	CTGGTTGCGCCTGGCCCTGGACTACCGG
Az	LadS-D80A R	CCGGTAGTCCAGGGCCAGGCGCAACCAG
Ba	LadS-ΔREC R	ATATCATGATCTTTATAATCACCGTCATGGTCTTTGTAGTCGAACACCGTGCACTCCTCGGGACG
Bb	$LadS_{cyt}$ F	GATCGAGCTCATGATCAACGCAATGAAGGAGG
Bc	M2-*Kpn*I F	GATCGGTACCTTCGACTACAAAGACCATG
Bd	M2-*Hin*dIII-*Xho*I R	GATCCTCGAGAAGCTTTTACTATTTATCGTCGTCATC

Be	LadS-Pf5 F	GATCGAGCTCGAGAGCTGTAGATATTCATGG
Bf	LadS-Pf5 R	GATCGGTACCAATGTCGGCGCTTTCGCCCTG
Bg	HB1 F	AGCAACCGCCTGAAGGACGAG
Bh	HB1 R	GAGGAACTCGTCCTTCAGGCGGTTGCTGTGGGCCAGTTGCTGGTTCAAC
Bi	HB2 F	GGAAGCGCTGAACCAGGAACTGGCCAACAGCAACCGGCTCAAGGACG
Bj	HB2 R	GTTGGCCAGTTCCTGGTTCAGC
Bk	HB4 R	GGACCACAGGCTCACCGGCG
Bl	HB4 F	CGCCGGTGAGCCTGTGGTCCCCCAAGGCCTACCTGGAGGAGC
Bm	HB5 R	GCGTTGAGTGAACTCGTCGAACTCG
Bn	HB5 mid F	CGAGTTCGACGAGTTCACTCAACGCCTGCCGCTGGGGCAGTCCAT
Bo	HB5 mid R	GGCTTCCAGGTAAGCACTGCTCGACCACAGGGTCAGCGGCG
Bp	HB5 F	AGCAGTGCTTACCTGGAAGCC
Bq	P_{T3SS} R	CTCCTTATAAAGTTAAGCTTGCGCGGCGGAGGAACTCTGG
Br	Ven,Cer F	AAGCTTAACTTTATAAGGAGGAAAACCTATGGTGAGCAAGGGCGAGGA
Bs	Ven R	CGATACCGTCGACCTCGAACTTACTTGTACAGCTCGTCCATGC
Bt	T_0,T_1, R	GATCCATATGCCGGGCCGCAAGCTCCTAGC
Bu	LadS-F113 F	GATCGAGCTCCGATATCTGCCTGCAAACCG
Bv	LadS-F113 R	GATCGGTACCAATATCGGCGCTTTCACCCTG
Bw	LadS-Pf-01 F	GATCGAGCTCGATATTCATGGTGCGCGAGC
Bx	LadS-Pf-01 R	GATCGGTACCGCAACTCAACACCCGCCGCTG
By	LadS-SBW25 F	GATCGGATCCCATGGTGCGCGAGCTTACC
Bz	LadS-SBW25 R	GATCGGTACCTTAAAACAGTGGCATACCC
Ca	Δpsl up F	GATCGAATTCTACCGCAACCGCAGCTACGACG
Cb	Δpsl up R	GGTCATGATGTTCACTTCCAGTAGCCTGCAGGCTATCTACCGACTTCGAATGC
Cc	Δpsl down F	CATTCGAAGTCGGTAGATAGCCTGCAGGCTACTGGAAGTGAACATCATGACC
Cd	Δpsl down R	GATCAAGCTTGACTTGGGCACGAAGACGATGTCG

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5 ADDITIONAL RESULTS

The following chapter comprises results, which are beyond the scope of the paper manuscript, but still contribute to the overall picture and/or might be seminal for future studies.

5.1 Cyclic-di-GMP has no effect on the dual acute-chronic reporter expression

The Gac/Rsm cascade has been shown to inversely regulate the T3SS and T6SS associated with acute and chronic infections, respectively [60], [77]. Likewise, c-di-GMP was suggested to reciprocally regulate these two systems in an RsmY- and RsmZ-dependent manner [193]. Along the lines of this report we tested the expression of our Gac-Rsm responsive dual reporter in cells harboring low (Δ4xDGCs) or high (p*wspR19* and Δ*yfiR*) c-di-GMP levels. However, contrary to the previous findings all strains exhibited a similar expression profile as compared to the parental strain (Fig 14) indicating that c-di-GMP mediated regulation might not represent a general mechanism of RsmA-regulated targets or that c-di-GMP operates at the post-translational level.

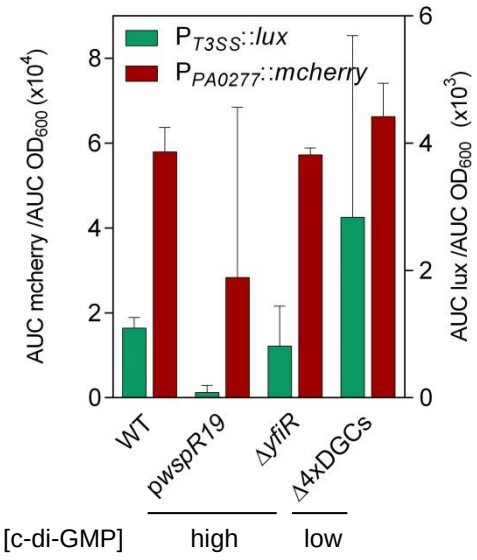

Fig 14. Cyclic-di-GMP has no effect on the dual acute-chronic reporter expression
Expression profiles of dual acute-chronic reporter IV (acute: P$_{T3SS}$::*lux* and chronic: P$_{PA0277}$::*mcherry*) in *P. aeruginosa* strains harboring low (Δ4xDGCs) or high (p*wspR19*/Δ*yfiR*) c-di-GMP levels. Strains were grown overnight in a microplate reader. Absorbance and fluorescence was measured every 30 min. Bars represent the area under the curve (AUC) normalized for growth. Standard deviations are indicated.

5.2 Calcium stimulates the activity of the Gac/Rsm cascade

The observation that calcium inversely regulates the expression of the dual Gac/Rsm-responsive acute-chronic reporter (acute: P_{T3SS}::*lux* and chronic: P_{PA0277}::*mcherry*) was the starting point of this project. The first aspect we addressed was to exclude that this observation is specific to our strain background. Moreover, preceding the construction of the transcriptional *rsmY* reporter, which provided direct evidence that calcium stimulates the Gac/Rsm cascade, we could show that calcium affects two other known RsmA-regulated targets besides the "chronic" reporter. We analyzed the effect of different divalent cations on the expression of the dual acute-chronic reporter. Furthermore we demonstrated that even though *P. aeruginosa* cells seem to rapidly capture calcium ions, calcium needs to be continuously present to maximally activate the signaling cascade. Moreover, we show that 50-100 μM $CaCl_2$ are sufficient to induce the acute-to-chronic switch.

5.2.1 Calcium-induced regulation of the acute-chronic reporter is a common phenomenon of different *Pseudomonas aeruginosa* strains

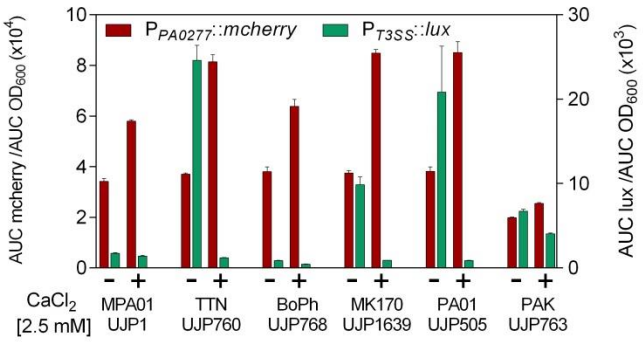

Fig 15. Calcium regulates the acute-chronic reporter in all tested *P. aeruginosa* wild-type strains
Calcium-dependent expression profiles of dual acute-chronic reporter IV (acute: P_{T3SS}::*lux* and chronic: P_{PA0277}::*mcherry*) in different *P. aeruginosa* wild-type strains (internal reference numbers are indicated). Strains were grown overnight in a microplate reader. Absorbance and fluorescence was measured every 30 min. Bars represent the area under the curve (AUC) normalized for growth. Standard deviations are indicated.

In order to rule out that the calcium-induced lifestyle switch is specific to our *P. aeruginosa* strain PA01 we analyzed other wild-type strains in terms of their calcium-dependent acute-chronic reporter profile. Despite the fact that the T3SS expression (green bars) is variable, all strains show increased expression levels of the "chronic" marker (red bars) in response to calcium (Fig 15). This indicates that calcium-mediated activation of the Gac/Rsm cascade is a general feature of all *P. aeruginosa* strains tested.

5.2.2 Calcium affects other RsmA-regulated targets

Next, we tried to gather more evidence that calcium indeed alters the activity of the Gac/Rsm cascade and is not simply an inherent property of the promoters used for the reporter system. Therefore, we analyzed other known RsmA-regulated targets by fluorescence microscopy-based readouts. Cells harboring PA2781::RBS-*gfp* (shown to be RsmA regulated by Tina Jaeger, unpublished) or ClpV1-GFP, ATPase associated with the T6SS (strain provided by Marek Basler, [6]) show markedly reduced expression when grown in Ca^{2+}-free minimal medium (MM) as compared to medium supplemented with 2.5 mM $CaCl_2$ (Fig 16). This observation strengthened the hypothesis that calcium globally affects the activity of the Gac/Rsm cascade.

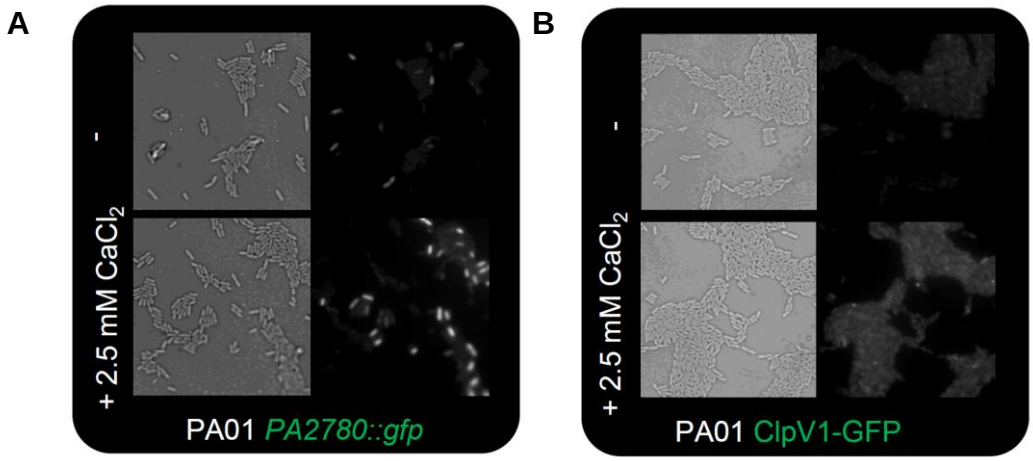

Fig 16. Calcium affects the expression of other known RsmA-regulated targets
Fluorescence microscopy analysis of cells grown in Ca^{2+}-free minimal medium (MM) or medium supplemented with 2.5 mM $CaCl_2$ (A) WT PA2781::RBS-*gfp* (B) WT ClpV1-GFP. Image contrast adjusted to the same levels.

5.2.3 Calcium ions specifically stimulate the Gac/Rsm cascade

In line with the findings presented in the paper manuscript we observe that only calcium ions are able to repress the expression of the T3SS while simultaneously stimulating the "chronic "marker expression (Fig 17A).

Interestingly, Zn^{2+} ions seem to have the opposite effect from Ca^{2+} ions with increasing concentrations activating acute traits while simultaneously repressing the "chronic" marker. However, this effect seems to be promoter-intrinsic and not mediated via altering the activity of the Gac/Rsm cascade as *rsmY* levels remain unaffected by Zn^{2+} (Figure 2D). All other ions have no effect on dual reporter expression. The data in Fig 17B indicate that the maximal stimulatory effect of calcium on the Gac/Rsm cascade is achieved with concentrations as low as 50-100 μM.

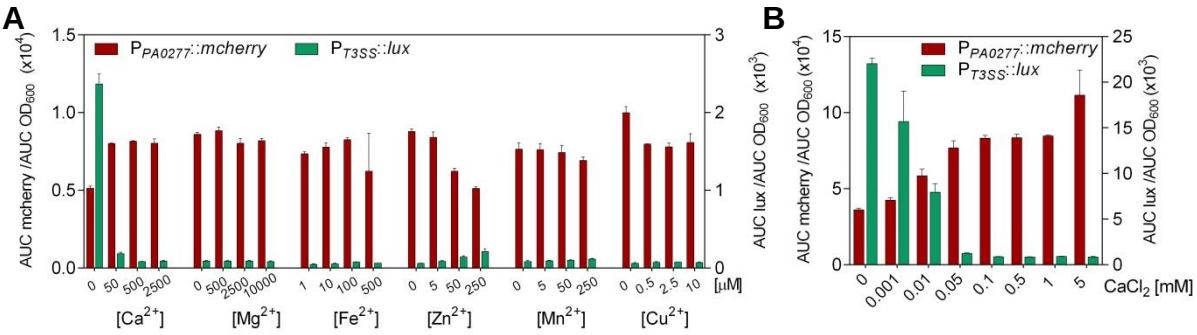

Fig 17. Calcium ions trigger inverse dual acute-chronic reporter expression
(A) Effect of increasing cation concentrations on dual acute-chronic reporter IV expression (acute: P_{T3SS}::*lux* and chronic: P_{PA0277}::*mcherry*) (B) Effect of small step-wise increasing calcium concentrations on dual acute-chronic reporter IV expression. Strains were grown overnight in a microplate reader. Absorbance, fluorescence and luminescence was measured every 30 min. Bars represent the area under the curve (AUC) normalized for growth ±SD

5.2.4 Continuous presence of calcium is required to maximally induce the Gac/Rsm cascade

Next, we addressed temporal aspects of calcium-mediated activation of the Gac/Rsm cascade. Is calcium a “trigger” that kick-starts the signaling cascade, thereby producing a long-lasting response through a positive feedback loop? Or is calcium rather like a “button” that needs to be continuously pushed to uphold the response? To answer this question *rsmY* expression was compared in cells first grown in Ca^{2+}-free medium, exposed for 10 min to 2.5 mM $CaCl_2$ and then transferred back to different media. This included Ca^{2+}-free medium without (3) or with a PBS washing step (4), medium containing EGTA (5) or calcium-rich medium (2) (Fig 18A). Interestingly, a 10-min exposure to $CaCl_2$ is sufficient to almost maximally activate the Gac/Rsm cascade indicating that calcium ions are rapidly captured by *P. aeruginosa* cells. Even shorter incubation times were tested with the same outcome (data not shown). Moreover, the fact that washing the cells does not efficiently remove the ions illustrates how well they are trapped. However, the presence of the calcium-chelator EGTA completely abolished the calcium-dependent stimulation of the Gac/Rsm cascade suggesting that free calcium ions need to be continuously present to fully stimulate the cascade. This finding is in favor of the “button theory”. The observation that also prolonged growth in calcium-rich medium (~4h) followed by growth in the presence of EGTA does not result in maximal activation of the Gac/Rsm cascade further strengthens this finding (Fig 18B).

