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DNA alternate polymerase PolB mediates inhibition of type III secretion in *Pseudomonas aeruginosa*

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Abstract

Opportunistic pathogen *Pseudomonas aeruginosa* uses a variety of virulence factors to cause acute and chronic infections. We previously found that alternate DNA polymerase gene *polB* inhibits *P. aeruginosa* pyocyanin production. We investigated whether *polB* also affects T3SS expression. *polB* overexpression significantly reduced T3SS transcription and repressed translation of the master T3SS regulator ExsA, while not affecting *exsA* mRNA transcript abundance. Further, *polB* does not act through previously described genetic pathways that post-transcriptionally regulate ExsA. Our results show a novel T3SS regulatory component which may lead to development of future drugs to target this mechanism.

Keywords

Pseudomonas aeruginosa; Type III secretion; stress

1. Introduction

Pseudomonas aeruginosa is a deadly pathogen that can be acquired from the environment or hospitals and can cause a vast array of infections, including keratitis, acute pneumonia, bacteremia, and burn infections [1]. One of the worst manifestations of *P. aeruginosa* infections is in CF lungs where it causes acute and chronic infections and is nearly impossible to eradicate due to its intrinsic antimicrobial resistance and ability to form biofilms [2, 3]. The versatility and severity of *P. aeruginosa* infections is attributed to the many virulence factors that are used to destroy host cells, including pyoverdine, pyocyanin, and the type III secretion system (T3SS) [4]. During the acute phase of infection, *P. aeruginosa* uses T3SS, a macromolecular syringe-like structure, to inject toxins that ultimately destroy the host cell. The T3SS effector toxins ExoU, ExoS, ExoT, and ExoY induce cell death by disrupting the host cell cytoskeleton, cell membrane, and cAMP levels thereby enhancing disease severity [1].

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5. Conflict of Interest

The authors state that they have no conflicts of interest.

Regulation of T3SS activity consists of complicated genetic networks that tightly regulate this critical virulence factor. The T3SS regulon in *P. aeruginosa* contains ~40 genes that are all controlled by the master regulatory protein ExsA [5]. During non-inducing conditions, ExsA is bound and inhibited by ExsD, the anti-activator, while ExsC is inhibited by ExsE [5]. Low calcium concentration, the presence of serum, and host cell contact are factors known to increase ExsA levels and initiate T3SS expression [5]. Activation of this system leads to a partner-switching mechanism where ExsD binds to the anti-anti-activator ExsC, therefore liberating ExsA to bind to T3SS promoter sites and initiate transcription [5]. It is important to note that ExsA also regulates its own transcription, via the $P_{exsCEBA}$ promoter site, resulting in a positive feedback loop [5]. Regulation of *exsA* transcriptional activation is also stimulated by Vfr, a global regulator of virulence factor expression that is controlled by cAMP concentrations [6]. Additionally, *exsA* expression is post-transcriptionally regulated by the RNA binding protein RsmA. RsmA levels in the cell are in turn regulated by small non-coding RNAs RsmY and RsmZ, which bind to this protein and titrate it away from its mRNA targets [7]. Environmental factors also influence ExsA inhibition. For example, the magnesium transporter MgtE can inhibit *exsA* post-transcriptional activity via inhibition of RsmA through RsmY and RsmZ [8]. Additionally, stress response genes such as the alternative sigma factor AlgU (AltT) has been shown to downregulate T3SS expression [9]. Overall, a general hallmark of T3SS gene expression is that it is downregulated under stressful conditions [9].

Previously, our laboratory found that mutation of *polB* (*PA14_40120*), encoding a stress-induced DNA polymerase, decreases biofilm formation and increases cytotoxicity by 70% compared to wild type by enhancing pyocyanin levels [10]. While the full impact of PolB on *P. aeruginosa* behavior is unclear, one study showed that it is homologous to the DNA repair system Pol II in *Escherichia coli* and is activated during DNA damage [11]. Considering that conditions for T3SS inhibition and PolB activation match (*i.e.* stress), we hypothesized that PolB could also inhibit T3SS activity. This study shows that *polB* overexpression leads to a decrease in T3SS promoter activity via post-transcriptional inhibition of the T3SS activator ExsA. This inhibition is *exsD* and *rsmYZ* independent, suggesting an alternate post-transcriptional ExsA inhibitory pathway.

