

## RESEARCH ARTICLE

# Global Regulator MorA Affects Virulence-Associated Protease Secretion in *Pseudomonas aeruginosa* PAO1

Ayshwarya Ravichandran<sup>1,2</sup>, Malarmathy Ramachandran<sup>1☯<sup>‡</sup>a</sup>,  
Tanujaa Suriyanarayanan<sup>1,4☯<sup>‡</sup>b</sup>, Chui Ching Wong<sup>1,2<sup>‡</sup>c</sup>, Sanjay Swarup<sup>1,2,3,4\*</sup>

**1** Metabolites Biology Lab, Department of Biological Sciences, National University of Singapore, Singapore 117543, **2** Mechanobiology Institute, National University of Singapore, 5A Engineering Drive 1, Singapore 117411, **3** NUS Environmental Research Institute (NERI), National University of Singapore, 5A Engineering Drive 1, Singapore 117411, **4** Singapore Centre on Environmental Life Sciences Engineering (SCELSE), Nanyang Technological University 60 Nanyang Drive, SBS-01N-27 Singapore 637551

☯ These authors contributed equally to this work.

<sup>‡</sup>a Current address: Myeloma Research Group, Central Clinical School, Alfred Hospital/Monash University, Level 1 AMREP Building, Commercial Road, Melbourne- 3004, Australia<sup>‡</sup>b Current address: Faculty of Dentistry, National University of Singapore, 11 Lower Kent Ridge Road, Singapore 119083<sup>‡</sup>c Current address: Singapore General Hospital, 20 College Road, Academia, Level 7 Singapore 169865\* [sanjay@nus.edu.sg](mailto:sanjay@nus.edu.sg) OPEN ACCESS

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## Abstract

Bacterial invasion plays a critical role in the establishment of *Pseudomonas aeruginosa* infection and is aided by two major virulence factors – surface appendages and secreted proteases. The second messenger cyclic diguanylate (c-di-GMP) is known to affect bacterial attachment to surfaces, biofilm formation and related virulence phenomena. Here we report that MorA, a global regulator with GGDEF and EAL domains that was previously reported to affect virulence factors, negatively regulates protease secretion via the type II secretion system (T2SS) in *P. aeruginosa* PAO1. Infection assays with mutant strains carrying gene deletion and domain mutants show that host cell invasion is dependent on the active domain function of MorA. Further investigations suggest that the MorA-mediated c-di-GMP signaling affects protease secretion largely at a post-translational level. We thus report c-di-GMP second messenger system as a novel regulator of T2SS function in *P. aeruginosa*. Given that T2SS is a central and constitutive pump, and the secreted proteases are involved in interactions with the microbial surroundings, our data broadens the significance of c-di-GMP signaling in *P. aeruginosa* pathogenesis and ecological fitness.

## Introduction

*Pseudomonas aeruginosa* is a highly versatile opportunistic pathogen for humans and is a major cause of nosocomial infections in immunocompromised patients such as those suffering from cystic fibrosis, pneumonia and skin-burn. It mainly colonizes the respiratory tract,

urinary tract, skin and surgical implants leading to high mortality rates in many cases [1]. Clinical isolates of *P. aeruginosa* are invasive or cytotoxic, with some cytotoxic strains also being inherently capable of invasion to some extent [2, 3]. The three classical stages of infection are (i) bacterial attachment to host cell and its colonization, (ii) local infection by tissue penetration and internalization, followed by (iii) dissemination via bloodstream [4]. The initial stages of tissue penetration and cellular invasion are especially critical for survival of bacteria and establishment of infection [5]. The non-mucoid *P. aeruginosa* PAO1 strain is known to effectively invade host cells and its efficiency of invasion is independent of lipopolysaccharide production or cytotoxicity [6]. While tissue penetration requires cleavage of extracellular matrix proteins and tight junctions, cellular invasion happens mostly through receptor-mediated response by the host [7]. Pathogenic bacteria accomplish these by releasing an arsenal of diffusible factors into the surrounding environment and delivering effector proteins directly into the host cytosol, through virulence-associated secretion systems on the surface. Extracellular proteins including toxins, proteases, lipases and lysins, which get secreted into the culture supernatant, are collectively referred to as the 'secretome'. Given the flexible lifestyles and adaptability of *P. aeruginosa*, it is not surprising that it possesses five out of the six secretion machineries described to date in Gram-negative pathogens [8]. However their copy numbers and functional organization vary depending on the strain and its environment. Hence, it is a good model to study secretion processes and their control mechanisms.

The general secretory pathways in *P. aeruginosa* generally employ a two-step process to secrete proteins into the extracellular medium via a transient periplasmic intermediate. The first step of inner membrane translocation is carried out by the Sec and Tat (co-factor bound proteins) systems [9, 10]. The second step, subsequent transport beyond the periplasm via the type II secretion system (T2SS) is a well-known mechanism [11]. Since the substrates of T2SS include both virulent factors and degradative enzymes, it plays a central role in pathogenesis and adaptation [12–15]. The T2S multi-protein nanomachine, also termed 'secretin', spans both the inner and outer membranes across the periplasm and is highly conserved among Gram-negative bacteria [16, 17]. It is a complex, typically composed of 12 proteins that make-up four subassemblies namely the pseudopilus, the outer-membrane complex, the inner-membrane platform and the secretion ATPase [18, 19]. However, the molecular model of the secretion mechanism is yet to be established [20]. There are four potential T2SS systems in *P. aeruginosa* [21–23], of which the Xcp system is the most studied [24]. In *P. aeruginosa*, the number of assembled secretion machineries is estimated at 50–100 complexes per cell [25] that are polar-localized [26]. Powered by ATPase activity at the inner membrane, the pseudopilus acts as a piston to export proteins from the periplasm through the outer-membrane pore [18, 27]. Exoproteins that use the T2SS are characterized by the presence of a signal peptide at their N-terminus, which gets proteolysed at the periplasm before getting secreted [28–30].