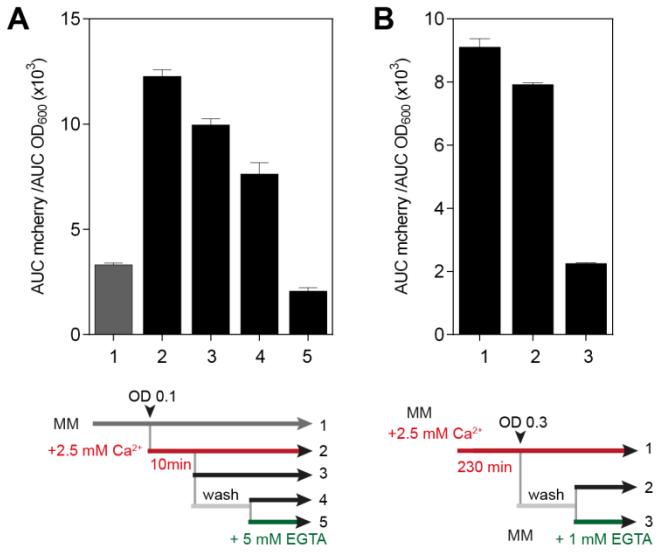

Fig 18. Temporal aspects of calcium-induced activation of the Gac/Rsm cascade
Expression profiles of *rsmY* in a *ladS* mutant strain expressing *ladS* under the control of an IPTG-inducible promoter (A) Bacteria were grown in Ca^{2+}-free MM until an OD_{600} of 0.1 followed by 10 min exposure to 2.5 mM $CaCl_2$. The cells were then either directly transferred back to Ca^{2+}-free MM (3), once washed with PBS (4) or washed and cultured in the presence of 5 mM EGTA (5). Cells not exposed to calcium (1) or continously cultured in calcium-rich medium (2) serve as reference points. The experimental design is schematically outlined. (B) Similar experimental design as in (A), though the cells were cultured from the beginning in calcium-rich medium until an OD_{600} of 0.3 after which they were washed once with PBS followed by culturing in Ca^{2+}-free MM (2) or Ca^{2+}-free MM with 5 mM EGTA (3). Unwashed cells serve as reference point (1). Strains were grown overnight in a microplate reader. Absorbance and fluorescence was measured every 30 min. Bars represent the area under the curve (AUC) normalized for growth. Standard deviations are indicated.

5.3 In search of the calcium-sensing unit

After demonstrating that calcium not only inversely affects the dual acute-chronic reporter expression but also induces the expression of *rsmY*, the evidence solidified that calcium is indeed able to stimulate the activity of the Gac/Rsm cascade. This led to the next question concerning the underlying mechanism of calcium-mediated activation of the signaling cascade.

5.3.1 Calcium does not affect the transcription of genes encoding for Gac/Rsm associated components

The first hypothesis we tested was that calcium alters the transcription of one of the main cascade components upstream of *rsmY* or even *rsmA* itself. Such transcriptional changes would most likely have a global impact on the activity of the cascade. Therefore, we created transcriptional promoter fusions of *gacA, gacS, ladS* and *rsmA* and tested the promoter activities in cells cultured under low and high calcium conditions. However, as illustrated in Fig 19A and B calcium has no significant impact on any of the promoter fusions tested. Based on this finding we started to look for a dedicated calcium-sensing unit.

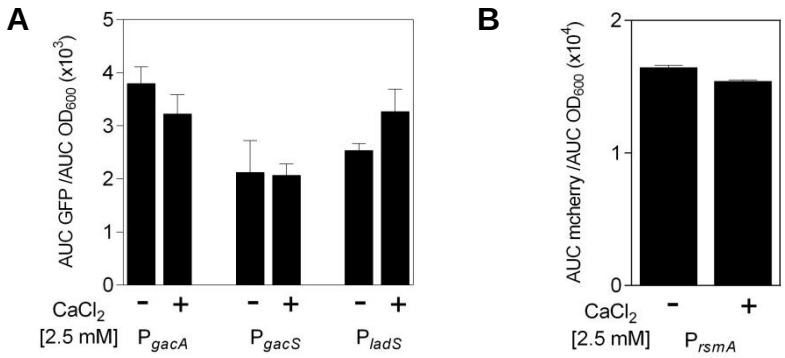

Fig 19. Calcium has no effect on transcription of core Gac/Rsm components
Calcium-dependent expression profiles of transcriptional promoter fusions. Strains were grown overnight in a microplate reader. Absorbance and fluorescence was measured every 30 min. Bars represent the area under the curve (AUC) normalized for growth. Standard deviations are indicated.

5.3.2 Mutational analysis of the central histidine kinase GacS

In the paper manuscript we provide evidence that all core components of the Gac/Rsm cascade, comprising RsmA, RsmY and RsmZ, GacA as well as GacS are essential for calcium sensing. This is probably due to the simplistic fact that without a functional cascade no signal transduction is possible and does not necessarily exclude that one of the components is directly involved in calcium sensing. Therefore, we tested if the disruption of different domains in *gacS* abolish the calcium-dependent *rsmY* promoter activation.

While deleting *gacS* renders the cascade irresponsive to calcium, chromosomal complementation restores the calcium-dependent expression profile (Fig 20). In line with previously published results we find that deletion of the cytoplasmic HAMP domain leads to a constitutively active signaling cascade [46], irrespective of the calcium levels. This is most likely attributable to the loss of interaction with RetS, which has been shown to be established via the HAMP and the histidine kinase domain [45], [194]. Cells harboring GacS with no periplasmic loop (Δ126) or harboring the loop of Env*Z* (ZacS) were still calcium-responsive. It even seemed that calcium is more potent in stimulating the Gac/Rsm cascade, especially in the case of ZacS.

Overall, our findings indicate that despite the fact that GacS is absolutely crucial for calcium-mediated signal transduction GacS is unlikely to be directly involved in calcium sensing.

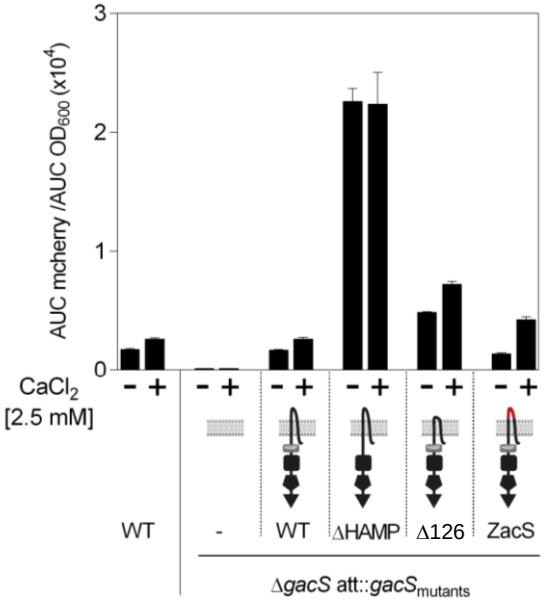

Fig 20. Mutational analysis of GacS
Calcium-dependent activity of the *rsmY* promoter in strains expressing different *gacS* mutants. All *gacS* alleles were chromosomally integrated in a *ΔgacS* strain. Δ126 = deletion of the periplasmic loop, ZacS = GacS with the periplasmic loop of EnvZ. Strains were grown overnight in a microplate reader. Absorbance and fluorescence was measured every 30 min. Bars represent the area under the curve (AUC) normalized for growth. Standard deviations are indicated.

5.3.3 Deletions in potential calcium-sensor candidates

In the continuous search of the calcium-sensing unit we expanded our analysis to other potential interesting candidates, either because they have been shown to be associated with the Gac/Rsm cascade such as LadS, PA1611, PA3462 [83], [94] or because they have been directly implicated in calcium-recognition such as the recently identified calcium-binding EF-hand like protein (EfhP) or the cation-sensing PhoQ histidine kinase [105], [168]. The expression analyses of the dual acute-chronic reporter (A) or the *rsmY* promoter (B) are illustrated in Fig 21 with the most important finding being that only LadS is essential for calcium-sensing.

Deleting *PA1611* reduces the activity of the Gac/Rsm cascade, which stands in line with previous reports [94] and is most likely caused by the loss of the inhibitory effect on RetS. The absence of *phoQ* leads to a strong increase in the expression of the T3SS, though this effect is independent from the Gac/Rsm cascade as the expression of *rsmY* remains unaffected. Mutations in *efhP* and in *PA3462* do not have any effect on the phenotypes assessed here.

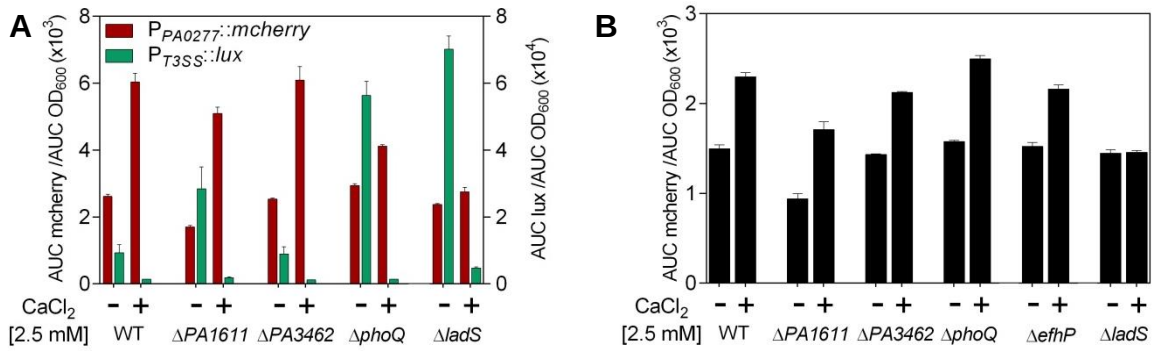

Fig 21. In search for the calcium-sensor: mutational analysis of different candidate proteins
Calcium-dependent expression profiles of different strains harboring clean deletions in putative calcium-sensing proteins. (A) Dual acute-chronic reporter IV (acute: P_{T3SS}::*lux* and chronic: P_{PA0277}::*mcherry*) (B) *rsmY* promoter activity. Strains were grown overnight in a microplate reader. Absorbance, fluorescence and luminescence was measured every 30 min. Bars represent the area under the curve (AUC) normalized for growth. Standard deviations are indicated.

5.4 LadS is essential for the calcium-dependent Gac/Rsm cascade activation

The identification of LadS being essential for calcium-sensing was key for the further proceedings of this project. We dissected various aspects of LadS such as expression levels, protein stability and domains essential for calcium-sensing.

We assessed the *rsmY* promoter activity in wild-type (black) and Δ*ladS* (red) cells grown in the presence of increasing cation concentrations. As illustrated in Fig 22 both strains show similar expression profiles (increasing or decreasing, respectively) in response to increasing Mg^{2+} and Fe^{2+} levels. This indicates that this effect is independent of LadS. In contrast LadS is essential for calcium-dependent induction of *rsmY* expression, as we have observed before.

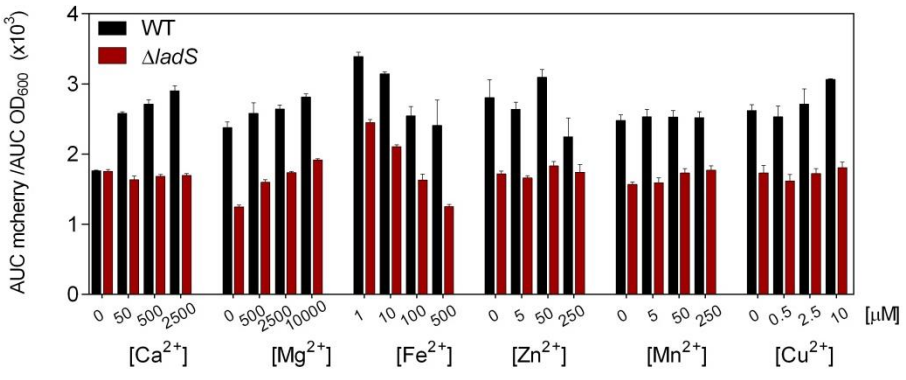

Fig 22. LadS is crucial for calcium-induced *rsmY* expression
Effect of increasing cation concentrations on *rsmY* promoter activity in wild-type and *ladS* mutant strain. Strains were grown overnight in a microplate reader. Absorbance and fluorescence was measured every 30 min. Bars represent the area under the curve (AUC) normalized for growth. Standard deviations are indicated.

5.4.1 LadS levels are increasing over time

Using a chromosomal FLAG-tagged copy of *ladS* we assessed LadS protein levels over the entire growth curve. Therefore, the cells were grown in LB and at the indicated time points samples were taken and analyzed by immunoblot. The data are shown in Fig 23 and indicate that LadS levels are steadily increasing with increasing cell density. This suggests that elevated LadS levels potentially contribute to the activation of the Gac/Rsm cascade during the transition from exponential to stationary phase, especially in a calcium-rich environment.

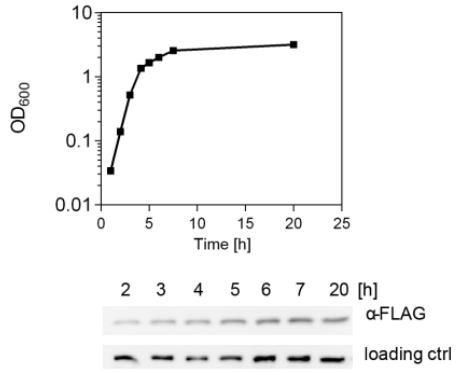

Fig 23. LadS levels are increasing over time
Immunoblot analysis using α-FLAG antibodies of a chromosomal FLAG-tagged *ladS* copy in a Δ*ladS* strain. Cells were grown in LB and samples were collected at the different time points indicated. The corresponding absorbance reading is indicated above. RNA-Pol subunit β served as loading control.

5.4.2 Mutational analysis of LadS

In order to dissect which domains of LadS are important for calcium sensing we analyzed several chromosomally integrated *ladS* mutant version in terms of calcium-dependent *rsmY* expression (Fig 24). The *P. aeruginosa* reference strain PA14 was found to harbor a 49 bp duplication in the *ladS* gene resulting in aberrant cytoplasmic domains [86]. As expected, expression of $LadS_{PA14}$ renders the cells calcium-blind, similarly as a C-terminally truncated version ($LadS_{7TM}$). In line with our previous observations we find that the periplasmic DISMED2 domain is essential for calcium-mediated signal transduction. However, unlike before no increase in overall *rsmY* promoter activity is observed. This is most likely due to the fact that the two DISMED2 mutants were constructed differently. The chromosomally-encoded DISMED2 mutant is deleted for amino acids 28-172 which renders the protein completely instable (Fig 24). On the other hand the more recent plasmid-encoded mutant is deleted for amino acids 19-226 which preserves the orientation of the transmembrane domain architecture and thereby leads to increased protein stability.

In line with previous observations, we find that the histidine residue H428 is absolutely crucial whereas the receiver domain is negligible for calcium-sensing. Lastly, the phenotype of mutants carrying two additional amino acids flanking the DISMED2 domain (RG_{DISM2} and $_{DISM2}QL$) could also be recapitulated. If cells are grown in calcium-free medium both *ladS* variants are partially degraded. However, LadS-RG_{DISM2} in contrast to LadS-$_{DISM2}QL$, can be stabilized by supplementing the growth medium with calcium, which coincides with an increase in *rsmY* promoter activity.