2. Materials and Methods

2.1 Bacterial strains and culture conditions

Experiments were carried out in *P. aeruginosa* strains PA14 and PA103 [8]. Strains and plasmids are described in Table 1. Expression of *polB* was performed by transforming the strains with plasmid p*polB*, containing the *polB* gene downstream from the P_{araBAD} promoter on vector pMQ72 [10]. This plasmid is maintained at high copy number in *P. aeruginosa* [12], and induction of the P_{araBAD} promoter is not required for expression in *P. aeruginosa* [10]. Plasmids were maintained in *E. coli* S17 [13] cultured on LB agar plates or LB containing 10 μg/mL gentamicin. Isolation of the plasmid from *E. coli* was accomplished by using the QIAprep spin miniprep kit (Qiagen) according to the manufacturer's instructions, and plasmids were transformed into *P. aeruginosa* strains using electroporation. Transformed *P. aeruginosa* strains were cultured on Vogel Bonner minimal (VBM) medium

plates with 60 μg/mL gentamicin [14]. Plasmid presence was confirmed by PCR primers p729 and p730 [15].

2.2 β-Galactosidase assays

Strains were grown to an OD_{600} of 1.0 and β-galactosidase activity was measured as previously described [14], using T3SS transcriptional reporters PA14 P_{exsD}-*lacZ* and PA103 P_{exsD}-*lacZ* (each containing the *exsD* promoter fused to a promoterless *lacZ*) [13, 14] and ExsA translational reporter PA103 P_{lacUV5}-*exsCEBA'-'lacZ* (containing *lacZ* fused in-frame at *exsA* codon 77) [14]. The substrate orthonitrophenyl-β-D-galactopyranoside (ONPG) was used in all β-galactosidase assays involving transcriptional reporters. For translational reporters, chlorophenol red β-D-galactopyranoside (CPRG) was used as the substrate. 2mM EGTA was added in all experiments to induce T3SS activity [8]. Data were normalized to the average pMQ72 value (as 100%); there was very little variation in pMQ72 samples, and thus error bars are very small.

2.3 RNA isolation and qRT-PCR

Strains were cultured as described above and 1 mL of culture was collected at an OD_{600} of 1. Cells were pelleted and washed with 1X PBS and RNA was isolated using the RNeasy Plus Kit (Qiagen) according to the manufacturer's instructions. Modifications were made to the protocol as described earlier [15]. cDNA was synthesized using the Superscript III First-Strand Synthesis for RT-PCR kit (Invitrogen; Eugene, OR), according to the manufacturer's instructions. DNA contamination was tested by performing cDNA synthesis in the absence of reverse transcriptase; this control indicated the absence of DNA in our RNA samples (not shown). Quantitative Real-time-PCR (qRT-PCR) was performed as previously reported using the LightCycler 480 Instrument (Roche) [15]. Primers exsARTfor and exsARTrev [8] were used to amplify *exsA* from nucleotides 436 to 676. Samples were normalized to the *fbp* transcript using primers PA5110for and PA5110rev, as described [15]. For semiquantitative RT-PCR, cDNA was subjected to standard PCR with primer pairs QpolBfor/QpolBrev and P5110for/5110rev [10], with the following thermocycler conditions: 95°C 5 min., 25X(95°C 1 min., 55°C 30 sec., 72°C 1 min.), 72°C 10 min. PCR reaction was then electrophoresed through a 1% agarose gel.