*P. aeruginosa* employs multiple regulatory mechanisms such as two-component systems, transcriptional regulators, sigma factors and small molecule signaling for the coordinate control of its virulence determinants in response to a wide range of environmental cues [31]. These can act at transcriptional, translational or post-translational levels. One such mechanism is the cell-cell communication system called quorum sensing [32], which regulates expression of a considerable number of genes in response to a critical concentration of signal molecules representative of the density of bacterial population [33, 34]. Expression of genes encoding T2SS machinery (*xcp*) [35] and substrate proteins exported through it have been reported to be under the control of two QS systems namely *lasRI* and *rhlRI* [36, 37]. Correspondingly, the extracellular levels of several secreted proteins including T2SS substrates are governed by these QS systems as well [38]. The regulation via QS is complex and is controlled by Vfr, a homologue of *Escherichia coli* cyclic AMP receptor protein (CRP) [39].

Likewise, the signal transduction pathway mediated by second messenger cyclic diguanylate (c-di-GMP) has well-established impact on multifarious virulence mechanisms in a wide variety of bacteria [40–42] including surface transport systems such as flagella biogenesis [43], adhesin production [44, 45] and type III secretion system (T3SS) [46] in *P. aeruginosa*. Evidence exists for varied modes of regulation by c-di-GMP namely transcriptional, post-transcriptional and translational [47–51]. Some recent studies have also established its involvement in regulating secretion machineries. C-di-GMP levels have been demonstrated to be critical for periplasmic processing of adhesin LapA in *P. fluorescens* [52], switching bacterial lifestyles by modulating T3SS and T6SS [53] as well as the Type I secretion machinery of a phytopathogen [54] and linked to type VI secretion system in a fish pathogen [55].

Previously, we have described a membrane-localized motility regulator, MorA, which possesses domains that are involved in the turnover of c-di-GMP, namely diguanylate cyclase (GGDEF motif) and phosphodiesterase (EAL motif). We have shown that MorA controls the timing of flagellar development by restricting flagellin (*fliC*) expression and hence affects motility, chemotaxis, and biofilm formation in *P. putida* PNL-MK25 [56]. In *P. aeruginosa* PAO1, the absence of MorA (PA4601) led to a reduction in biofilm formation [56]. Further investigation by expression profiling revealed the possibility that many surface-associated phenomena, particularly secretion-related genes might be under the control of MorA in *P. aeruginosa* [57]. MorA has also been linked to fimbriae formation in a clinical strain [58]. A recent report has confirmed that MorA is predominantly a diguanylate cyclase (DGC) with some phosphodiesterase (PDE) activity *in vitro* [59]. It is believed that the PDE domain could have a regulatory role *in vivo* likely through dimerization. Thus, we explored whether MorA plays a significant role in regulating *P. aeruginosa* secretome. In this study, we have investigated the effect of MorA on protein secretion in planktonic *P. aeruginosa* PAO1 cultures and show evidence suggesting that MorA negatively controls T2SS-mediated protease secretion, which in turn impacts infection efficiency of *P. aeruginosa*. While the GGDEF domain of MorA is predominantly involved in this regulation, interestingly, the EAL domain also seems to have a similar but lesser effect and both likely occur at the post-translational level.

## Materials and Methods

### Bacterial strains, plasmids and growth conditions

The bacterial strains and plasmids used in this study are described in Table 1. *P. aeruginosa* PAO1 [60] cultures were grown aerobically at 37°C in Luria-Bertani (LB) medium (1.0 Tryp- tone, 0.5% Yeast Extract, 1.0% NaCl; pH 7.0) unless stated otherwise.

A markerless *morA* null mutant was generated by allelic replacement using *pk18mobsacB* [61]. The 5' and 3' flanking chromosomal regions of *morA* gene were amplified using primers with overlapping *Xba*I restriction site and ligated into a single fragment, which was in-frame to exclude the ORF. The resulting fragment was cloned into the *pk18mobsacB* resulting in a suicide construct (pK-MorAflank) and was transformed into *E. coli*. Mobilization into *P. aeruginosa* PAO1 WT was performed by triparental conjugation in 1x M9 minimal medium using pRK 2013 [62] as the helper strain. Selection for single cross-over recombination was done on LB supplemented with Gentamycin. These primary Gm<sup>R</sup> transconjugants were verified by PCR and again grown in the absence of Gm to allow double cross-over recombinations. Upon counter selection on medium containing 5–10% sucrose, the colonies were further tested for loss of Gm resistance. Mutants that were both sucrose resistant and Gm sensitive were screened by PCR using primers that flank the *morA* gene. The products were sequenced to confirm the markerless deletion of *morA*, denoted as  $\Delta morA$  in this study.

**Table 1. Bacterial strains and plasmids used in this study.**

Strain/ Plasmid	Characteristics	Source/ Reference
<b><i>P. aeruginosa</i> strains</b>		
PAO1 WT	Wild-type <i>P. aeruginosa</i> strain	[60]
<i>morA</i> KO	PAO1 with insertional mutation in <i>morA</i> ( <i>morAPa::aacC1</i> ); Gm <sup>r</sup>	[56]
$\Delta$ <i>morA</i>	Markerless deletion mutant of <i>morA</i> in PAO1	This study
$\Delta$ <i>morA</i> -pU	$\Delta$ <i>morA</i> complemented with full length <i>morA</i> (pUPMR)	This study
$\Delta$ <i>morA</i> -pUG*	$\Delta$ <i>morA</i> complemented with full length <i>morA</i> containing GGDEF domain mutation (pUE1060K)	This study
$\Delta$ <i>morA</i> -pUE*	$\Delta$ <i>morA</i> complemented with full length <i>morA</i> containing EAL domain mutation (pUE1189K)	This study
<i>fliC</i> KO	PAO1 with insertional mutation in <i>fliC</i> ( <i>fliC:: Gm<sup>r</sup></i> )	[67]
<i>xcpQ</i>	PAO1 mutant strain PW6222 ( <i>xcpQ</i> -C07:: ISphoA/hah)	PA two-allele library [74]
<b><i>E. coli</i> strains</b>		
BL21	F <sup>-</sup> , <i>ompT</i> , <i>hsdS</i> (r <sub>B</sub> , m <sub>B</sub> ), <i>gal</i> , <i>dcm</i> ; host for protein expression	Laboratory collection
DH5 $\alpha$	F <sup>-</sup> $\Phi$ 80 <i>lacZ</i> $\Delta$ M15 $\Delta$ ( <i>lacZYA-argF</i> ) U169 <i>recA1 endA1 hsdR17</i> (rK <sup>-</sup> , mK <sup>+</sup> ) <i>phoA supE44</i> $\lambda$ - <i>thi-1 gyrA96 relA1</i> ; host for pK18 <i>mobsacB</i> and pK- <i>morA</i> flank	Laboratory collection
<b>Plasmids</b>		
pUPMR	Full-length <i>morA</i> <sub>Pa</sub> gene with native promoter cloned into pUCP19; Amp <sup>r</sup>	[56]
pRK2013	Vector that aids mobilization of plasmid in triparental conjugation, kan <sup>r</sup> Gm <sup>r</sup>	[62]
pK18 <i>mobsacB</i>	Allelic exchange suicide plasmid, sucrose-sensitive, kan <sup>r</sup> Gm <sup>r</sup>	[61]
pK- <i>morA</i> flank	pK18 <i>mobsacB</i> with fusion product containing 5' upstream and 3' downstream regions of <i>morA</i> , kan <sup>r</sup> Gm <sup>r</sup>	This study
pUE1060K	pUPMR with E1060K mutation; Cb <sup>r</sup>	This study
pUE1189K	pUPMR with E1189K mutation; Cb <sup>r</sup>	This study
pETM	Modified pET32 (Novagen) expression vector lacking Trx and S tags	Laboratory collection
pETM-LasB	pETM carrying partial <i>lasB</i> (A <sub>198</sub> -L <sub>498</sub> )	This study