Overall, the finding using chromosomally encoded *ladS* mutant alleles is in accordance with the conclusions from the plasmid-borne alleles, described in the paper draft.

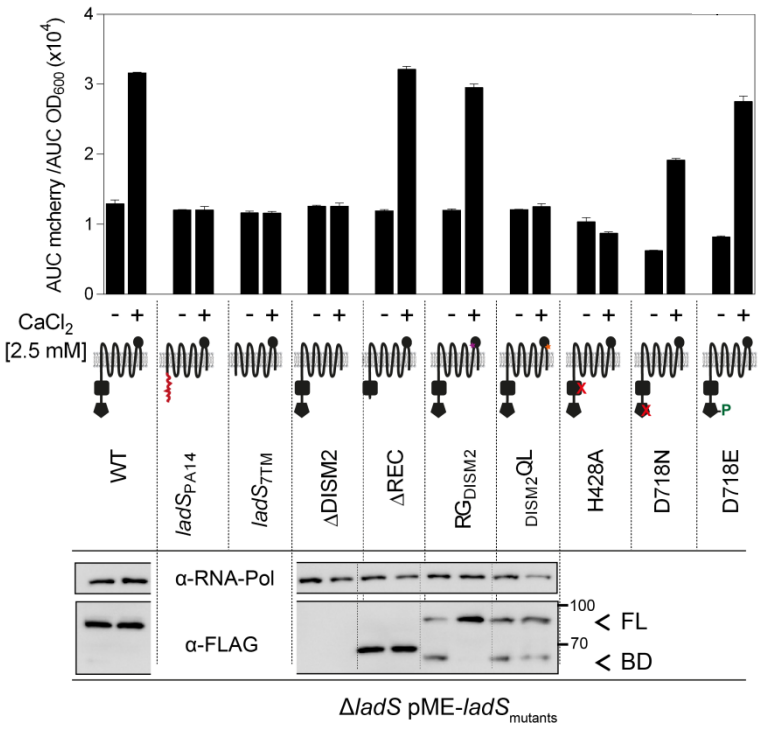

Fig 24. Mutational dissection of LadS
Activity of the *rsmY* promoter in strains expressing different *ladS* mutants under low and high calcium conditions. All *ladS* alleles were chromosomally integrated in a *ladS* mutant strain under the control of the native promoter. Mutant variants are outlined schematically below the graph. Strains were grown overnight in a microplate reader. Absorbance and fluorescence was measured every 30 min. Bars represent the area under the curve (AUC) normalized for growth. Standard deviations are indicated. Immunoblot analysis with α-FLAG antibodies of the corresponding strains is indicated below. Arrows mark full-length LadS (FL) and a breakdown product (BD). RNA-polymerase subunit β served as loading control.

5.4.3 LadS is prone to degradation in the absence of calcium

Calcium induced protein stabilization is to some extent also observable for wild-type LadS. Upon overexpression of *ladS*-FLAG in calcium-free medium we observe a similar degradation product as for LadS-RG_{DISM2}. This breakdown product is absent if the growth medium is supplemented with $CaCl_2$ (Fig 25A).

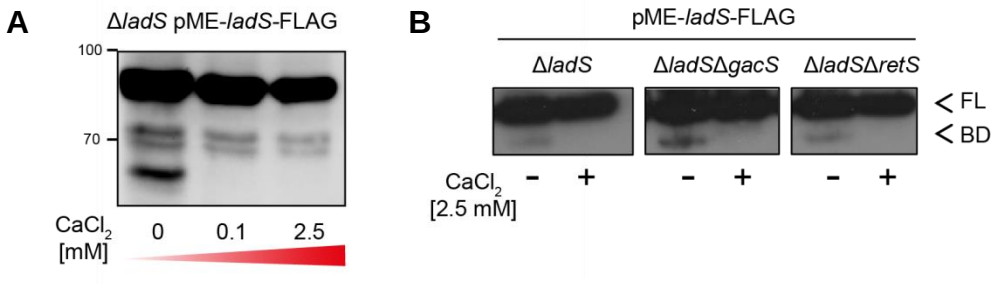

Fig 25. Calcium-mediated stabilization of wild-type LadS
Immunoblot analysis with α-FLAG antibodies of *P. aeruginosa* strains expressing wild-type *ladS* in different strain backgrounds grown in Ca^{2+}-free medium supplemented with 1 mM IPTG and $CaCl_2$ as indicated. Arrows mark full-length LadS (FL) and a breakdown product (BD)

To check if the degradation and/or stabilization is dependent on any of the Gac/Rsm associated kinases, we analyzed the levels of LadS-FLAG in different mutant strain backgrounds. However, the degradation as well as the calcium-dependent stabilization occurs independent from GacS and RetS (Fig 25B).

5.5 Unraveling the molecular details of LadS-mediated calcium-induced signal transduction

We have shown that the histidine kinase activity as well as the periplasmic DISMED2 domain of LadS are absolutely crucial for calcium-mediated signal transduction. In contrast the receiver domain is not required. This suggests that upon signal perception the histidine most likely undergoes autophosphorylation. Further signal transmission then occurs independent of its own receiver domain either by phosphotransfer to another response regulator or by modulating protein-protein interactions. The latter mechanism has been demonstrated for other members of the Gac/Rsm signaling network [94], [194]. To probe for possible connectors between LadS and the Gac/Rsm pathway, several potential interesting candidates were analyzed for calcium-dependent expression of *rsmY.*

Apart from the downstream signal transduction we also lack information about how calcium is sensed and if any co-factors are required. As the DISMED2 domain was shown to adopt a jelly-roll like fold [91], which is typically associated with carbohydrate binding modules, we tested the effect of a variety of carbohydrates on calcium-induced signal transduction.

5.5.1 The ambiguous role of RetS in calcium-mediated signal transduction

RetS is next to LadS one of four proteins in *P. aeruginosa,* which harbors a periplasmic DISMED2 domain. RetS negatively regulates the Gac/Rsm cascade by directly interacting with GacS. Moreover, it has been suggested that RetS is epistatic to LadS as a double mutant phenocopies a *retS* deletion. We made similar observation on the level of dual acute-chronic reporter expression (Fig 26A) as well as on the *rsmY* promoter activity (Fig 26B). In both cases the phenotype of a *retS* mutant is similar to a *retS ladS* double mutant. Overexpression of *ladS* in the presence of calcium, which usually strongly induces the Gac/Rsm cascade has no impact on *rsmY* expression in a *retS* mutant background. This is in line with an epistatic relationship of the two proteins. However, it is also possible that the inactivation of *retS* already maximally activates the signaling cascade. We also tested the impact of *retS* overexpression on calcium-dependent *rsmY* promoter activity and found that the induction strength is reduced compared to wild-type (Fig 26C). Lastly, we performed a mutational analysis of RetS. However, we found that neither the conserved phosphoryl residues nor the DISMED2 domain are required to induce the Gac/Rsm cascade upon calcium stimulation (Fig 26D). Mutating the conserved histidine residue (H424A) as well as deletion of the periplasmic DISMED2 domain led to an increased *rsmY* expression, though this might also be caused by altered protein stability.

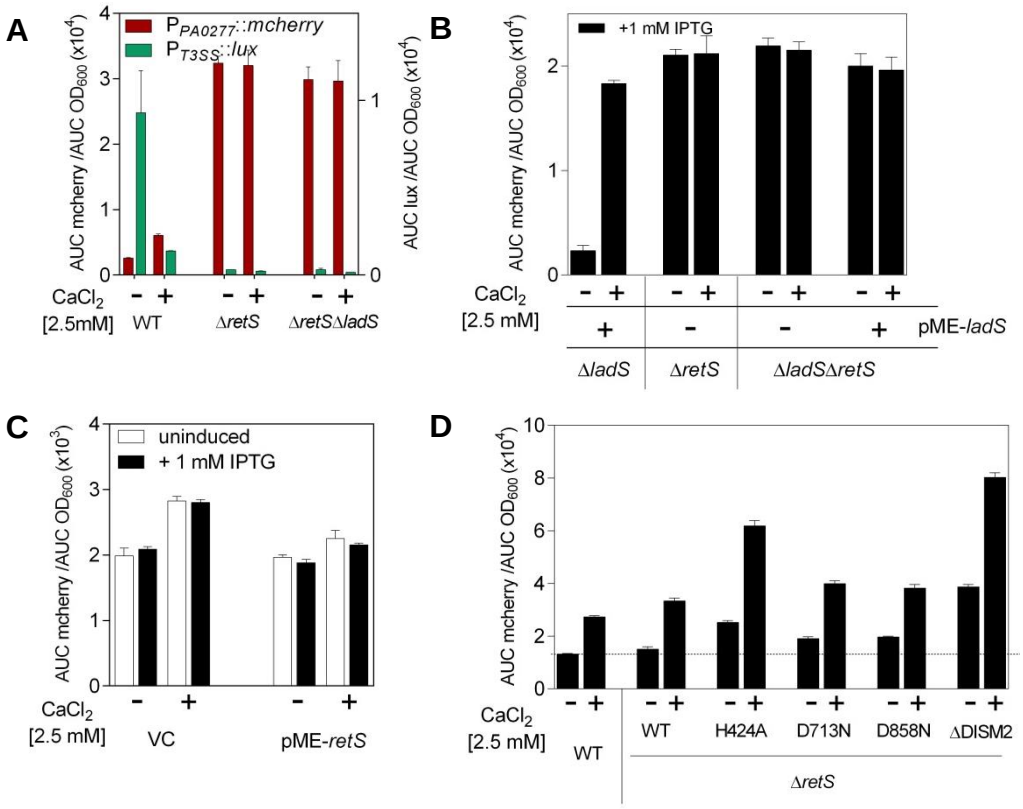

Fig 26. Epistasis analysis of RetS and LadS

(A) Expression profiles of dual acute-chronic reporter IV (acute: P_{T3SS}::*lux* and chronic: P_{PA0277}::*mcherry*) in WT, Δ*ladS* and Δ*retS*Δ*ladS* strains grown under low and high calcium conditions. (B) Calcium-dependent *rsmY* promoter activity upon overexpression of *ladS* in different strain backgrounds. (C) Calcium-dependent *rsmY* promoter activity upon overexpression of *retS* in wild-type cells with the empty vector serving as control (VC). (D) Activity of the *rsmY* promoter in strains expressing different RetS mutants under low and high calcium conditions. All *retS* alleles were expressed under the control from the native promoter chromosomally integrated in a Δ*retS* strain.

Strains were grown overnight in a microplate reader. Absorbance, fluorescence and luminescence was measured every 30 min. Bars represent the area under the curve (AUC) normalized for growth. Standard deviations are indicated.

PA1611 codes for a hybrid histidine kinase, which has been shown to stimulate the Gac/Rsm cascade by directly interacting with RetS. We tested the effect of expressing different *ladS* alleles on the calcium-dependent expression of *rsmY* in a *PA1611* mutant background. Fig 27 shows the expression profiles in a wild-type and a Δ*PA1611* strain background. The absence of *PA1611* causes a decrease in *rsmY* expression compared to wild-type cells, most likely due to the interaction loss with RetS. Overexpression of the different *ladS* mutant alleles resulted qualitatively in very similar expression profiles in both strain backgrounds. A catalytic histidine mutation is calcium blind and point mutations in the receiver domain slightly reduce *rsmY* levels compared to wild-type *ladS*. However, what is striking is that the overall *rsmY* promoter activity in Δ*PA1611* is significantly increased compared to the wild-type background, for all different *ladS* alleles tested. From this we conclude that LadS is more potent to fulfill its function in the absence of PA1611. One possible explanation is that RetS is better accessible for LadS. This would stand in line with LadS being epistatic to RetS.

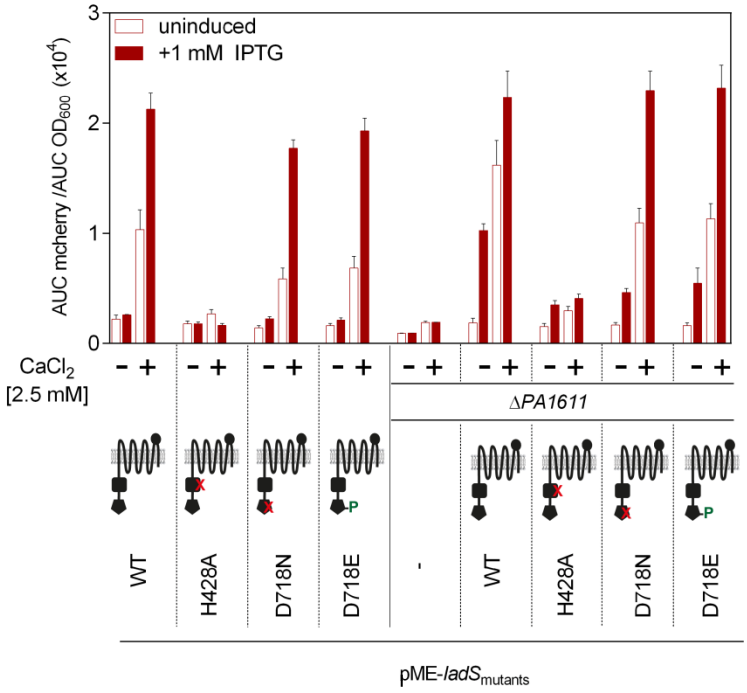

Fig 28. Deletion of *PA1611* renders the cells more responsive to LadS-mediated activation of the Gac/Rsm cascade

Activity of the *rsmY* promoter in wild-type and Δ*PA1611* strain backgrounds expressing different LadS mutants under low and high calcium conditions. All *ladS* alleles were expressed from an IPTG-inducible promoter. Mutant variants are outlined schematically below the graph. Strains were grown overnight in a microplate reader. Absorbance and fluorescence was measured every 30 min. Bars represent the area under the curve (AUC) normalized for growth. Standard deviations are indicated.

Lastly, we tested an alternative readout of the Gac/Rsm cascade activity in order to gather more evidence if RetS is involved in calcium-mediated signal transduction or not. The presence of RetS does not seem to be required for calcium-dependent regulation of ClpV1-GFP (ATPase of the T6SS), as slightly less foci seem to be present in calcium-free medium (Fig 28). However, more quantitative data are necessary to confirm this preliminary result and exclude that it is a T6SS-specific effect.

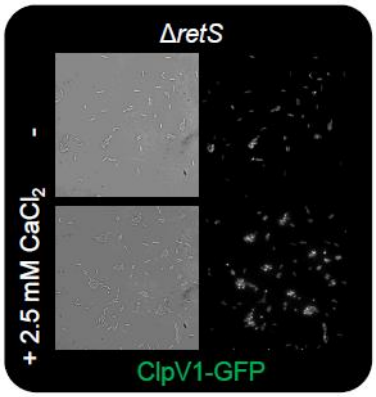

Fig 27. Calcium affects ClpV1-GFP expression in the absence of RetS

Fluorescence microscopy images of Δ*retS* ClpV1-GFP in Ca^{2+}-free minimal medium or medium supplemented with 2.5 mM $CaCl_2$. Image contrast is adjusted to the same levels.