2.4 Strain construction of PA103 Δ*exsD*

Isogenic *exsD* deletion was carried out using plasmid pSMC296, as previously described [16]. This plasmid was maintained in *E. coli* S17 and conjugated into PA103 P_{exsD}-*lacZ*. Exoconjugants were selected on LB plates containing 80 μg/mL gentamicin and 20 μg/mL nalidixic acid. Isolated colonies were grown overnight in LB and then plated on LB agar containing 10% sucrose to select for excision of the plasmid. Deletion mutants were confirmed using primers ExsDfor (GCGACATGAGCATCGTCGAC) and ExsDrev (CAGCAACAGGACGCTCTGTC).

3. Results

3.1 *polB* inhibits T3SS activity in PA14 and PA103

β-galactosidase assays were used to measure transcriptional activity of T3SS in PA14 P_{exsD}-*lacZ* and PA103 P_{exsD}-*lacZ*. Though ExsD can inhibit T3SS activation through protein-protein interactions, its gene is the first in one of the longest T3SS operons, and the P_{exsD}-*lacZ* construct has been used extensively to report T3SS transcriptional activation [5, 6, 8, 14, 16]. In PA14, *polB* overexpression resulted in a 20% reduction in β-galactosidase activity at the T3SS promoter site compared to PA14 with empty control vector pMQ72 (Fig. 1A). Similarly, in PA103, a strain with hyperactive T3SS activity, *polB* overexpression led to 45% less β-galactosidase activity from the T3SS promoter site than PA103 with empty control vector (Fig. 1B). Importantly, expression from p*polB* led to dramatic increase in *polB* transcript levels, compared to strains with vector control (Fig. 1C).

3.2 *polB* inhibits T3SS activity via post-transcriptional regulation of *exsA*

To determine the mechanism by which *polB* inhibits *exsA* activity, qRT-PCR was used to measure relative *exsA* transcript levels. Compared to PA103 with empty vector, *polB* overexpression did not lower *exsA* transcript abundance (Fig. 2A). To measure *exsA* translation, β-galactosidase activity was determined from a PA103 P_{lacUV5}-*exsCEBA'-'lacZ exsA* translational reporter [14]. ExsA translational activity was significantly reduced when *polB* was overexpressed in PA103 P_{lacUV5}-*exsCEBA'-'lacZ* (Fig. 2B). These results indicate that *polB* expression inhibits ExsA synthesis by affecting *exsA* translation while not impacting *exsA* mRNA turnover.

3.3 *polB* bypasses the *rsmYZ* and *exsD* mediated inhibition of ExsA

Post-transcriptional inhibition of ExsA is mediated through the RsmYZ/RsmA pathway. RsmA can be sequestered by RsmYZ, leading to decreased ExsA translation. However, overexpression of *polB* in PA103 Δ*rsmYZ* with the T3SS P_{exsD}-*lacZ* transcriptional reporter still led to inhibition of T3SS activity (Fig. 3A). To investigate another potential effect of *polB* on ExsA post-transcriptional activity, *exsD* was isogenically deleted in PA103 containing the T3SS P_{exsD}-*lacZ* transcriptional reporter. ExsD binds and sequesters ExsA, so Δ*exsD* strains exhibit highly active T3SS [5, 16]. When *polB* was overexpressed in the PA103 Δ*exsD* strain, T3SS transcriptional activity was still inhibited (Fig. 3B). This data further indicates that *polB* acts on ExsA synthesis, rather than acting through functional ExsA activators/inhibitors.

4. Discussion

Virulence in *P. aeruginosa* is tightly regulated. It is not surprising that 8% of the *P. aeruginosa* genome codes for multiple regulatory genes and pathways, including those that respond to various environmental signals [17]. Some of these regulatory mechanisms include downregulation of virulence factors in response to stress, most likely as a survival mechanism for this microorganism. For example, the alternative sigma factor AlgU (AlgT), which is expressed during chronic infection state and oxidative stress, downregulates T3SS expression [9, 18]. Similarly, *P. aeruginosa* isolates from CF patients exhibit a high mucoid