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For the generation of point mutants of *morA* domains (E1060K and E1189K), primers introducing the site-directed mutations were used to generate partial *morA* fragments, which were subsequently fused via PCR amplification using forward and reverse primers for full-length *morA* gene. Primer and gene positions of the partial fragments can be found in [S1 Table](#). Amplicons carrying these mutations were cloned into pUPMR [56] at the *AscI* and *NcoI* restriction sites. These pUPMR plasmids carrying point mutations were subsequently introduced into  $\Delta$ *morA*.

### Extraction of cell-associated protein fraction

Bacterial pellets from cultures used for extra-cellular protein extraction were washed once and resuspended in a maximum volume of 3 ml extraction buffer (50 mM Tris, 1 mM EDTA, 20 mM DTT) with Complete, Mini Protease Inhibitor Cocktail (Roche). Homogenization was carried out in a cell disrupter Micro Smash MS-100 (Tomy Seiko Co. Ltd., Japan) with 0.1 mm glass beads in screw cap tubes at 20 sec pulses of 4,000 rpm repeated about 8–10 times until the pellet was completely disrupted. The tubes were then centrifuged at maximum speed on

tabletop centrifuge for 10 minutes, supernatant saved. This is referred to as the 'cellular fraction' in this article. Equal volumes of protein were loaded for SDS-PAGE.

### Secretome analysis

For analysis of secreted protein levels, the different strains were cultured at 37°C in equal volume Luria-Bertani (LB) medium till they reached the same optical density (OD<sub>600</sub>). For late-log and mid-log phase cultures, the growth time was about 13 hrs and 9 hrs respectively. A physical cell count was also performed using hemocytometer to verify the OD<sub>600</sub> measurement. Trichloroacetic acid (TCA) precipitation method was used to extract extracellular proteins (ECP) from culture supernatants as described previously [63]. Briefly, TCA was added to the cell-free supernatants to a final concentration of 10% w/v and incubated at 4°C for 2–16 hours. The precipitated protein was recovered by centrifuging at 20,000 x g for 5 minutes at 4°C and the pellet washed thrice with ice-cold acetone to remove salts. Upon air-drying, all the pellets were dissolved in equal volume of a denaturing buffer containing 40 mM Tris, 40 mM Dithiothreitol (DTT) and 2% SDS and loaded using a buffer devoid of SDS for PAGE analysis.

Equal volumes of ECP samples were loaded for SDS-PAGE. Further, to account for inaccuracies and ensure protein loading from equal number of cells, minor volume adjustments (5–10%) were made based on the intensity of RNA polymerase band (immunoblot) from the respective cellular fractions. Densitometry of relative levels of protein bands was performed using the image analysis tool ImageJ 1.43 (<http://rsbweb.nih.gov/ij/>). Bands were selected for quantification based on contrast difference with respect to the background. Intensity values obtained as area under curve were converted to relative proportions. Gel strips were then sent to Protein and Proteomics Centre, Department of Biological Sciences, National University of Singapore for identification of individual proteins by using 4800 MALDI TOF/TO Analyzer (Applied Biosystems). Peptides were matched using Mascot search against *P. aeruginosa* PAO1 database of NCBI.

### Elastolytic activity assay

Cell-free supernatants were concentrated 200 times using centrifugal filters (Ultracel-10k, Millipore) and total ECP concentration estimated by Bradford method (Bio-Rad). Elastase activity assay was performed using a modification of a method described previously [64]. For each sample, 5µg protein was added to 20 mg Elastin-Congo red (Sigma) suspended in 1ml of reaction buffer (25 mM Tris pH 7.8, 0.15 mM NaCl, 10 mM CaCl<sub>2</sub>) and incubated with slow rotation at 37°C for 6 hrs. The assay tubes were then centrifuged at low speed to remove insoluble material and absorbance of the supernatants measured at 495nm. *Pseudomonas aeruginosa* elastase (Elastin Products Company, Inc., USA) was used as standard to calculate activity (S1 Fig).

### Membrane protein preparation

The membrane proteins were prepared as described previously [65] with some optimization. Overnight *P. aeruginosa* culture in LB was pelleted down at 5000 g at 4°C for 10 minutes. The cell pellet was washed once with 50mM sodium phosphate buffer (pH 8) and resuspended in the same buffer (8ml per 50ml culture) containing Complete, Mini Protease Inhibitor Cocktail (Roche). Homogenization was optimized to 4 min (12 x 20 sec pulse) of sonication at maximum intensity to ensure complete cell disruption. The homogenized suspension was centrifuged at 1,500 x g at 4°C for 10 minutes to remove cell debris. The clear supernatant was centrifuged again at 125,000 x g at 4°C for 30–45 minutes to separate the membrane fraction as pellet and the cytoplasmic and periplasmic fractions in the supernatant. The pellet was then



dissolved in the extraction buffer with protease inhibitor cocktail (Roche) and stored in  $-80^{\circ}\text{C}$  until further use.