5.5.2 Analysis of putative candidates involved in calcium-sensing or signal transduction

To probe for possible connectors between LadS and the Gac/Rsm pathway, several potential interesting candidates were ordered from the Washington transposon library [195] and analyzed for calcium-dependent expression of *rsmY* (Fig 29). However, none of the strains tested showed an impaired response to calcium, which allows us to exclude several possible signal transduction mechanisms that will be briefly discussed. LadS belongs to the family of hybrid histidine kinases, however signal transmission does not rely on any of the three histidine phosphotransfer proteins (HptA, HptB or HptC). Likewise, the 13 transporters involved in maintaining calcium homeostasis [147] are not required for calcium-mediated signaling. In contrast, respective deletion strains show a slightly enhanced response to calcium.

Moreover, none of the following proteins is required for the calcium-mediated signal transduction:

- PA3973, a transcriptional regulator residing directly downstream of *ladS* on the *P. aeruginosa* chromosome
- HsbR/HsbA (downstream targets of HptB)
- Vfr (global virulence factor regulator)
- Zn^{2+}-responsive TCS CzcSR
- Carbohydrate-binding lectins PA-IL and PA-IIL
- NicD, belonging to the 7TMR-DISMED2 protein family
- Response regulator RocA1
- Periplasmic glucans (NdvB, OpgGH),

Interestingly, mutations in *rocA1, hptB* and *nicD* show elevated *rsmY* expression levels, irrespective of the calcium concentrations. However, the underlying mechanism by which they interfere with the Gac/Rsm cascade is unclear.

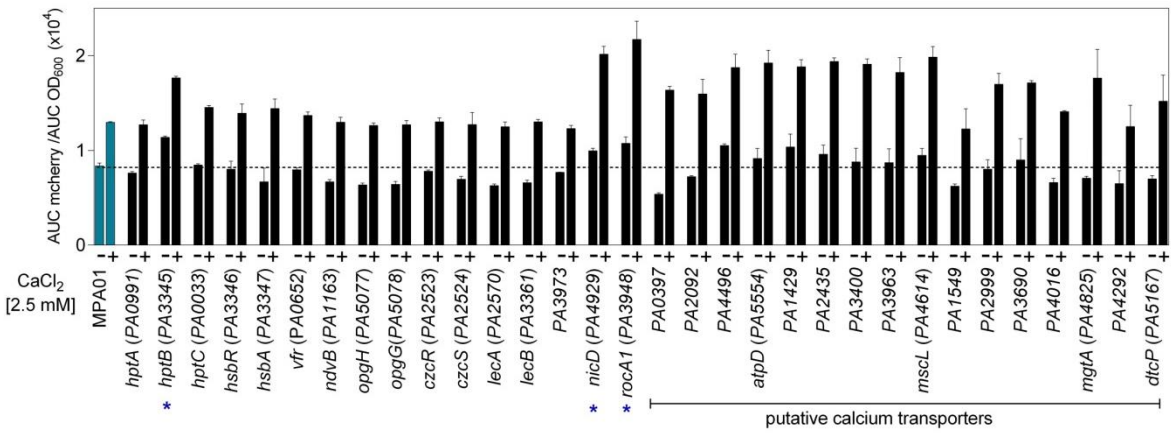

Fig 29. Analysis of potential candidates involved in calcium-induced signal transduction
Activity of the *rsmY* promoter in different transposon mutants under low and high calcium conditions. The parental MPA01 strain serves as reference point (indicated in blue). Asterisks indicate strains with increased *rsmY* promoter activity. Strains were grown overnight in a microplate reader. Absorbance and fluorescence was measured every 30 min. Bars represent the area under the curve (AUC) normalized for growth. Standard deviations are indicated.

5.5.3 Does calcium-induced activation of the Gac/Rsm cascade rely on a co-factor?

The DISMED2 domain of LadS is thought to adopt a similar fold to carbohydrate-binding modules (CBMs) [90], [91]. However, the classical binding pocket is obstructed by a helix generally absent in CBMs. Nevertheless, based on the observation that calcium can enhance the binding of carbohydrates to lectins [175] we tested a range of different carbon sources (provided by Julien Buyck, Bumann lab) in respect to calcium-induced activation of the *rsmY* promoter (Fig 30). One of the supplements was particularly interesting. The addition of L-lysine activated the Gac/Rsm cascade also in the absence of calcium, which we corroborated using L-lysine from our chemical stock. However, supplementing the medium with freshly ordered L-lysine showed no effect on the promoter activity of *rsmY* (data not shown). Together with the fact that the addition of EGTA suppressed the L-lysine mediated activation of the *rsmY* promoter suggests that the old stocks probably contained calcium impurities *e.g.* from the production process.

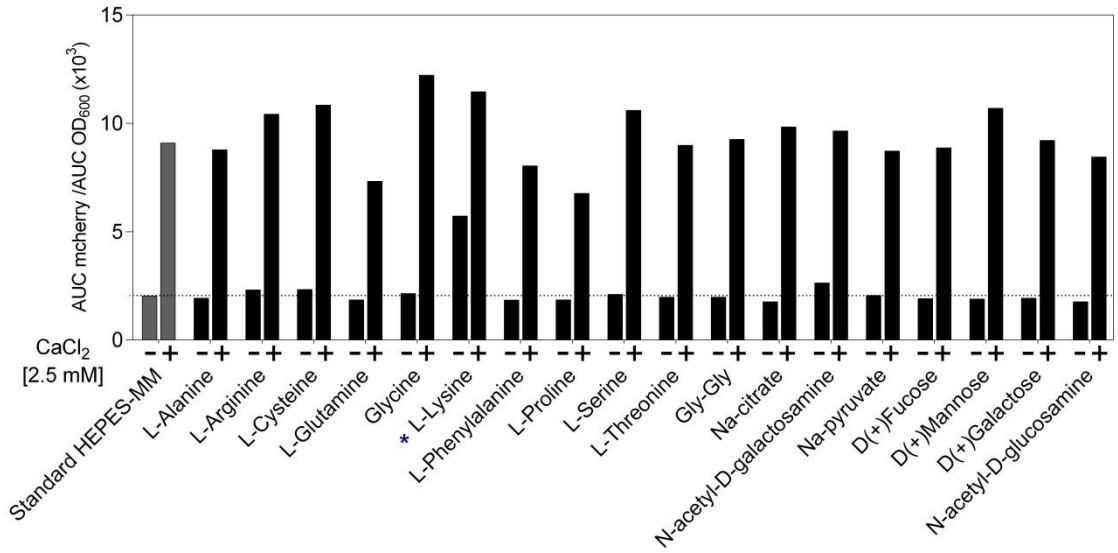

Fig 30. Effect of different carbon sources on the calcium-induced Gac/Rsm cascade activation
Calcium-dependent *rsmY* promoter activity upon expression of *ladS* in Ca^{2+}-free minimal medium supplemented with different carbon sources (10 mM). Strains were grown overnight in a microplate reader. Absorbance and fluorescence was measured every 30 min. Bars represent the area under the curve (AUC) normalized for growth. Standard deviations are indicated.

Previous reports suggested that the Gac/Rsm cascade is induced by high-cell density associated signals, independent from known QS molecules [62]. In line with this study we observe that especially growth-limiting conditions are able to strongly induce the Gac/Rsm cascade (Fig 31A). Comparing the activity profile of the *rsmY* promoter in rich medium (LB) and minimal medium supplemented with sodium succinate (15 mM) shows that the abrupt cessation of growth, illustrated by the kink in the absorbance reading (blue), coincides with a strong induction of *rsmY* expression (red). In contrast, in a nutrient rich environment *rsmY* expression is steadily increasing. We do not think that differences in calcium can attribute for this effect as LB was shown to contain about 150 μM [146] and we have demonstrated that this is sufficient to maximally induce the acute-to-chronic switch (Fig 17B). If the expression levels are normalized for growth it is evident that the Gac/Rsm cascade is less active in cells grown in LB (Fig 31B).

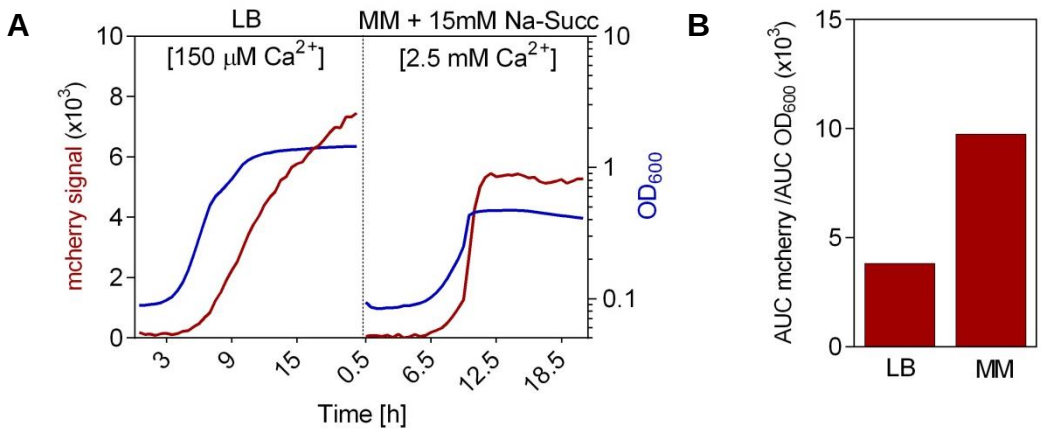

Fig 31. Growth-limiting conditions strongly induce the Gac/Rsm cascade
(A) Raw expression profiles of strains carrying *rsmY* promoter fusions over time of cells grown either in LB (left, calcium levels determined to be 150 µM [146]) or MM + 2.5mM $CaCl_2$ (right). Blue: absorbance, red: fluorescence. (B) Quantification *rsmY* promoter activity. Bars represent the area under the curve (AUC) normalized for growth. Strains were grown overnight in a microplate reader. Absorbance and fluorescence was measured every 30 min.

On the basis of our observation that LadS levels correlate with the strength of calcium activation of the Gac/Rsm cascade, we assume that the protein levels might be adjusted under certain conditions to tune *P. aeruginosa* calcium sensitivity. The identification of transcriptional or translational regulators of *ladS* might be a promising step towards a better understanding in which environmental niches LadS is operative. Using random transposon (Tn) mutagenesis and the following screening strain: Δ*ladS* ctx::*ladS-FLAG* att::P_{ladS}::*gfp-lacZ* we tried to address this question (Fig 32). The transcriptional *ladS* promoter fusion to *lacZ* allows visual screening on X-Gal plates for blue colonies after Tn mutagenesis. The rate of false positive hits can be reduced by analyzing *gfp* expression in the plate reader and LadS protein levels by immunoblot before identification of the insertion site in strains demonstrating both elevated protein levels and transcriptional activity.

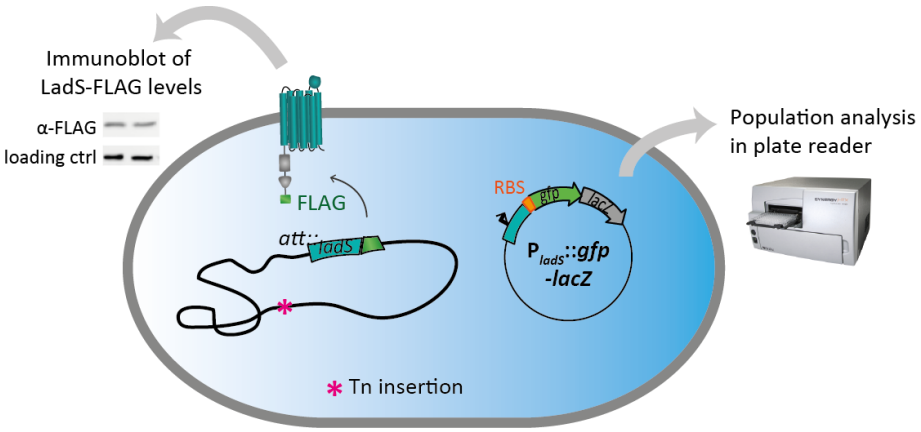

Fig 32. Screening strain to identify regulators of *ladS* transcription by transposon mutagenesis
Genotype of screening strain: Δ*ladS* ctx:*ladS-FLAG* att::P_{ladS}::*gfp-lacZ*. Transcriptional *ladS* promoter fusion to *lacZ* allows an easy visual screening on X-Gal plates for blue colonies after Tn mutagenesis. The rate of false positive hits can be reduced by analyzing *gfp* expression in the plate reader and LadS protein levels by immunoblot before identification of the insertion site.

Several rounds of Tn mutagenesis were performed, however only few blue colonies were obtained. Despite of the fact that none of them showed elevated LadS levels we determined the Tn insertion site for some of the strains. We mainly found genes involved in LPS biosynthesis or periplasmic chaperones confirming the foreboding that all of them account for false-negative hits, as they most likely alter the membrane permeability leading to increased uptake of X-Gal and hence bluer colonies.

5.6 Cell biological aspects of the Gac/Rsm cascade

Cell biological aspects of *P. aeruginosa* are generally not very well studied. Single cell analysis using our developed Gac/Rsm-responsive reporter tools led to the discovery of several interesting features.

5.6.1 Heterogeneous activity of the *rsmY* promoter

The *rsmY* promoter fusion provides a very useful tool to study regulatory mechanisms concerning the Gac/Rsm cascade. Interestingly, analyzing wild-type cells by fluorescence microscopy with a chromosomal integrated version revealed that the expression level varies widely from cell to cell (Fig 33). Similar trends are observable for *rsmZ* transcription (data not shown). This suggests that the heterogeneous activation of the Gac/Rsm cascade is the basis for the observed effect.

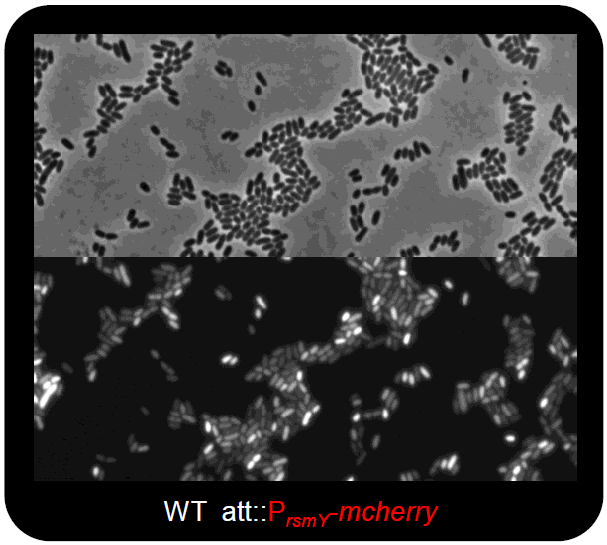

Fig 33. *rsmY* promoter activity varies widely among different cells
Fluorescence microscopy images of wild-type *P. aeruginosa* cells harboring transcriptional *rsmY* promoter fusion integrated into the chromosome. Cells were grown overnight in LB.

5.6.2 Overexpression of LadS does not alter the stochasticity of the *rsmY* promoter

Using population measurements we previously observed that increasing levels of *ladS* in the presence of calcium led to a strong increase in *rsmY* expression. Analysis of single cells by fluorescence microscopy corroborated this finding as the overall *rsmY* promoter activity of a strain overexpressing

ladS (right) is strongly increased compared to a *ladS* mutant (left) (Fig 34). Of note, the heterogeneous activity of the *rsmY* promoter is retained.