phenotype due to mutations in *mucA*, further enabling this microorganism to survive and cause chronic infections [19]. Furthermore, metabolic dysregulation and nutritional stress also inhibit T3SS [20]. This current study reveals a novel role for stress-related gene *polB* in downregulating T3SS expression. *polB* encodes an alternate DNA polymerase that is activated during DNA damage. Prior research showed that *polB* mutation decreased biofilm formation and increased cytotoxicity through stimulation of pyocyanin levels [10]. Here, we found that overexpression of *polB* in *trans* showed a downregulation of T3SS activity in PA14 and PA103 (Fig. 1) [14]. It is important to note that deletion of *polB* in PA14 and PA103 had no effect on T3SS expression (not shown), most likely because *polB* has minimal expression under homeostatic conditions (Fig. 1C). Thus, we used plasmid based *polB* expression as a surrogate to mimic what happens under stressful conditions when *polB* expression is high. It is possible that use of antibiotics to maintain plasmids could confer a level of intrinsic stress to the system, though we found very little *polB* transcription in pMQ72 samples, which were also treated with antibiotics (Fig. 1C). In contrast, transforming *P. aeruginosa* with p*polB* resulted in high *polB* transcript levels (Fig. 1C).

This study highlights an important *exsA* inhibitory pathway. *polB*-mediated downregulation of T3SS activity is due to post-transcriptional ExsA inhibition (Fig. 2). The exact mechanism of this inhibition is unclear. Though RsmY and RsmZ inhibit ExsA translation [10, 14], ExsA inhibition was still seen in the Δ*rsmYZ* strain (Fig. 3). ExsD also failed to show an effect in this process (Fig. 3). Because *polB* inhibited translation (Fig. 2), it likely does not additionally influence *exsA* activity through Vfr or the autoregulatory transcriptional pathway. Due to PolB's role in error prone DNA replication during stress [11], and the accumulation of mutations in *P. aeruginosa* during prolonged stress exposure [9, 21, 22], we speculate that PolB-induced mutation during stress could be activating expression of regulatory factors that repress ExsA translation, or it could be inhibiting expression of genes that encode ExsA translational activators. It would be interesting in future experiments to test whether *polB* inhibits *exsA* post-transcriptionally through PtrB, which inhibits T3SS in response to DNA damage [23]. Alternatively, *polB* might be affecting expression of some upstream *exsA* regulators.

Many CF *P. aeruginosa* isolates exhibit non-functioning DNA repair mechanisms, thereby enhancing the genetic mutations in a stressful environment [24, 25]. These mutations presumably increase survival under these conditions. By understanding how *P. aeruginosa* regulates virulence in stress, we can design better therapeutics to fight these infections.