### LasB antibody production

Recombinant LasB corresponding to A<sub>198</sub>-L<sub>498</sub>, which lacks the signal peptides, was expressed in *E. coli* BL21 carrying a modified version of the expression plasmid pET32 (Novagen), pETM. Primer details can be found in [S1 Table](#). A mixture of this recombinant partial protein and secreted LasB extracted from culture supernatants were used for polyclonal antibody production by LAMPIRE Biological Labs. Inc., USA.

### Immunoblot analyses

The XcpP, XcpY and XcpZ rabbit antibodies were generous gifts from Dr. Gerard Michel, CNRS, France [65, 66]. Mouse monoclonal antibody against alpha subunit of *E. coli* RNA polymerase was purchased from Neoclone Biotechnology, WI, USA and used as control. LasB antibody was produced as part of this study. All secondary antibodies were anti-rabbit/mouse IgG conjugated with alkaline phosphatase (Sigma). Immobilon Western chemiluminescent AP substrate (Millipore) was used as substrate.

### Transcriptional analysis by qRT-PCR

Total RNA were extracted from bacterial pellets using TRIzol Reagent (Invitrogen Corp., USA) according to manufacturer's instructions with some modifications. Bacterial cell pellets were mixed by vortexing in TRIzol and heated at  $50^{\circ}\text{C}$  for 10 min prior to RNA extraction to lyse the cells. RNA samples were prepared from early-, mid- and late-logarithmic phases. cDNA was prepared using the Superscript II First-Strand Synthesis System (Invitrogen) as per manufacturer's instructions. RNA concentrations were determined using a Nanodrop (Nanodrop Technologies, USA). Real-time quantitative PCR was performed on an ABI Prism 7700 thermal cycler using SYBR Green PCR Master Mix (Applied Biosystems) as per manufacturer's instructions. Gene-specific primers designed using Primer Express (Applied Biosystems) were used ([S1 Table](#)) and *RpsL*, a gene that encodes a ribosomal protein, was used as the endogenous control. The comparative  $C_T$  method ( $\Delta\Delta C_T$ ) for relative quantification of gene expression was used for calculating fold change. All amplifications were done with three independent growth experiments (biological replicates) and two RNA samples (technical replicates) from each of them.

### Cell culture conditions and invasion assay

Lung fibroblast line MRC-5 (American Type Culture Collection CCL-171) monolayer was grown in Eagle's Minimum Essential Medium containing 10% fetal bovine serum (Hyclone) and penicillin (100 IU/mL)- streptomycin (100  $\mu\text{g}/\text{mL}$ ) at  $37^{\circ}\text{C}$ . For infection with *P. aeruginosa* PAO1 strains, bacterial cells at log phase ( $\text{OD}_{600}$  0.8–1.2) were collected, washed and re-suspended in PBS. Immediately, a cell count was performed to determine the volume of bacterial suspension to infect the host cell culture. After allowing infection for 1 hr, unattached swimming bacteria were washed off using PBS.

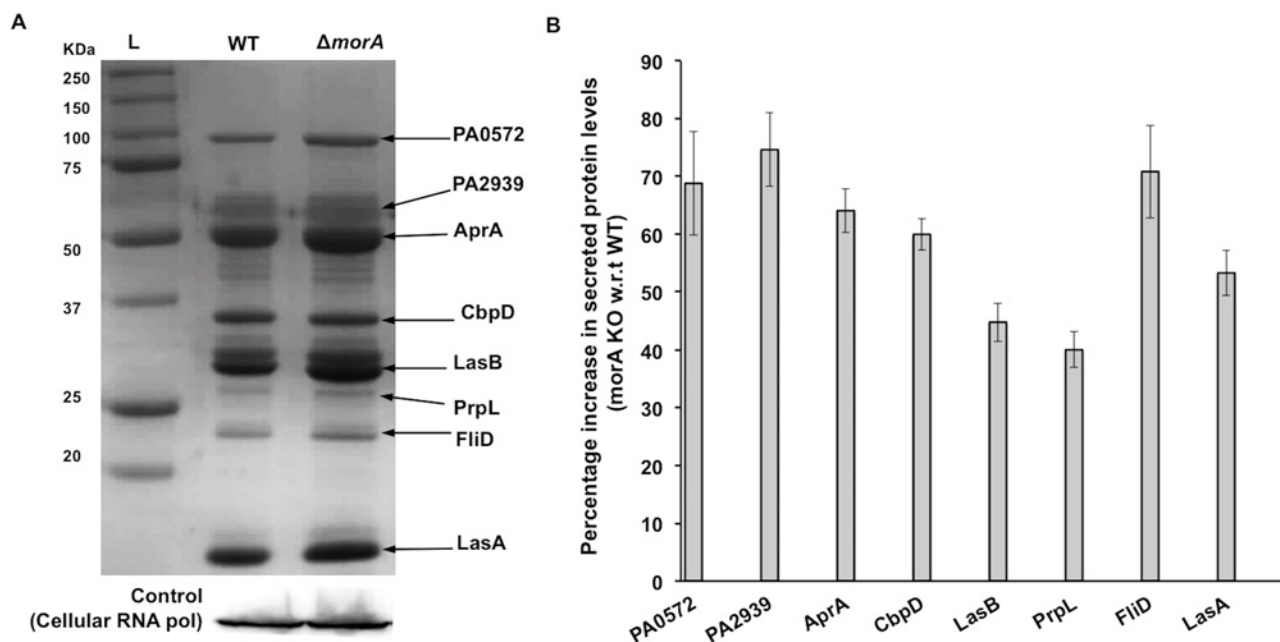
For invasion assay, antibiotics of higher concentration, namely 250 IU/mL penicillin-250  $\mu\text{g}/\text{mL}$  streptomycin was used for efficient killing of bacteria attached to the surface of the host cells. After incubation at  $37^{\circ}\text{C}$  for 1 hr, the cells were washed twice with PBS to remove antibiotics, lysed with 100  $\mu\text{l}$  of 1% TritonX-100 and the contents homogenized by pipetting. This suspension containing the internalized bacteria was serially diluted and plated on LB agar

plates. After ~24 hr incubation in 37°C, viable colony count was performed. Efficiency of invasion was calculated as the difference between the number of bacteria used for infection and the number internalized divided by number of bacteria used for infection. Infection time and multiplicity of infection (MOI) were standardized to achieve optimal conditions that resulted in significant difference in invasion. However, the percentage range of invasion efficiency between the two experimental sets varied, likely due to difference in their scale. Infection was performed in 24-well plates for the set with *fliC* KO control [67] and in 12-well plates for the set with different infection conditions. Hence, the absolute numbers of host and bacterial cells were used based on OD values, which could have led to the variability.