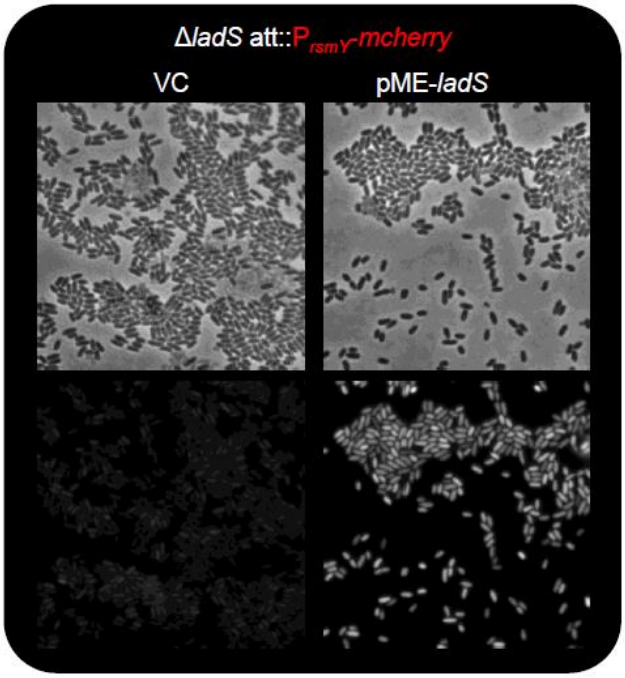

Fig 34. LadS expression leads to overall increased *rsmY* promoter activity
Fluorescence microscopy images of chromosomally integrated *rsmY* promoter fusion (*mcherry*) in Δ*ladS* upon overexpression of *ladS* (right) with the empty vector (VC) serving as control (left). Cells were grown overnight in LB supplemented with the appropriate antibiotics.

As a central player involved in the regulation of virulence factors as well as biofilm formation, the highly variable activity of the Gac/Rsm cascade is very intriguing and might be crucial to rapidly react to environmental changes by priming a subpopulation of the cells. However, so far any knowledge about the biological significance or which factors contribute to the emergence of this variability is missing.

5.6.3 GacS localizes to distinct foci

In order to obtain more information about the regulation of the Gac/Rsm signaling cascade we aimed to identify the subcellular localization of GacS and LadS by fluorescent tags. Unfortunately, the signal for LadS-YFP was below the detection limits. In contrast, GacS-YFP seems to localize to distinct foci (Fig 35A), which dynamically reside close to the poles, but also often move towards mid-cell during cell division. However, a more thorough analysis is required to draw any conclusions about the biological significance of the localization pattern of GacS.
Based on the fact that the intensity of the GacS-YFP foci is very variable and not all cells show foci we asked the question if this might be the missing link to explain the heterogeneous expression of *rsmY*.

However, analysis of a strain expressing a chromosomally encoded *rsmY* promoter fusion and GacS-YFP shows that there is no correlation between GacS foci and *rsmY* expression (Fig 35A). Moreover, also calcium has no impact on the localization pattern (data not shown).

Another question we addressed was if the formation of GacS-YFP foci relies on another Gac/Rsm-associated kinase. As illustrated in Fig 35B the following histidine kinases can be excluded as localization factors: RetS, LadS and PA1611.

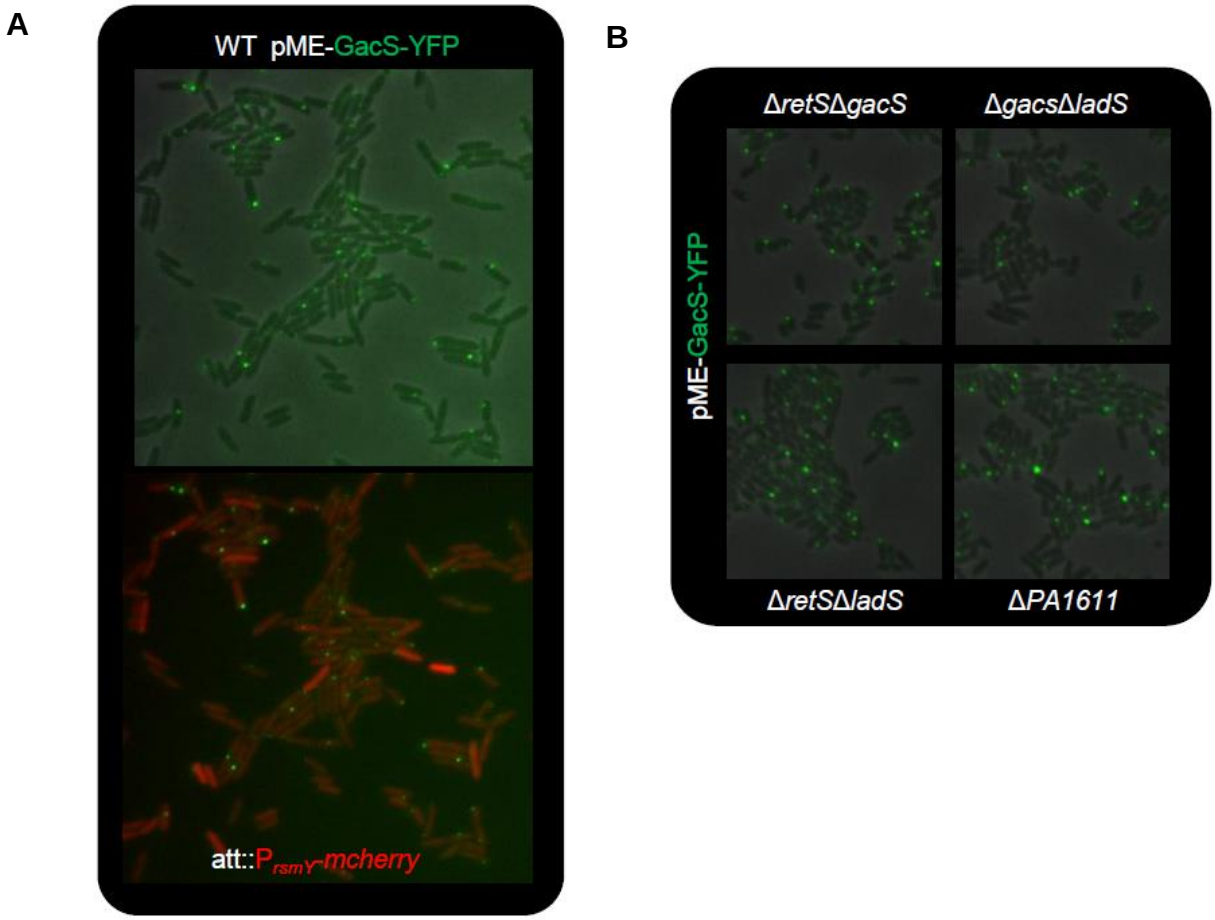

Fig 35. GacS-YFP localizes to distinct foci
Fluorescence microscopy images of strains expressing GacS-YFP (green) (A) Wild type *P. aeruginosa* cells harboring a chromosomal *rsmY* promoter fusion (red) (B) Expression pattern in different mutant strain backgrounds.

Overall, our preliminary observations illustrate the importance of analyzing single cells, which potentially leads to the emergence of new concepts and a better understanding of the behavior of entire populations.

5.7 Supplementary material and methods

Materials and methods not covered in the paper manuscript are summarized in the following.

Bacterial strains and plasmids

Strains used in this study are listed in Table 1 and were grown as described previously. The E79tv2 [196] transducing lysates to create *ΔgacSΔretS* and *ΔgacSΔretS* strains were prepared and used as described previously [197]. Plasmids and oligonucleotides used in this study are listed in Table 1 and Table 2, respectively. Cloning was carried out in accordance with standard molecular biology techniques. Deletion constructs were constructed by SOE-PCR using the following primers: Δ*PA1611*: A-D, Δ*PA3462*: E-H, Δ*efhP*: I-L, Δ*phoQ*: M-P and ligated between *Bam*HI and HindII (Δ*PA1611*, *PA3462*, Δ*phoQ*) or *Eco*RI and HindII (Δ*efhP)* of pEX18-Tc. Deletions were achieved by two-step allelic exchange. After transformation of the resulting vectors into PA01, Tc^R colonies were plated on 5%-sucrose plates to force the formation of double crossovers [198]. Tc^S colonies were tested for successful deletion by colony PCR.

The screening strain for transposon mutagenesis was created via integration of mini-CTX-*ladS-FLAG* into *ΔladS* and subsequent FLP-mediated excisions of the Tc-resistance cassette. Next, the transcriptional P_{LadS}-*gfp-lacZ* was integrated using the pTNS2 helper plasmid into the attTn*7* site.

P_{T6SS}-(RBS)-Cer fusions were produced by SOE PCR using the following primers pairs (and templates) to amplify the individual components for translational or transcriptional fusions, respectively: Q/R or Q/S (gDNA of PA01), T/V or U/V (pCERC-2), W/X for both fusions (pUC18T-mini-Tn*7*T-Gm). Outside primers Q/X were used to create final reporter fusions. PCR products were phosphorylated and cloned blunt into *Eco*RI-*Bam*HI-digested and blunted pME6032. Translational P_{PA0277}-Cer fusion was created by amplifying the *PA0277* promoter region using primers R/S (see section 4.8.2.2) from gDNA of PA01, digest with *Eco*RI and *Hin*dIII and ligation with the *Eco*RI-*Hin*dIII fragment of P_{T6SS}-Cer. P_{rsmA}-mcherry-Tc was created by amplifying the *T6SS* promoter region using primer Aa/Ab from gDNA of PA01, digest with *Xba*I and *Sph*I and ligation with the *Xba*I-*Sph*I fragment of P_{rsmY}-mcherry-Tc. mini-CTX-based promoter fusions were created by amplifying the respective promoter regions using primer pairs Ac/Ad (*ladS),* Ae/Af (*gacS),* Ag/Ah *(gacA)* from gDNA of PA01 followed by digest with *Not*I and *Bam*HI and ligation into the *Not*I-*Bam*HI fragment of mini-CTX-PilV-*gfp*. pME-GacS-YFP and pME-LadS-YFP were created in two steps by first amplifying the respective coding regions using primers Ai/Aj (*gacS)* and Ak/Al (*ladS)* from gDNA of PA01 followed by digest with *Bam*HI and *Kpn*I and ligation into pME6032. Secondly, eYFP was amplified using primers Am/An from pRVYFPC-2, cloned via *Kpn*I into the intermediate vectors and checked for the correct orientation via colony PCR.

pTn*7*T-LadS-PA14 and pTn*7*T-LadS-7TM were created using the following primer pairs (and corresponding templates) Ak/Ap (gDNA of PA14) and Ak/Aq (gDNA of PA01), followed by digest with *Bam*HI and *Kpn*I and ligation into pUC18T-mini-Tn*7*T-Gm. Different LadS mutations were introduced by SOE-PCR with Ak/Ao and pTn*7*T-LadS-FLAG as template. The following internal primers were used: H428A: Ar/As; D718N: At/Au; D718E: Av/Aw; ΔDISM2: Ax/Ay; $_{DISM2}$QL: Az/Ba; RG_{DISM2}: Bb/Bc. After SOE-PCR the inserts were digested with *Bam*HI and *Hin*dIII and ligated into pUC18T-mini-Tn*7*T-Gm. mini-CTX-LadS-FLAG was created by subcloning the cassette via *Bam*HI + *Hin*dIII digest from pTn*7*T-LadS-FLAG into mini-CTX.

pTn*7*T-RetS-His was constructed using primers Bd/Be, followed by digest with *Sac*I and *Hin*dIII and ligation into pUC18T-mini-Tn*7*T-Gm. pTn*7*T-RetS-HA was created by amplifying the insert using Bd/Bf and pTn*7*T-RetS-His as template, followed by digest with *Sac*I and *Hin*dIII and ligation into pUC18T-mini-Tn*7*T-Gm. Different RetS were mutations were introduced by SOE-PCR with Bd/Bf and pTn*7*T-RetS-HA as template. The following internal primers were used: H424A: Bg/Bh; D713N: Bi/Bj; D858N: Bk/Bl; ΔDISM2: Bm/Bn. After SOE-PCR the inserts were digested with *Sac*I and *Hin*dIII and ligated into pUC18T-mini-Tn*7*T-Gm. Release of RetS-HA from pTn*7*T-RetS-HA by *Sac*I and *Kpn*I followed by ligation into pME6032 resulted in pME-RetS-HA.

pTn*7*T-GacS was constructed using primers Bo/Bp, followed by digest with *Bam*HI and *Hin*dIII and ligation into pUC18T-mini-Tn*7*T-Gm. Different GacS mutations were constructed as following using either gDNA of PA01 (♦) or gDNA of *E.coli* MG1655 (◊) as template. Primers to create final fusion are underlined. Δ126: Bo/Bq (♦) and Br/Bp (♦); ΔHAMP: Bo/Bs (♦) and Bt/Bp (♦); ZacS: Bo/Bq (♦) Bu/Bv (◊) and Bw/Bp (♦). All fusions were digested with *Bam*HI and *Hin*dIII and ligated into pUC18T-mini-Tn*7*T-Gm.

pTn*7*T-P_{ladS}::*gfp-lacZ* was created by first amplifying the respective promoter region using primers Bx/By from gDNA of PA01 followed by exchange of P_{cdrA} from P_{cdrA}-*gfp*(Mut3) for P_{ladS} via digest with *Sph*I and *Xba*I. The final vector was constructed by releasing the P_{ladS}::*gfp* cassette via XmaI and *Hin*dIII digest and ligation into pUC18mini-Tn*7*T-Gm-lacZ.

Determination of LadS protein levels over the growth curve

Overnight cultures of Δ*ladS* att::*ladS*-FLAG were 1:200 diluted in the morning in 50 ml LB medium and incubated shaking at 37°C. Cells were harvested at intervals, normalized for cell number and stored in SDS sample buffer at -20°C until immunoblot analysis.

Timing experiment

Stationary cells grown in LB medium (Δ*ladS* att::P_{rsmY}::*mcherry* pME-*ladS*-FLAG) were diluted in the morning in Ca^{2+}-free medium supplemented if necessary with 2.5 mM $CaCl_2$. At the indicated time point the culture was split into Eppendorf tubes, the cells were collected by centrifugation for 1 min at 12'000 rpm and the pellet resuspended in the appropriate medium followed by incubation at 37°C for the desired time. Afterwards, cells were washed once with 1x PBS, resuspended in the desired medium or directly aliquoted (200 µl/well) in triplicates in 96 flat bottom clear plates (Falcon) and fluorescence (mCherry: 580 nm / 620 nm) and OD_{600} were recorded every 30 min for 20 h using a Synergy 2 plate reader (Biotek). The data were analyzed using GraphPad Prism by integrating the area under the recorded curves and subsequent normalization for growth.