## Results

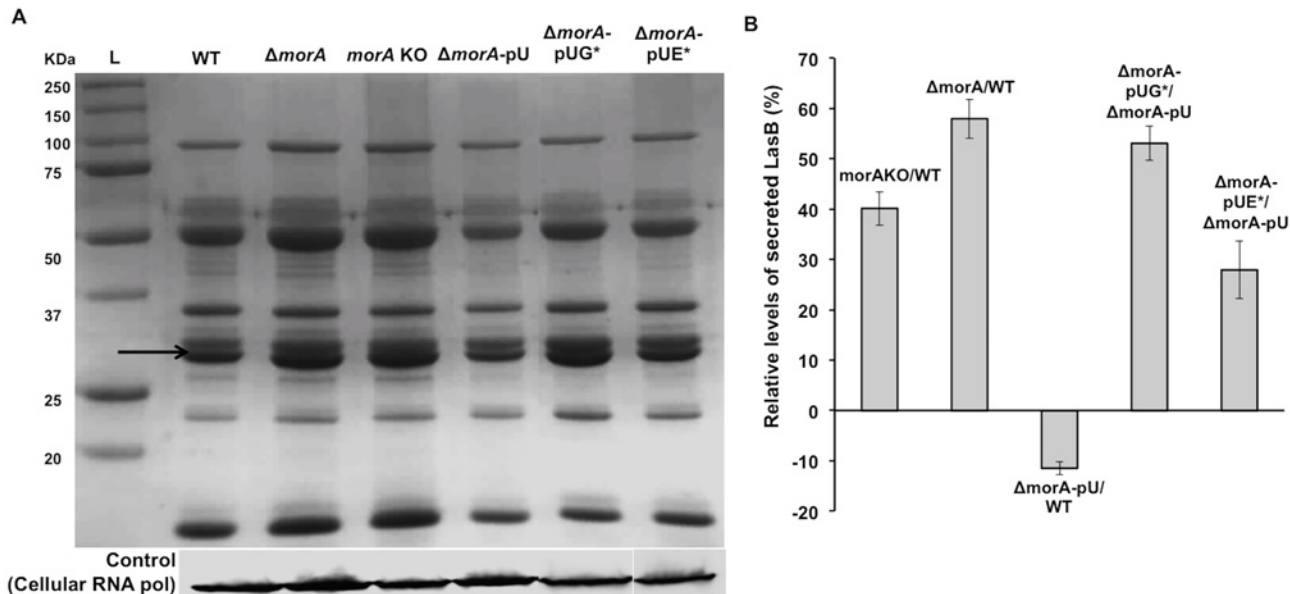
### MorA affects levels of extracellular proteins in *P. aeruginosa* PAO1

Based on our previous findings from gene expression profiling of MorA mutant [57] and other reports showing evidence that c-di-GMP regulating proteins control bacterial secretion systems [45, 46, 52–55], we tested the effect of MorA on the *P. aeruginosa* secretome. Profiles of extracellular proteins of *P. aeruginosa* PAO1 WT and *morA* KO were compared. RNA polymerase  $\alpha$ -subunit levels in cellular fractions from the same samples were used as controls for biomass. Strains lacking MorA had higher overall levels of extracellular proteins than WT in planktonic culture supernatants (Fig 1A). In order to quantitate the levels of extracellular proteins, densitometry analysis of individual bands from the protein profiles was performed. As shown in Fig 1B, there was at least 50% increase in secretion due to *morA* mutation in six out of eight proteins observed. Decrease in levels of extracellular proteins upon *morA* complementation (Fig 2) further validated that changes in their levels were due to MorA expression.



**Fig 1. Levels of secreted proteins are affected by MorA in *P. aeruginosa*.** (A) Top panel—Profiles of total extracellular protein (ECP) from *P. aeruginosa* PAO1 WT and *morA* KO culture supernatants. Samples were loaded based on protein secreted from equal number of cells. Protein bands were identified by MALDI-ToF-ToF. Bottom panel—Immunoblot of RNA polymerase (loading control) from cellular fractions of respective cultures. L—Protein ladder (Bio-Rad). (B) Percentage increase in levels of secreted proteins in *morA* KO compared to WT. Error bars represent mean +SE (n = 3). Student's t-test, p-value < 0.05.

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**Fig 2. Effect on protein secretion by MorA in *P. aeruginosa* is c-di-GMP signaling dependent.** (A) Top panel—Total extracellular protein (ECP) from culture supernatants of *P. aeruginosa* strains loaded based on protein secreted from equal number of cells. Black arrow indicates the position of elastase (LasB) band. Bottom panel—Immunoblot of RNA polymerase (loading control) on cellular fractions of respective cultures in top panel. L—Ladder (Bio-Rad). (B) Percentage increase in levels of secreted LasB based on protein band quantification by densitometry. Band intensity values of MorA insertion (*morA* KO) and deletion ( $\Delta$ *morA*) mutants are compared with wildtype (WT) while those of strains expressing MorA with mutations in GGDEF and EAL motifs (represented as  $\Delta$ *morA*-pUG\* and  $\Delta$ *morA*-pUE\* respectively) are compared with complementation strain ( $\Delta$ *morA*-pU) containing full length *morA* expressed in  $\Delta$ *morA* background. Error bars represent mean +SE (n = 5). Student's t-test, p-value<0.05.

doi:10.1371/journal.pone.0123805.g002

Identity of the extracellular protein bands that showed differences in intensities between strains was established by MALDI-ToF-ToF. Spectra and peptide information can be found in [S2 Fig](#) and [S2 Table](#). Five out of eight identified proteins were proteases. All the five proteases and the chitin binding protein are known to be secreted by the Type II secretion system [24, 68–70]. We, therefore, refer these proteins collectively as secreted proteins or secretome. Elastase LasB, the major protease secreted via T2SS showed nearly 40% increase in *morA* KO than WT.