Transposon mutagenesis

Random transposon mutagenesis of Δ*ladS* ctx::*ladS*-FLAG att::P_{LadS}::*gfp-lacZ* was performed using the plasmid pALMAR3 carrying a mariner transposon with a tetracycline resistance gene. Strains for bi-parental mating were grown overnight in LB medium, the donor strain (*E. coli* ST18 harboring pALMAR3) at 37°C and supplemented with levulinic acid (50 µg/ml) and the recipient strain (Δ*ladS* ctx::*ladS*-FLAG att::P_{LadS}::*gfp-lacZ*) at 42°C. 1 ml of each culture was collected by centrifugation, washed with LB, combined, washed again and spotted onto a LB plate containing levulinic acid (50 µg/ml). After incubation for 6 h at 37°C, successful transposon insertions were selected by plating on LB plates containing tetracycline (100 µg/ml) and X-Gal (80 µg/ml). Transposon-flanking sequences were obtained by two-step arbitrarily primed PCR. A tenfold diluted culture of the respective strains, boiled for 10 min at 99°C, served as a template for the initial PCR using primers Bz and Ca with a a cycle of 5 min at 96°C followed by 30 cycles of 30 s denaturation at 96°C, 45 s annealing at 38°C, 90 s extension at 72°C and an additional cycle of 7 min final extension at 72°C. Three µl of this PCR reaction was used as template to perform a second PCR using the primer set Bz and Cb, beginning with a cycle of 5 min denaturation at 96°C followed by 30 cycles of 30 s denaturation at 96°C, 45 s annealing at 59°C, 90 s extension at 72°C and a final cycle of 7 min extension at 72°C. PCR products were directly purified using NucleoSpin® Gel and PCR Clean-up (Macherey-Nagel) and sequenced using primer Cc.

5.7.1 Table 1 Bacterial strains and plasmids

Strains	Description	Reference
PA01	WT *P. aeruginosa*	[199]
MPA01	WT *P. aeruginosa* from B. Iglewski (Rochester, NY)	[195]
TTN	WT *P. aeruginosa* received from Tim Tolker-Nielsen	[199] / [200]
BoPh	WT *P. aeruginosa* received from B. Phillip (University of Konstanz)	B. Phillip
MK170	WT *P. aeruginosa* received from Mattick lab	[199]
PAK	WT *P. aeruginosa* from Lory lab	D. Bradley
Δ*phoQ*	Clean deletion of *phoQ* in PA01	This study
Δ*PA1611*	Clean deletion of *PA1611* in PA01	This study
Δ*efhP*	Clean deletion of *efhP* in PA01	This study
Δ*PA3462*	Clean deletion of *PA3462* in PA01	This study
Δ*ladS*	Clean deletion of *ladS* in PA01	This study
Δ*retS*	Clean deletion of *retS* in PA01	This study
Δ*retS*Δ*ladS*	Clean deletion of *retS* and *ladS* in PA01	This study
Δ*gacS*	Transduction with E79tv2 of gacS:: Ω Sp^R/Sm^R from PAO6327 [66] into PA01	T. Jaeger
Δ*retS*Δ*gacS*	Transduction with E79tv2 of gacS:: Ω Sp^R/Sm^R from PAO6327 [66] into Δ*retS*	This study
*ΔladS*Δ*gacS*	Transduction with E79tv2 of gacS:: Ω Sp^R/Sm^R from PAO6327 [66] into *ΔladS*	This study
ΔyfiR	Clean deletion of *yfiR* in PA01	[197]
Δ4xDGCs	Clean deletion of 4 diguanylate cyclases in PA01 (Δ*PA4332* Δ*PA0847* Δ*PA5487* Δ*PA0338*)	T. Jaeger
WT ClpV1-GFP	WT harboring ClpV1-GFP at the native locus	[201]
Δ*retS* ClpV1-GFP	Δ*retS* harboring ClpV1-GFP at the native locus	[201]
WT PA2781::RBS-*gfp*	Transcriptional promoter fusion of *PA2780/1* at the native locus	T. Jaeger
hptA::Tn	Tn insertion in *PA0991* (*hptA*) (phoAwp01q4B04)	[195]
hptB::Tn	Tn insertion in *PA3345* (lacZwp05q4E08)	[195]
hptC::Tn	Tn insertion in *PA0033* (lacZwp06q3B06)	[195]
hsbR::Tn	Tn insertion in *PA3346* (lacZwp04q4B01)	[195]
hsbA::Tn	Tn insertion in *PA3347* (phoAwp07q4C03)	[195]
PA0652::Tn	Tn insertion in *PA0652* (lacZbp03q2G11)	[195]
PA1163::Tn	Tn insertion in *PA1163* (lacZwp05q1G09)	[195]
PA5077::Tn	Tn insertion in *PA5077* (phoAbp02q4H03)	[195]
PA5078::Tn	Tn insertion in *PA5078* (phoAbp01q3C10)	[195]
PA2523::Tn	Tn insertion in *PA2523* (lacZwp01q2G04)	[195]
PA2524::Tn	Tn insertion in *PA2524* (phoAwp01q2D04)	[195]
PA2570::Tn	Tn insertion in *PA2570* (lacZwp03q4H08)	[195]
PA3361::Tn	Tn insertion in *PA3361* (phoAwp07q1E02)	[195]
PA3973::Tn	Tn insertion in *PA3973* (lacZwp07q3E06)	[195]
nicD::Tn	Tn insertion in *PA4929* (phoAwp05q3A12)	[195]
rocA1::Tn	Tn insertion in *PA3948* (phoAwp09q1G08)	[195]
PA0397::Tn	Tn insertion in *PA0397* (lacZbp01q3H11)	[195]
PA2092::Tn	Tn insertion in *PA2092* (lacZwp06q4F01)	[195]
PA4496::Tn	Tn insertion in *PA4496* (phoAbp02q3F02)	[195]
PA5554::Tn	Tn insertion in *PA5554* (lacZwp06q2B01)	[195]
PA1429::Tn	Tn insertion in *PA1429* (lacZbp03q2E05)	[195]
PA2435::Tn	Tn insertion in *PA2435* (phoAwp03q3A02)	[195]
PA3400::Tn	Tn insertion in *PA3400* (phoAwp08q2H11)	[195]
PA3963::Tn	Tn insertion in *PA3963* (phoAwp05q4E02)	[195]

Strains (continued)	**Description**	**Reference**
PA4614::Tn	Tn insertion in *PA4614* (phoAwp09q1E07)	[195]
PA1549::Tn	Tn insertion in *PA1549* (phoAwp01q3G12)	[195]
PA2999::Tn	Tn insertion in *PA2999* (lacZwp06q2D10)	[195]
PA3690::Tn	Tn insertion in *PA3690* (lacZbp02q2C04)	[195]
PA4016::Tn	Tn insertion in *PA4016* (phoAbp01q1E06)	[195]
PA4825::Tn	Tn insertion in *PA4825* (phoAwp10q1F12)	[195]
PA4292::Tn	Tn insertion in *PA4292* (phoAbp03q1A03)	[195]
PA5167::Tn	Tn insertion in *PA5167* (phoAbp01q3F06)	[195]
DH5α	General *E.coli* strain used for cloning	[202]
ST18	pro thi hsdR$^+$ Tp^r Sm^r, chromosome::RP4-2 Tc::Mu-Kan::Tn7/λIpir ΔhemA	[203]

Plasmids	**Description**	**Reference**
pME6032	P_K, 9.8 kb pVS1 derived shuttle vector, Tc^R	[204]
pUC18T-mini-Tn*7*T-Gm	Tn*7* insertion vector, Amp^R, Gm^R	[205]
pUC18mini-Tn*7*T-Gm-lacZ	Tn*7* insertion vector to create transcriptional *lacZ* fusions, Amp^R, Gm^R	[205]
pCERC-2	Template for *cerulean*, Kan^R	[206]
pXVENN-2	Template for *venus*, Kan^R	[206]
pRVYFPC-2	Template for *eyfp*, Kan^R	[206]
pRSET FLIPglu-600u Delta11 Ares-Aphrodite	Template for codon-diversified form of *venus* (=*aphrodite*), Amp^R	[207]
pTNS2	helper plasmid for Tn*7* integration events, Amp^R	[205]
pFLP2	FRT cassette excision vector, Amp^R	[208]
pALMAR3	Insertion vector for Tc^R Mariner transposon	A. Levi
pEX18-Tc	*oriT*+, *sacB*+, gene replacement vector, Tc^R	[198]
Reporter tools		
pME-acu-chro (VI)	pME6032 carrying dual translational promoter fusion (acute: P_{T3SS}::*lux* and chronic: P_{PA0277}::*mcherry*), Tc^R	This study
pME-acu-chro-2 (IV)	pME6032 carrying dual translational promoter fusion (acute: P_{T3SS}::*aphrodite* and chronic: P_{PA0277}::*cerulean*), Tc^R	This study
pME-acu-chro-3 (III)	pME6032 carrying dual translational promoter fusion (acute: P_{T3SS}::*cerulean* and chronic: P_{PA0277}::*aphrodite*), Tc^R	This study
pME-acu-chro-4 (V)	pME6032 carrying dual translational promoter fusion (acute: P_{T3SS}::*gfp*(Mut3) and chronic: P_{PA0277}::*mcherry*), Tc^R	This study
pME-P_{T6SS}-RBS-*Cer*	pME6032 carrying transcriptional *T6SS* promoter fusion to *cerulean*, Tc^R	This study
pME-P_{T6SS}-*Cer*	pME6032 carrying translational *T6SS* promoter fusion to *cerulean* , Tc^R	This study
pME-P_{T3SS}- RBS-*Ven*	pME6032 carrying transcriptional *T3SS* promoter fusion to *venus*, Tc^R	This study
pME-P_{T3SS}-*Ven*	pME6032 carrying translational *T3SS* promoter fusion to *venus*, Tc^R	This study
pME-P_{PA0277}-*Cer*	pME6032 carrying translational *PA0277* promoter fusion to *cerulean* , Tc^R	This study
P_{cdrA}::*gfp*(Mut3)	pUCP22-*Not*I based cyclic di-GMP level reporter, Gm^R	[209]
P_{rsmY}::*mcherry*	pUCP22-*Not*I carrying transcriptional *rsmY* promoter fusion, Gm^R	T. Jaeger
P_{rsmY}::*mcherry*-Tc	pUCP22-*Not*I carrying transcriptional *rsmY* promoter fusion, Tc^R	This study
pTn*7*T-P_{rsmY}::*mcherry*	Tn*7*-integratable transcriptional *rsmY* promoter fusion, Amp^R, Gm^R	This study
P_{rsmA}::mcherry-Tc	pUCP22-*Not*I carrying transcriptional *rsmA* promoter fusion, Tc^R	This study
mini-CTX-PilV::*gfp*	self-proficient integration vector with PilV fragment for constitutive labeling of cells, Tc^R	I. Attree
mini-CTX-P_{gacA}::*gfp*	mini-CTX carrying transcriptional *gacA* promoter, Tc^R	This study
mini-CTX-P_{gacS}::*gfp*	mini-CTX carrying transcriptional *gacS* promoter, Tc^R	This study
mini-CTX-P_{ladS}::*gfp*	mini-CTX carrying transcriptional *ladS* promoter, Tc^R	This study
pTn*7*T P_{ladS}::*gfp-lacZ*	Tn*7*-integratable vector carrying *ladS* promoter fusions to gfp and lacZ, Gm^R	This study

Expression constructs		
pTn*7*T-*gacS*	Tn*7*-integratable vector carrying *gacS* under control of its native promoter, Gm^R	
pTn*7*T-*gacS*-ΔHAMP	Tn*7*-integratable vector carrying *gacS* with a deletion of the HAMP domain under control of its native promoter, Gm^R	This study
pTn*7*T-*gacS*-Δ126	Tn*7*-integratable vector carrying *gacS* with a deletion of the periplasmic loop under control of its native promoter, Gm^R	This study
pTn*7*T-*zacS*	Tn*7*-integratable vector carrying *gacS* with the periplasmic loop of *envZ* under control of its native promoter, Gm^R	This study
pTn*7*T-*ladS*-FLAG	Tn*7*-integratable vector carrying *ladS* from *P. aeruginosa* PA01 under control of its native promoter, 3xFLAG, Gm^R	This study
mini-CTX-*ladS*-FLAG	mini-CTX carrying *ladS* from *P. aeruginosa* PA01 under control of its native promoter, 3xFLAG, Tc^R	This study
pTn*7*T-*ladS*-PA14	Tn*7*-integratable vector carrying *ladS* from *P. aeruginosa* PA14 under control of IPTG-inducible ptac promoter, 3xFLAG, Gm^R	This study
pTn*7*T-*ladS*-7TM	Tn*7*-integratable vector carrying *ladS* without cytoplasmic domains, 3xFLAG, Gm^R	This study
pTn*7*T-*ladS*-H428A-FLAG	Tn*7*-integratable vector carrying *ladS* with a kinase-inactivating H428A point mutation, 3xFLAG, Gm^R	This study
pTn*7*T-*ladS*-D718N-FLAG	Tn*7*-integratable vector carrying *ladS* with inactivating D718N point mutation in REC domain, 3xFLAG, Gm^R	This study
pTn*7*T-*ladS*-D718E-FLAG	Tn*7*-integratable vector carrying *ladS* with phospho-mimetic D718E point mutation in REC domain, 3xFLAG, Gm^R	This study
pTn*7*T-*ladS*-ΔREC-FLAG	Tn*7*-integratable vector carrying *ladS* with a deletion of the receiver domain, 3xFLAG, Gm^R	This study
pTn*7*T-*ladS*-RG_{DISM2}-FLAG	Tn*7*-integratable vector carrying *ladS* with an insertion of 'RG' N-terminally flanking 7TMR-DISMED2 domain, 3xFLAG, Gm^R	This study
pTn*7*T-*ladS*-$_{DISM2}$QL-FLAG	Tn*7*-integratable vector carrying *ladS* with an insertion of 'QL' C-terminally flanking 7TMR-DISMED2 domain, 3xFLAG, Gm^R	This study
pTn*7*T-*ladS*-ΔDISMED2-FLAG	Tn*7*-integratable vector carrying *ladS* with a deletion of the periplasmic 7TMR-DISMED2 domain, 3xFLAG, Gm^R	This study
pME-*ladS*-FLAG	pME6032 carrying *ladS* from *P. aeruginosa* PA01 under control of IPTG-inducible ptac promoter, 3xFLAG, Tc^R	This study
pME-*ladS*-H428A-FLAG	pME6032 carrying *ladS* with a kinase-inactivating H424A point mutation, 3xFLAG, Tc^R	This study
pME-*ladS*-D718N-FLAG	pME6032 carrying *ladS* with inactivating D718N point mutation in REC domain, 3xFLAG, Tc^R	This study
pME-*ladS*-D718E-FLAG	pME6032 carrying *ladS* with phospho-mimetic D718E point mutation in REC domain, 3xFLAG, Tc^R	This study
pTn*7*T-*retS*-His	Tn*7*-integratable vector carrying carrying RetS under control of its native promoter, 6xHis-tag, Gm^R	This study
pTn*7*T-*retS*-HA	Tn*7*-integratable vector carrying carrying *retS* from *P. aeruginosa* PA01 under control of its native promoter, HA-tag, Gm^R	This study
pME-*retS*-HA	pME6032 carrying *retS* from *P. aeruginosa* PA01 under control of IPTG-inducible ptac promoter, HA, Tc^R	This study
pTn*7*T-*retS*-H424A	Tn*7*-integratable vector carrying *retS* with a kinase-inactivating H424A point mutation, Gm^R	This study
pTn*7*T-*retS*-D713N	Tn*7*-integratable vector carrying *retS* with inactivating D713N point mutation in REC1 domain, Gm^R	This study
pTn*7*T-*retS*-D858N	Tn*7*-integratable vector carrying *retS* with inactivating D858N point mutation in REC2 domain, Gm^R	This study
pTn*7*T-*retS*-ΔDISMED2-HA	Tn*7*-integratable vector carrying *retS* with a deletion of the DISMED2 domain, HA-tag, Gm^R	This study
p*wspR19*	pBBR-MCS4 with *wspR19* as *EcoRI-HindIII* fragment	[197]
Deletion constructs		
pEX18-Tc-Δ*ladS*	pEX18-Tc carrying *ladS* deletion cassette as *Eco*RI-*Hin*dIII fragment	This study
pEX18-Tc-Δ*phoQ*	pEX18-Tc carrying *phoQ* deletion cassette as *Bam*HI-*Hin*dIII fragment	This study
pEX18-Tc-Δ*PA1611*	pEX18-Tc carrying *PA1611*deletion cassette as *Bam*HI-*Hin*dIII fragment	This study
pEX18-Tc-Δ*efhP*	pEX18-Tc carrying *efhP* deletion cassette as *Eco*RI-*Hin*dIII fragment	This study
pEX18-Tc-Δ*PA3462*	pEX18-Tc carrying *PA3462* deletion cassette as *Bam*HI-*Hin*dIII fragment	This study
pEXG2-Δ*retS*	*retS* deletion construct	[201]

Localization constructs		
pME-GacS-YFP	pME6032 carrying a GacS-YFP fusion , Tc^R	This study
pME-LadS-YFP	pME6032 carrying a LadS-YFP fusion, Tc^R	This study

5.7.2 Table 2 Primers

Primer	Description	Sequence	No°
A	PA1611 up F	GATCGGATCCCAACGACGACACCCGCCTGC	7093
B	PA1611 up R	CTCGTCCGGTTTCGTTCGACGTACGGATGTCCCAGCCGTG	7094
C	PA1611 down F	CACGGCTGGGACATCCGTACGTCGAACGAAACCGGACGAG	7095
D	PA1611 down R	GATCAAGCTTCTTAGAAGCTGTCAGTGGAG	7096
E	PA3462 up F	GATCGGATCCGGAGCCGCCGATCGACTGCC	7099
F	PA3462 up R	GCTGGCCGAGCAGGCGTTCGCAGCAGAGCAACAGCAGGAC	7100
G	PA3462 down F	GTCCTGCTGTTGCTCTGCTGCGAACGCCTGCTCGGCCAGC	7101
H	PA3462 down R	GATCAAGCTTGTTGGAGACGGAGGAGGGTG	7102
I	EfhP up F	GATCGAATTCCACCAGGTCCGATGCATGCG	7105
J	EfhP up R	CTGGCCTTGCTCCAGCTTGCCGAGCAGGCTGGCGGAAGTC	7106
K	EfhP down F	GACTTCCGCCAGCCTGCTCGGCAAGCTGGAGCAAGGCCAG	7107
L	EfhP down R	GATCAAGCTTCATGGGTGTCGATCTTCAGG	7108
M	PhoQ up F	GATCGGATCCCGAATACCACCACGACCTGG	7123
N	PhoQ up R	GACTGTAGCGAAACGTATGCGGATCACCGGCAGCGCTCGG	7124
O	PhoQ down F	CCGAGCGCTGCCGGTGATCCGCATACGTTTCGCTACAGTC	7125
P	PhoQ down R	GATCAAGCTTGCAGGAGCATCGCGTTGTCC	7126
Q	P_{T6SS} F	GATCGAATTCGGGGGTCAGCTTGTGGTAGC	4841
R	P_{T6SS} R	TCCTCGCCCTTGCTCACCATAAGCTTTACGGCAGCCAGCAAAACGG	4842
S	P_{T6SS} R-2	CTCCTTATAAAGTTAAGCTTACGGCAGCCAGCAAAACGGG	4707
T	Ven/Cer F	ATGGTGAGCAAGGGCGAGGA	4840
U	Ven,Cer F	AAGCTTAACTTTATAAGGAGGAAAACCTATGGTGAGCAAGGGCGAGGA	4705
V	Ven R	CGATACCGTCGACCTCGAACTTACTTGTACAGCTCGTCCATGC	4706
W	T_0,T_1 F	GTTCGAGGTCGACGGTATCG	4709
X	T_0,T_1 R	GATCCCTAGGCCGGGCCGCAAGCTCCTAGC	5656
Y	P_{PA0277} F	GACTGAATTCTCGGCGCCGACCTCGCCTGG	5246
Z	P_{PA0277} R	AAGCTTGGCGGAAAGGGAAAGACGGA	5247
Aa	*Xba*I-pRsmA-F	GATCTCTAGACGGCGCGACAGGGTGAGTGA	6769
Ab	pRsmA-R-*Sph*I	GATCGCATGCGAATCAGCATTCCTTTCTCC	6770
Ac	pLadS-F-*Not*I	GATCGCGGCCGCCATGGCGCGTGAGCTTACC	7442
Ad	pLadS-R-*Bam*HI	GATCGGATCCCAGCCAGTGCCGCATGATGC	7443
Ae	pGacS-F-*Not*I	GATCGCGGCCGCGAACATGGCAGCCAAATAAG	7444
Af	pGacS-R-*Bam*HI	GATCGGATCCCTTGAACACACGTCTCTCC	7445
Ag	pGacA-F-*Not*I	GATCGCGGCCGCGTAGCCGCCGGTGCCAGTGG	7446
Ah	pGacA-R-*Bam*HI	GATCGGATCCCTTAATCACGCTGCACCTCG	7447
Ai	GacS-FP F	GATCGGATCCGAACATGGCAGCCAAATAAG	6753
Aj	GacS-FP R	GATCGGTACCGAGTTCGCTGGAGTCGAGGC	7322
Ak	LadS-FP F	GATCGGATCCCATGGCGCGTGAGCTTACC	7234
Al	LadS-FP R	GATCGGTACCGGCGGACTTGGTGACGATCG	7320
Am	eYFP F	ATTCTTGGTACCATGGTGAGCAAGGGCGAGGAGC	6240
An	eYFP R	ATTCTTGGTACCTTACTTGTACAGCTCGTCCATGCCG	6241
Ao	FLAG R	GATCAAGCTTTTACTATTTATCGTCGTCATCTTTGTAGTCGATATCATGATCTTTATAATC	6509
Ap	LadS-PA14 R	GATCGGTACCTCAGGCGGACTTGGTGACG	7235
Aq	LadS-7TM R	GATCGGTACCTCACCGGTCGGCCAGTGCCAGC	7298
Ar	LadS H428A F	CGCCACCGTCACCGCCGAACTGCGCACC	7404
As	LadS H428A R	GGTGCGCAGTTCGGCGGTGACGGTGGCG	7405
At	LadS D718N F	CGGCGTACTGCTCAACTGCCAGATGCCG	7406
Au	LadS D718N R	CGGCATCTGGCAGTTGAGCAGTACGCCG	7407

Av	LadS D718E F	GGCGTACTGCTCGAGTGCCAGATGCCGG	7408
Aw	LadS D718E R	CCGGCATCTGGCACTCGAGCAGTACGCC	7409
Ax	LadS-ΔDISM2 R	GATCGGATCCCCGCTGGGGCAGTCCATCGAC	7858
Ay	LadS-ΔDISM2 F	GATCAAGCTTTTACTCCTCCAGGTAGGCCTTGG	7859
Az	DISM2-QL R	CCTTCAGCTGGGGCGACCACAGGGTCAG	7588
Ba	DISM2-QL F	CTGACCCTGTGGTCGCCCCAGCTGAAGGCCTACCTGGAGG	7586
Bb	RG-DISM2 R	GACTGCCCCAGCGGCAGGCGGCCGCGCTCGACCTGCTCGTTGAAG	7585
Bc	RG-DISM2 F	CGAGCGCGGCCGCCTGCCGCTGGGGCAGTC	7587
Bd	RetS F	GATCGAGCTCGAGCGCGGCGAGTCCTGACC	7305
Be	RetS-His R	GATCAAGCTTTCAGTGGTGGTGGTGGTGGTGGGAGGGCAGGGCGTCGCCCTGG	7306
Bf	RetS-HA R	GATCAAGCTTTCAGGCGTAGTCCGGCACGTCGTACGGGTAGGAGGGCAGGGCGTCGCCCTGG	7449
Bg	RetS H424A F	GGCCAAGATCAGCGCCGAGATCCGCACGC	7410
Bh	RetS H424A R	GCGTGCGGATCTCGGCGCTGATCTTGGCC	7411
Bi	RetS D713N F	CGTGGTCCTGCTCAACCAGGACATGCCC	7412
Bj	RetS D713N R	GGGCATGTCCTGGTTGAGCAGGACCACG	7413
Bk	RetS D858N F	GACCTGGTGCTGATGAACTGCGAGATGCCG	7414
Bl	RetS D858N R	CGGCATCTCGCAGTTCATCAGCACCAGGTC	7415
Bm	RetS ΔDISM2 R	GAAGGCGTAGGCGGGCTTGGGAACGCTGGAAACGCCG	8230
Bn	RetS ΔDISM2 F	CGGCGTTTCCAGCGTTCCCAAGCCCGCCTACGCCTTC	8231
Bo	GacS F	GATCGGATCCGAACATGGCAGCCAAATAAG	6753
Bp	GacS R	GATCAAGCTTTCAGAGTTCGCTGGAGTCGA	6755
Bq	GacS Δ126 R	CTGGACCCAGGTGAAGTAAC	6756
Br	GacS Δ126 F	GTTACTTCACCTGGGTCCAG CTGCTGCGCGGATATCGCAG	6757
Bs	GacS ΔHAMP R	CATGCGCAGGGCGAGGAGGG	6758
Bt	GacS ΔHAMP F	CCCTCCTCGCCCTGCGCATGGAGGCCTTCGAGCTGGACCTGGCGCGCAA	6759
Bu	ZacS up F	GTTACTTCACCTGGGTCCAGTTCGCGATTTTGCCGAGCCT	6760
Bv	ZacS up R	AGCAAGAGGCTGGTGAACAGCAGCGGAGAGAAATCGCCCT	6831
Bw	ZacS-down-R	AGGGCGATTTCTCTCCGCTGCTGTTCACCAGCCTCTTGCT	6762
Bx	LadS-F-*Xba*I	GATCTCTAGACATGGCGCGTGAGCTTACC	7719
By	LadS-R-RBS-*Sph*I	GATCGCATGCCATAGTTAATTTCTCCTCTTTTCAGCCAGTGCCGCATGATGC	7720
Bz	Arb	GGCCAGCGAGCTAACGAGACNNNNGTTGC	1365
Ca	Almar3-1	AATGCGCAAACCAACCCTTGGCAG	5734
Cb	Almar3-2	CATATCCATCGCGTCCGCCATCTC	5735
Cc	Almar3-3	CGCATCTCGGGCAGCGTTG	5736

6 DISCUSSION AND PERSPECTIVES

Pseudomonas aeruginosa is a clinical highly relevant opportunistic pathogen and the leading cause of morbidity and mortality in people suffering from cystic fibrosis (CF). Moreover, *P. aeruginosa* was classified in 2008 together with *Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii* and *Enterobacter* species as an ESKAPE organism. The ESKAPE bugs are of special importance, not only because they are responsible for the majority of all nosocomial infections, but also because the commonly used antibiotics are not effective against these pathogens anymore. This highlights the urgent need to develop new strategies of combat [210], [211].

The global Gac/Rsm signaling cascade in *P. aeruginosa* is one of the key factors regulating the switch from initial acute to long-term persistent infections of CF airways. Using Gac/Rsm responsive dual reporter tools we found that calcium acts as an environmental trigger activating the signaling cascade. Calcium-mediated stimulation resulted in reduced expression of genes involved in acute virulence such as the T3SS and increased expression of genes that are normally repressed by RsmA and contribute to chronic behavior. Furthermore, LadS one of the Gac/Rsm associated hybrid kinases is absolutely essential to mediate the calcium-induced acute-to-chronic lifestyle switch. In line with a direct effect on the Gac network we found that calcium increased the levels of phosphorylated GacS and induced the expression of the small RNA *rsmY* in a GacA dependent manner. To our knowledge this is the first defined input signal for the Gac/Rsm cascade.

These findings raise several questions. What is the role of calcium in *P. aeruginosa* host persistence? How is calcium sensed? And how is the signal transmitted intracellularly upon activation of LadS?

Calcium is the fifth most prevalent element in our biosphere [128] and a well-established signaling molecule in eukaryotic cells. Likewise, the evidence is growing that calcium also interferes with different processes in bacteria [150]–[154]. In *P. aeruginosa* calcium-binding proteins are involved in diverse processes including maintenance of calcium homeostasis [168], establishment of host cell contacts [174], [175], swimming and twitching motility [169]. Moreover, calcium serves as a co-factor for virulence factors [171] [172]. *P. aeruginosa* cells maintain strict calcium homeostasis involving several redundant calcium transport systems, which adjust the intracellular calcium concentration at about 150 nM, roughly four orders of magnitude below the concentrations found in the extracellular fluid of the human body [128], [130], [147]. We found that calcium-sensing does not represent a general feature of *Pseudomonas* species, but is rather an adaptation to the lifestyle of *P. aeruginosa*. Interestingly, a dysregulated calcium homeostasis is intimately linked to the pathology of CF and various body fluids show elevated calcium levels compared to healthy individuals [180], [181], [183]. Together with our findings that most *P. aeruginosa* isolates from CF airways remained calcium sensitive even after decade-long colonization of CF airways, we postulate that calcium is used as specific signal for *P. aeruginosa* in CF patients driving or maintaining the acute-to-chronic behavioral transition. In line with this, we find that cells with an active Gac/Rsm cascade tend to exit more slowly from stationary phase and have an increased tolerance to antibiotics, both traits that are beneficial for persistent infections. Considering this it is not surprising that under the selective pressure imposed by the host environment, *P. aeruginosa* tends to retain a functional Gac/Rsm cascade, which is also

crucial to ensure fitness and survival in rapidly changing environments. A putative acyl transferase (PA2537) serves as a prime candidate for the growth phenotype. PA2537 is part of the RsmA regulon and its overexpression has been previously shown to cause an increased lag phase [212].

We show that LadS, a Gac/Rsm associated hybrid histidine kinases is essential for calcium-mediated activation of the Gac/Rsm cascade. Increased levels of LadS directly translate into increased activity of the signaling cascade in the presence, but not in the absence of calcium, implying that LadS is directly involved in calcium recognition. Because LadS does not harbor any of the known calcium-binding domains or motifs, the mode of signal recognition remains unclear. LadS belongs to the family of 7TMR-DISMED2 proteins, which was originally identified by an *in silico* screen for eukaryotic G-protein coupled receptors (GPCRs) analogs [88]. CaSR, a member of the eukaryotic GPCRs is directly involved in calcium sensing and has been shown to bind calcium via its extracellular Venus flytrap-like module [139]. However, we did not detect any structural or sequence similarities among the CaSR and the DISMED2 domain of LadS. The periplasmic residing DISMED2 domain adopts a jelly-roll fold, which is reminiscent of carbohydrate-binding modules (CBMs) [90], [91] and is shared by three other *P. aeruginosa* proteins RetS, NicD and PA3462. CBMs are an integral part of carbohydrate-active enzymes such as glycoside hydrolases and help them to cleave insoluble polysaccharides [92], [213]. Apart from the fact that the proteins of the 7TMR-DISMED2 family in *P. aeruginosa* lack any enzymatic activity for carbohydrate degradation, another striking difference is that the DISMED2 domain contains an additional α-helix, which occupies the position where the carbohydrate would bind in classical carbohydrate-binding modules. This suggests that ligands different from carbohydrates might be recognized by DISMED2 [91]. PSIPRED [214] analysis indicates that this α-helix is conserved in all four proteins of the DISMED2 family in *P. aeruginosa*. For two of the 7TMR-DISMED2 family members in *P. aeruginosa* cognate input signals were discovered, which are thought to act via their periplasmic DISMED2 domains. NicD mediates biofilm dispersal in response to glutamate [89] and RetS senses signals related to kin cell lysis, however the physico-chemical nature of this cue is unknown [63].