### MorA controls T2SS secretome levels via its c-di-GMP signaling domains

In order to further ascertain the role of MorA-mediated c-di-GMP signaling in the differential secretion phenotype, secretome profiles of strains carrying MorA deletion strain ( $\Delta$ *morA*) or those expressing MorA with mutations in GGDEF or EAL motifs were analyzed. The band intensity of elastase, the representative protease, was quantified for comparison between the strains.

Both insertion (*morA* KO) and deletion ( $\Delta$ *morA*) mutants revealed similar trends of increase in secreted elastase levels (Fig 2A and 2B). Further comparison of point mutants of GGDEF and EAL domains in the *morA* null background-  $\Delta$ *morA*-pUG\* and  $\Delta$ *morA*-pUE\* respectively, with full length *morA* complementation ( $\Delta$ *morA*-pU) revealed that the GGDEF mutant had higher secreted protein levels than the EAL mutant. The percentage difference in LasB levels for G\* with respect to complementation strain was comparable to that observed for *morA* deletion strain with respect to WT, respectively (Fig 2B). Interestingly, E\* also showed a similar qualitative trend (increase in LasB levels) as that of G\*, albeit of lesser magnitude and



was comparable to that of *morA* insertion mutant with respect to WT. These results provide evidence that the effect of MorA on *P. aeruginosa* secretion is dependent on its enzymatic domains, thereby implicating a role of c-di-GMP signaling in controlling T2SS secretome levels.

### Extracellular elastase activity increases with the loss of MorA

We tested whether the increased levels of extracellular proteases are also associated with increased protease activity. Since elastase is the key activator protease of many other extracellular proteases, its elastolytic activity in the extracellular protein fraction was chosen to be a representative of the cumulative effect of the secreted protease activity. The results showed a statistically significant increase of nearly 33% in the elastase activity in *morA* mutant compared to that in WT, which is consistent with the increase in elastase protein levels (Fig 3A).

### Invasion efficiency correlates with altered secretion due to MorA loss

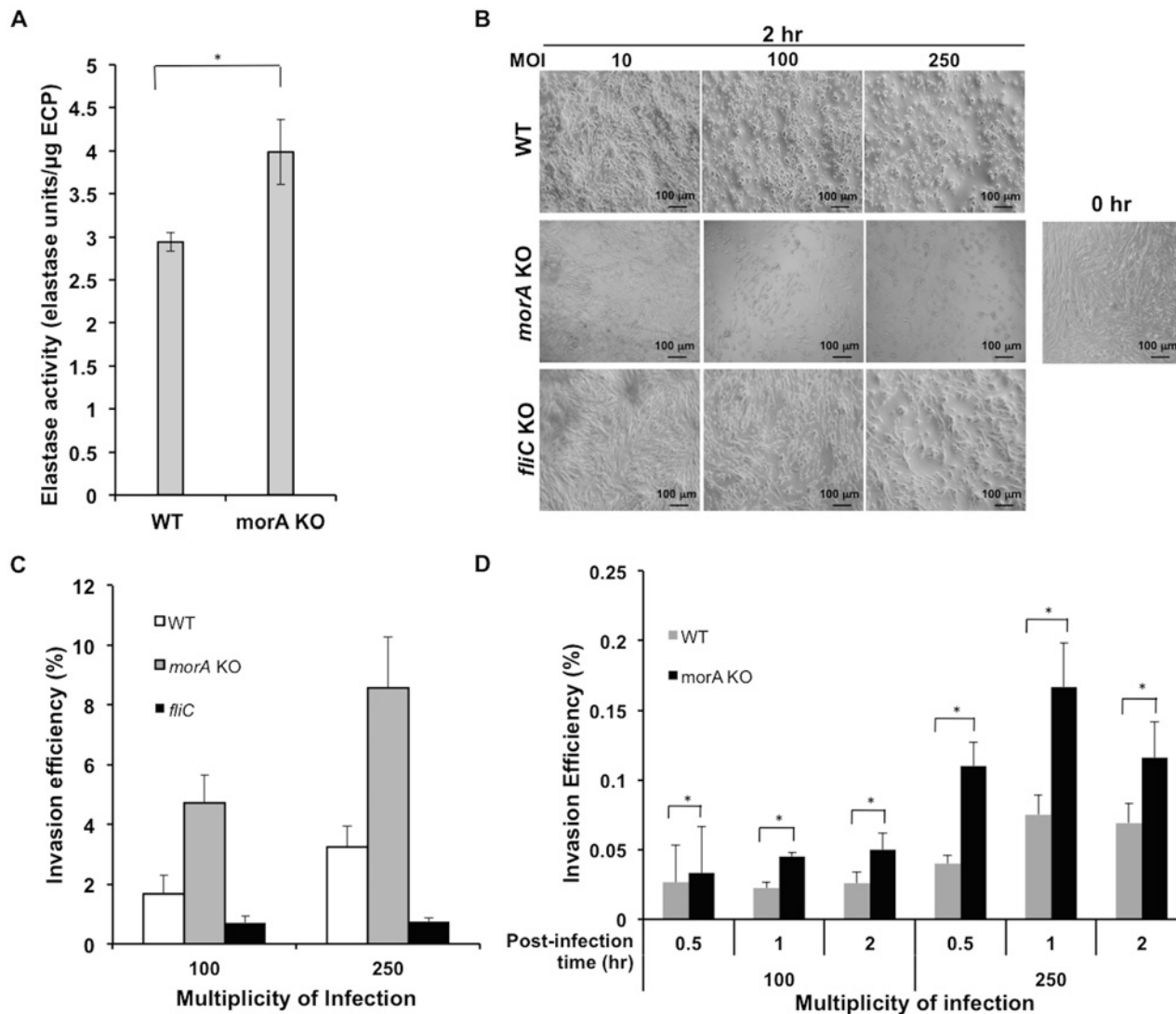
Since protease activity plays a critical role in tissue penetration and colonization of *P. aeruginosa* in the host [71, 72], we tested the biological significance of altered protease secretion by testing the efficiency of bacterial invasion. Lung fibroblast cell line (MRC-5) was chosen as the model for infection by *P. aeruginosa* PAO1. Marked changes in host cell phenotype were evident between adherent cultures infected with WT and *morA* KO. Cell rounding and surface detachment as the proteases act on extracellular matrix (ECM) proteins were higher in mutant infected cultures than WT infected ones (Fig 3B). Since flagellar attachment to the host is also a crucial event preceding invasion, a mutant lacking the flagellar filament (*fliC* KO) was used as negative control for invasion, which exhibited poor attachment and highly limited invasion capacity.

The invasion efficiency of *P. aeruginosa morA* KO was at least two-fold higher compared to WT (Fig 3C). Invasion efficiencies at two different multiplicities of infection over time were calculated to verify the consistency of the effect of MorA signaling on the invasion phenotype (Fig 3D). The range of invasion efficiency due to MorA perturbation varied from 1.5–3 folds in most conditions and was statistically significant ( $p$ -value $<0.05$ ) (Fig 3C and 3D). Thus, the overall trend remained similar across tested conditions.

### Mode of regulation of *P. aeruginosa* T2SS function by MorA signaling

We hypothesized that the possible ways in which MorA might regulate the T2SS secretome levels could be by coordinately affecting: a) the RNA levels of protease genes, b) the cell-associated protease levels, c) the number of T2SS assemblies per cell, or d) the secretion efficiency of the machinery. Each of these hypotheses was addressed. RNA and protein levels of LasB elastase were quantified at different stages of planktonic growth since elastase secretion is known not to be uniform through the growth curve. Under our experimental conditions, secreted elastase was detectable only at the late-log to stationary transition phase. Earlier time points were included to analyze whether proteases accumulate in the cell before effective secretion takes place.

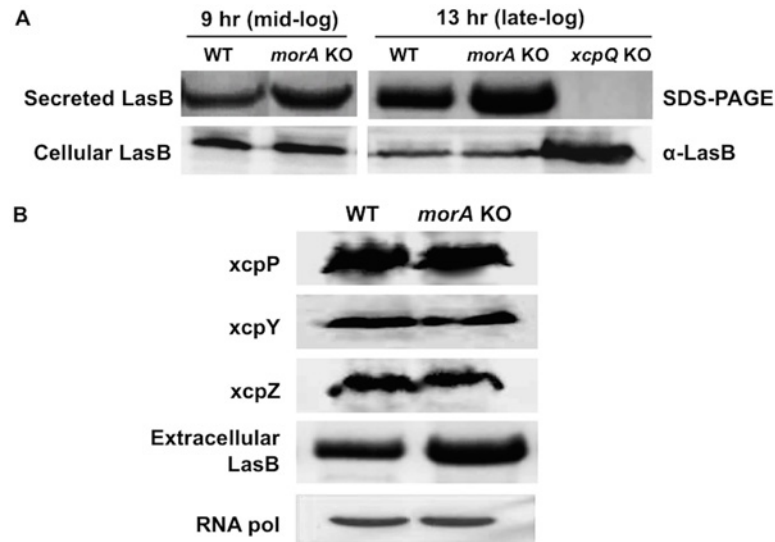
Comparison of expression levels of genes encoding two key secreted proteins (LasB and CbpD) between *P. aeruginosa* PAO1 WT and *morA* KO revealed only a small increase ( $< 2$ -fold change of RNA levels;  $p$ -value $<0.05$ ) due to MorA loss (S3 Fig). It is to be noted that at LasB transcript levels were much lower in *morA* mutant than WT especially at late log phase beyond which secreted elastase was detected in the culture supernatants. At late log phase, the secreted protein levels (~60% increase; Fig 1B) and transcript levels (~30% increase) of CbpD due to *morA* mutation are not directly correlating. Further, unlike the extracellular protein levels, no significant change in the cell-associated levels of LasB was observed over time due to



**Fig 3. Elastase activity, host cell morphological changes and invasion efficiency corroborate with differential protein secretion.** (A) Graph shows total active elastase per unit of total secreted extracellular proteins measured in *P. aeruginosa* PAO1 WT and *morA* KO. Activity was measured using elastin-congo red as substrate. Error bars represent mean +SE (n = 4). \*Student's t-test, p-value<0.05. ECP-extracellular protein. (B) Lung fibroblasts (MRC-5) infected with *P. aeruginosa* WT and mutant strains viewed by differential interference contrast microscopy. Images were captured 2 hours post-infection. MOI- Multiplicity of infection; 0 hr- No infection (control). (C) Graph shows difference in invasion efficiency of *P. aeruginosa* strains on lung fibroblasts (MRC-5) 2 hours post-infection. (D) Effect of MorA on invasion efficiency is consistent over a range of infection time and multiplicities of infection. Error bars represent mean +SE (n = 3). \*Student's t-test, p-value<0.05.

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MorA loss (Fig 4A). Quantification of immunoblot using polyclonal LasB antibody on the cellular fraction confirmed that the difference due to *morA* mutation was less than 5%. A mutant defective in the T2SS outer membrane pore complex protein XcpQ, which is incapable of secretion [73, 74], had significant cellular accumulation, as expected (negative control) (Fig 4A). As there are small differences at RNA level too, a minor contribution of regulatory control at RNA level cannot be ruled out. However, as this does not proportionally reflect in protease production as seen in the cellular fractions, post-translational control seems to be the major control step for T2SS secretion by MorA.



**Fig 4.** (A) MorA does not affect cellular levels of LasB. Top panel- Extracellular LasB from WT and *morA* KO culture supernatants at mid log and late log phases (SDS-PAGE). XcpQ mutant lacks functional T2SS and does not secrete any proteases; negative control. Bottom panel- LasB from cellular fractions of respective cultures immunoblotted using polyclonal antibody. Samples on both panels were loaded based on proteins from equal number of cells as described in methods. **(B) Levels of T2SS machinery proteins remain unaltered.** Immunoblots of the T2SS machinery component proteins from membrane fraction. Membrane proteins were loaded from equal number of bacterial cells. XcpY and XcpZ are inner membrane proteins while XcpP spans both the inner and outer membranes. Respective culture supernatants were loaded to compare secreted LasB levels. RNA pol- RNA polymerase from the cellular protein fraction was used as loading control.