The following experimental observations suggest that the DISMED2 domain of LadS is also of central importance for calcium-sensing and that calcium most likely does not act via modulating the histidine kinase activity, a mechanism observed for other sensors [215], [216]. i) The presence of calcium stabilizes LadS-RG_{DISM2} harboring two additional amino acids flanking the DISMED2 domain, which coincides with calcium-induced stimulation of the Gac/Rsm cascade; ii) Mutation of a single aspartic acid residue (D80A) renders LadS-RG_{DISM2} calcium-blind; iii) Wild-type LadS also seems to be destabilized in a calcium-free environment and iv) Deletion of the DISMED2 domain renders LadS calcium-blind and constitutively active. Interestingly, the last finding suggests that this domain is important to normally restrain the activity of LadS. Similar observations were made for RetS where deleting the DISMED2 domain renders the bacteria more virulent in an *in vivo* infection model [79]. Likewise, deleting the extracellular linker domain of the *Staphylococcus aureus* SaeS histidine kinase renders the protein constitutively active [217]. We demonstrate that closely related LadS homologues lack the ability to sense calcium. However, grafting the *P. aeruginosa* DISMED2 domain onto an otherwise calcium-blind LadS from *P. fluorescens* is sufficient to restore calcium-sensing. This

suggests that calcium-sensing is not a general feature of DISMED2 containing proteins but that the LadS DISMED2 domain of *P. aeruginosa* has diversified during evolution and adopted novel ligand binding capabilities. Alternatively, calcium-sensing is a more ancient property that was selectively lost in other *Pseudomonas* species.

Intriguingly, calcium ions are frequently associated with lectins, another class of carbohydrate binding proteins. Calcium ions are involved in coordinating the carbohydrate and are required to maintain the correct conformation of its binding site [174], [218]. Several reports also highlight the importance of calcium-binding in CBMs [219], [220]. Therefore, it is tempting to speculate that the DISMED2 domain of LadS has lost the sugar-binding capacity, but has retained the ability to recognize calcium ions. However, it is important to note that we have no evidence that calcium directly binds to LadS. Clearly, additional biochemical and biophysical studies are needed, like *e.g.* inductively coupled plasma optical emission spectrometry (ICP-OES), which assesses the general metal content of purified proteins. Moroever, techniques such as $^{45}CaCl_2$ radiography, isothermal titration calorimetry (ITC) or differential scanning fluorimetry (DSF) will help to substantiate ligand binding. In order to identify the exact binding site amide hydrogen/deuterium exchange mass spectrometry (HDXMS) or proteolytic thrombin cleavage profiles would provide alternative methods to classical site-directed mutagenesis. Thus, at this stage it cannot be excluded that LadS interacts with calcium indirectly.

However, the existence of a *P. aeruginosa* specific proteinaceous co-factor is unlikely, as calcium is able to stabilize the LadS-RG_{DISM2} mutant protein even when expressed in *E.coli.* Based on the homology of the DISMED2 domain to carbohydrate-binding modules and the observation that calcium can enhance the binding of carbohydrates [91], [175], we tested the effect of a variety of carbohydrates on calcium-dependent activation of the Gac/Rsm cascade. However, none of the supplements showed a significant effect on LadS activity. Another interesting co-factor candidate are membrane-derived oligosaccharides, which have been shown to affect the levels of free periplasmic calcium ions in *E. coli* [146]. However, our results indicate that the homologous molecules in *P. aeruginosa* are not required for LadS-mediated calcium sensing.

After signal perception, the second prominent question relates to the intracellular signal transduction mechanisms employed by LadS. While the receiver domain is negligible, all other domains of LadS are essential for signaling. To probe for possible connectors between LadS and the Gac/Rsm pathway, several potential interesting candidates were analyzed for calcium-dependent expression of *rsmY*. Hybrid histidine kinases like LadS generally rely on Hpt phosphotransfer proteins to activate their cognate RRs. However, our findings indicate that none of the three Hpt proteins identified in *P. aeruginosa* appears to be required for calcium-mediated signal transduction. Moreover, we tested several candidates, which are either themselves implicated in cation sensing or are functionally linked to the Gac/Rsm cascade. However, none of the following factors affected calcium-dependent expression of *rsmY*: a transcription factor residing directly downstream of *ladS* on the *P. aeruginosa* chromosome (PA3973), the virulence regulator vfr [221], the cation-sensing HK PhoQ [102], [105], the calcium-binding EF-hand like protein EfhP [168] or the DISMED2-family members NicD and PA3462 [89]. Another prominent candidate we analyzed is RetS, an accessory kinase of the Gac system. RetS, like LadS, harbors a periplasmic DISMED2 domain and interferes with GacS

activity via protein-protein interaction [194]. LadS is thought to be epistatic to RetS, as the phenotype of a double deletion resembles the single *retS* deletion [83]. However, because of the strong activating effect of a *retS* mutation on GacA activity, it is difficult to distinguish between a true epistatic relationship and parallel action of RetS and LadS. The following findings are consistent with the idea that LadS activates the Gac/Rsm signaling cascade via interfering with RetS function: i) Calcium induces GacS phosphorylation levels in a LadS-dependent manner; ii) Overexpression of *retS* reduces calcium stimulation, which is independent of the conserved phosphorylation sites and the periplasmic DISMED2 domain of RetS; iii) Maximal expression levels of *rsmY* in response to calcium are similar to levels reached in a *retS* mutant; iv) Deletion of *PA1611*, coding for another Gac/Rsm-associated kinase that directly represses RetS increases the dynamic range of LadS. Thus, it is possible that LadS acts on RetS, which in turn controls the phosphorylation state of GacS. How LadS phosphorylation contributes to this control and if LadS and RetS (or possibly GacS) interact directly, remains unclear. No interaction was observed between the cytoplasmic portions of LadS and RetS of *P. fluroescens* [45].

Alternatively to this signaling mode LadS might engage in a classical phosphorelay. Our preliminary observation that calcium regulates the levels of the T6SS ATPase ClpV1 independently from RetS is in favor of this possibility. As the receiver domain is negligible for calcium-mediated signal transduction we think that the signal is transmitted directly via the histidine kinase domain. In support of this notion is the fact that many LadS homologues do not contain a receiver domain. This further indicates that the receiver domain might be important to modulate LadS activity, possibly by acting as intramolecular phosphoryl sink or storage. Using Phos-Tag SDS-PAGE we assessed LadS phosphorylation of cells grown under low and high calcium conditions. However, only aspartate phosphorylation was stable enough to be detected. Interestingly, we observed phosphorylation of the receiver domain aspartate in a LadS mutant lacking the primary histidine phosphoryl acceptor. It is thus possible that an alternative HK can target the LadS receiver domain. Alternatively, unspecific phosphorylation via acetyl phosphate could be responsible for this phenomenon [37], [222]. Based on the observation that calcium reduces the phosphorylation of the receiver domain we propose the following signaling mechanism. Under low-calcium conditions LadS retains some basal activity with the receiver domain serving as a phosphate repository, which then in calcium-rich conditions donates its phosphate back to the histidine in the DHp domain and from there to another acceptor protein. Such phosphate sink systems where two RRs compete for the phosphoryl group from a single HK have been shown to accelerate signal termination and present a way to neatly fine-tune the signaling output [223]. Potential acceptor proteins containing a receiver domain include GacA, GacS or any other response regulator (Fig 36). However, based on our findings that GacS is absolutely required for calcium-mediated signal transduction and that calcium stimulation leads to enhanced GacS phosphorylation we exclude GacA as a direct target of LadS. Detrimental cross-talk between individual two-component systems is generally minimized via specific adaptive mechanisms [30], [224]. Nonetheless, the body of evidence for phosphorelay networks with complex signaling architectures is growing. The Roc system for instance comprises one kinase phosphorylating two response regulators [121]. Moreover, RetS seems to interfere with GacS activation through protein-protein interaction [225], which opens up the possibility for novel unconventional signaling modes. We are unaware of

any reports showing cross-phosphorylation between non-homologous histidine kinases, nevertheless we think that it is possible that LadS directly phosphorylates GacS. Alternatively, especially orphan response regulators are considered as likely downstream targets of LadS. Clearly, more experiments, like *e.g.* an unbiased co-immunoprecipitation approach, are required to disentangle the specific requirements for LadS-mediated calcium signaling.

Taken together, we propose that the periplasmic domain of LadS is able to sense calcium ions, either directly or indirectly, which results in signal transduction across the membrane and autophosphorylation activity of the cytoplasmic kinase domain. Phosphate groups may then be transferred to a receiver-domain containing protein, which in turn mediates signaling events finally resulting in the activation of GacS. Alternatively, LadS engages in protein-protein interactions to transduce the calcium-perceived stimuli (Fig 36).

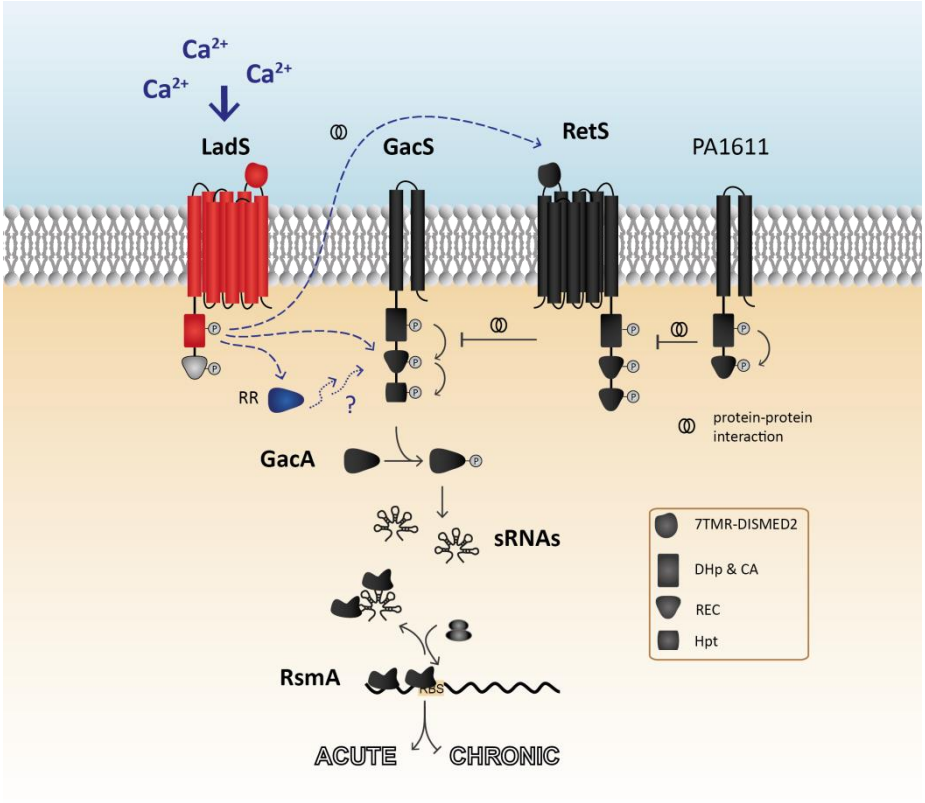

Fig 36. Schematic model of calcium-mediated activation of the Gac/Rsm cascade via LadS

We propose that calcium is sensed by the periplasmic domains of LadS (either directly or indirectly via a ubiquitous cofactor) inducing autophosphorylation of the histidine kinase core. Signal transduction occurs in a receiver-domain independent manner supposedly by phosphotransfer to another receiver domain containing protein *e.g.* a yet unidentified response regulator or to GacS directly. Alternatively LadS might act via modulating protein-protein interactions *e.g.* the interaction of RetS and GacS. All domains of LadS essential for calcium-mediated signal transduction are colored in red and comprise the periplasmic DISMED2 domain as well as the transmembrane helices and the histidine kinase domain.

On the basis of our observation that LadS levels correlate with the strength of calcium activation of the Gac/Rsm cascade, we assume that the protein levels might be adjusted under certain conditions to tune *P. aeruginosa* calcium sensitivity. The identification of transcriptional or translational regulators of *ladS* might be a promising step towards a better understanding in which environmental niches LadS is operative. One candidate could be the transcription factor AmpR, which was originally identified as a

regulator of AmpC β-lactamase [226]. The AmpR regulon was recently expanded to over 500 targets, including *ladS* as well as *rsmZ* that underlie positive control [227]. Based on this it would be interesting to test if the presence of β-lactam antibiotics affects the expression of *ladS*. Moreover, *ladS* was shown to be negatively regulated by the σ-factor RpoN (σ^{54}) in a KinB-dependent manner, however the physiological significance of this observation is unclear [124]. The KinB-AlgB TCS controls mucoidy and acute virulence traits [127], [126]. We tried to use random transposon mutagenesis to identify novel regulatory elements. However, the intrinsically weak *ladS* promoter renders the chromosomally integrated reporter construct unsuitable. Ideas for optimization comprise the use of a plasmid-borne reporter system in combination with a translational instead of a transcriptional readout, which presumably also increases the number of potential targets.

Single cell analysis using our developed Gac/Rsm-responsive reporter tools led to the discovery of several interesting features, which will be briefly discussed. First of all, we discovered that the T3SS promoter is only active in a subpopulation, which is in line with previous reports [228], [229]. Based on the fact that many clinical *P. aeruginosa* isolates are T3SS-negative, Czechowska and coworkers addressed the question if these cells experience a selective advantage *in vivo.* Intriguingly, in the presence of phospholipase A (ExoU) expressing cells they found that T3SS-negative cells have an advantage. As cheaters they can profit form the public good produced by others. About 15-20% of clinical isolates are ExoU positive [230], however, as the *P. aeruginosa* strain PA01 does not express ExoU the significance of the observed heterogeneous expression of the T3SS is unclear.

Interestingly, also the GacA-regulated small RNA *rsmY* is not homogeneously expressed across all cells. This phenotypic heterogeneity of the Gac/Rsm cascade in a clonal population is very striking and suggests that this might be essential to ensure fitness and survival in complex environments. One of the most intriguing questions associated with this finding concerns the underlying mechanism inducing this heterogeneity. Moreover, based on the observation that a derepressed Gac/Rsm cascade is associated with increased tolerance to antibiotics it would be very interesting to challenge *P. aeruginosa* with different drugs and observe their behavior at a single cell level. Our hypothesis would be that cells with higher *rsmY* expression have a higher tendency to survive the drug treatment. Overall, our findings highlight the importance of analyzing single cell behavior as otherwise interesting and potential significant observations might be missed.

Taken together, the suggested follow-up experiments will take us a step closer in understanding how LadS mechanistically activates the global Gac/Rsm cascade in response to calcium. Moreover, the developed reporter tools help us to identify the regulatory modules responsible for the heterogeneous activation of this intricate network. And a better understanding of the environmental factors triggering the acute-to-chronic switch in chronically infected CF airways will hopefully contribute to the discovery of new therapeutic approaches.

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