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We postulated that if MorA mutation was responsible for increased secretion by affecting the number of T2SS pumps, then it would be reflected in the corresponding level of T2SS component proteins. In such a case, the number of T2SS assemblies on the bacterial membrane per cell is expected to be higher than in WT. Hence, the levels of three key T2SS structural proteins, two on the cytoplasmic membrane- XcpY, XcpZ and one that spans across both the inner and outer membrane layers- XcpP, were compared in the membrane fraction. None of the proteins tested show significant change in their levels in membrane preparations of *P. aeruginosa* PAO1 WT and *morA* KO strains (Fig 4B). Densitometry analysis also confirmed similar levels of the secretion members (data not shown). Thus, increased expression of proteases or that of the secretion system cannot explain increased Type II secretome levels. These results, therefore, eliminate the above possibilities and strongly suggest that MorA-mediated c-di-GMP signaling likely acts at the level of secretion efficiency of T2SS.

## Discussion

The second messenger c-di-GMP signaling system is a well-known regulator of various cellular processes and virulence determinants in bacteria. Here, we report a novel mechanism for the control of protease secretion via T2SS by the c-di-GMP signaling domains of MorA, which operates largely at the post-translational level in *P. aeruginosa*. Notably, MorA loss results in a substantial increase of elastase (LasB), the major protease secreted by T2SS, in the extracellular fraction. Further, we have shown that the elastolytic activity is also correspondingly high in the extracellular fraction in *morA* mutants. Though the effect of MorA on elastase secretion is lesser compared to that due to loss of its transcriptional regulators [38], this might still have

significant biological effects; the reason being that LasB is critical for the functional activation of several other secreted proteases such as LasA and aminopeptidase [75, 76]. Taken together, these results indicate that effect of c-di-GMP sensor regulator MorA on protease secretion is significant and biologically relevant during *P. aeruginosa* pathogenesis.

All the proteases that show differential secretion levels in this study have well-established roles in the penetration of host tissue and spread of disease [70–72]. LasB, a zinc metalloproteinase, cleaves a variety of host proteins at multiple sites in addition to elastin. Thus, it ruptures the respiratory epithelium by damaging tight-junctions and facilitating neutrophil recruitment. LasA is also a zinc metalloendopeptidase with low elastolytic activity, but a key player as it enhances the elastolytic activity of LasB. It is activated upon processing by LasB, alkaline protease or PrpL (protease IV) [72]. PrpL (PvdS-regulated endoprotease; lysyl class) is a serine protease that can digest casein, lactoferrin, transferrin, fibrinogen, plasminogen and decorin [77, 78]. CbpD (Chitin binding protein) is a non-staphylococcal protein, which is cleaved by elastase and is suggested to act as an adhesion-mediating colonizing factor of eukaryotic cells, although this remains to be proven [68]. The aminopeptidase (PA2939), which is enriched in the outer membrane vesicles (OMVs) of *P. aeruginosa* clinical isolates, may have a role in colonization of the lungs since OMVs are known to activate a significant pro-inflammatory response in lung epithelial cells [79]. Thus this finding highlights that c-di-GMP signaling by MorA affects a major virulence factor in this pathogen, which confers the unique invasive characteristic to it.

Interestingly, in this study, the site-directed mutants of MorA enzymatic domains ( $\Delta morA$ -pUG\* and  $\Delta morA$ -pUE\*) show the same qualitative trend for protease secretion as that of their roles in c-di-GMP turnover [59]. Hence, it is clear that the effect of MorA on T2SS is consistent with its enzymatic function i.e. its ability to regulate c-di-GMP levels. Infection assays show that the increased secretion phenotype due to MorA loss leads to increased invasion efficiency and thus has relevance at its function level. It is well established that flagellar attachment is vital for host cell invasion; thus one may argue that the changes in host cell morphology and invasion efficiency due to MorA loss could have resulted through modulation of the flagellar pathway. However, we have previously demonstrated that *morA* perturbation only led to impairment of biofilm and had no effect on flagellum number [56].

Investigation on underlying mechanisms and intermediary players linking MorA and T2SS has ruled out the secretion machinery components as well as its secreted substrates at the transcriptional and translational level. Based on our current understanding, this is the first report on post-translational level regulation of protease secretion via type II machinery by a second messenger signaling system.

Though we do not know the exact post-translational mechanism of regulation, existing evidence from our laboratory and others enables us to speculate a variety of possibilities. Given that the T2SS secretome is polar-localized [26] in *P. aeruginosa*, it is likely that the effect of c-di-GMP turnover by MorA is local and the signal is transmitted directly to the secretion machinery i.e. at the level of secretion efficiency. This notion of spatial sequestration of c-di-GMP pools for regulation of specific cellular functions is well-known in many bacterial species [51, 80–85]. It is possible that changes in c-di-GMP levels may directly i) alter the activity of inner membrane transport machineries, *sec* and/or *tat* systems; ii) increase the efficiency of ATPase-mediated pseudopilin activity (pushing out the periplasmic proteins through outer membrane ring) or iii) control the periplasmic processing of secreted proteins as in the case of LapD [52, 86]. On the contrary, MorA signaling might have an indirect effect on T2SS through a signaling cascade in association with similar proteins [84, 85] or crosstalk with other regulatory virulence systems that control secretion, such as the pyoverdine system that regulates PrpL [72] and the quorum sensing system as in the case of TbpB in *P. aeruginosa* [87].

Further investigations are required to understand the mechanism by which MorA-cyclic-di-GMP signaling affects secretion. However, c-di-GMP receptors known are very eclectic in nature [47, 51, 88–91], it is difficult to predict possible intermediary players unless experimentally validated. Nevertheless, proteins with predicted c-di-GMP binding canonical motifs can be used to test their roles as receptors in this signaling process. Alternatively, cytoplasmic membrane-localized T2SS structural proteins could also be targeted to test c-di-GMP binding efficiency and/or any post-translational modification.

## Supporting Information

**S1 Fig. Standard curve for elastase activity.**

(PDF)

**S2 Fig. MALDI-ToF-ToF spectra of secreted proteins affected by MorA.**

(PDF)

**S3 Fig. RNA levels of major secreted proteases show no significant change due to MorA.**

(PDF)

**S1 Table. List of primers used in this study.**

(PDF)

**S2 Table. MALDI-ToF-ToF identification of *P. aeruginosa* secreted proteins affected by MorA.**

(PDF)

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## Author Contributions

Conceived and designed the experiments: AR SS. Performed the experiments: AR MR TS. Analyzed the data: AR MR TS. Contributed reagents/materials/analysis tools: CCW. Wrote the paper: AR SS.

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