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7. References

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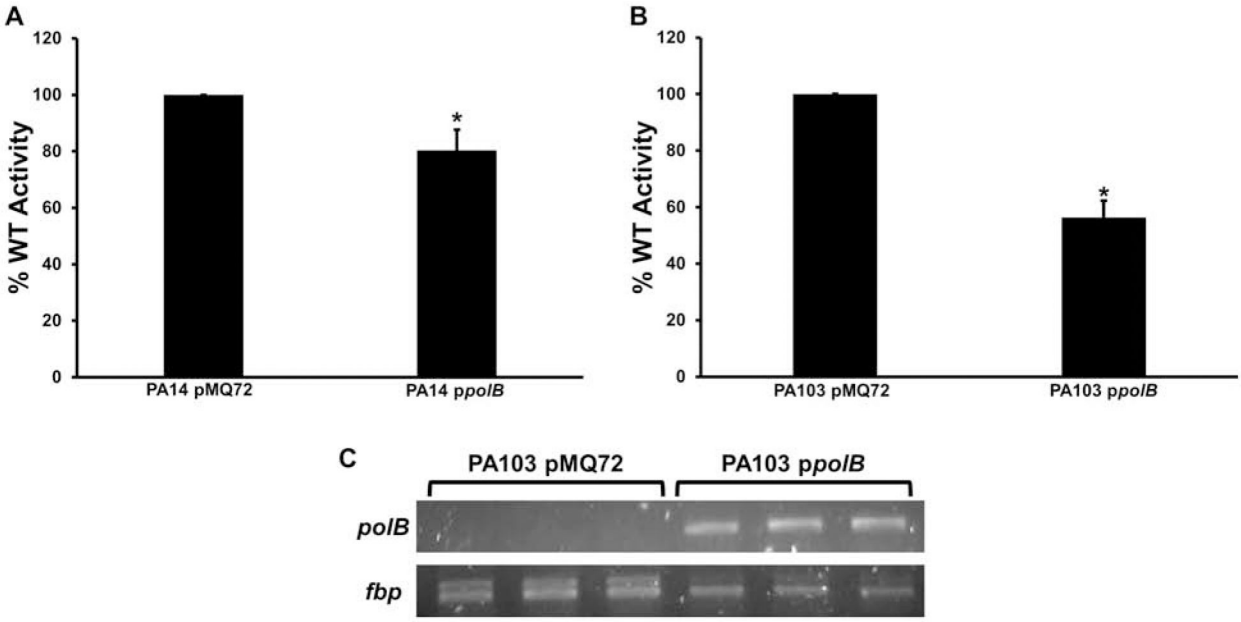

Figure 1. Overexpression of *polB* inhibits T3SS gene expression.

B-galactosidase activity was measured in (**A**) PA14 P_{exsD}-*lacZ* and (**B**) PA103 P_{exsD}-*lacZ*, compared to empty control vector (pMQ72). The results are representative of three independent experiments carried out in triplicate (n = 3) and error bars represent standard deviation. Error bars of pMQ72 samples are present but very small. *$p < 0.05$ compared to pMQ72. (**C**) Semiquantitative analysis of *polB* transcript levels in 3 independent samples of PA103 pMQ72 and PA103 p*polB*. *fbp* is our control transcript. All images were taken from the same agarose gel, with no alteration of brightness or contrast.

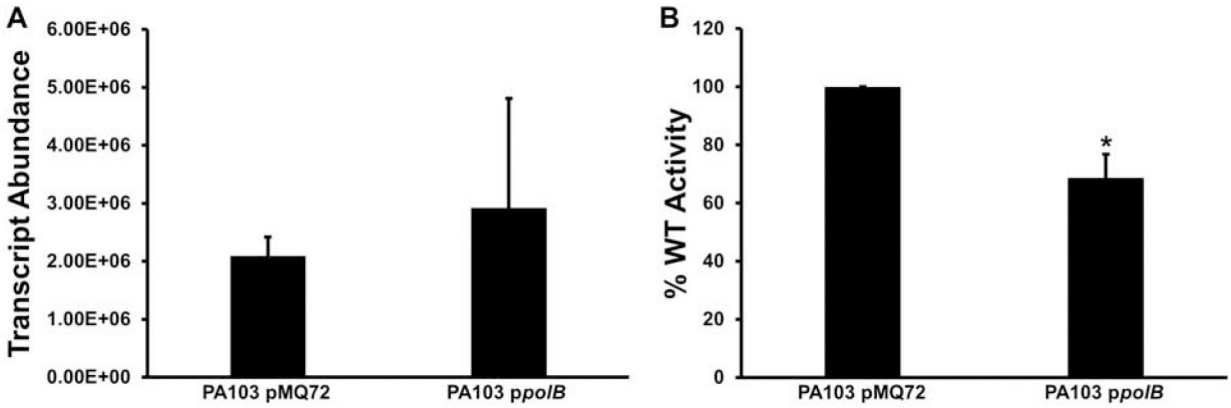

Figure 2. *polB* overexpression decreases *exsA* translation, but not transcript abundance.

Overexpression of *polB* (**A**) does not affect *exsA* transcript abundance in PA103 but (**B**) inhibits *exsA* translation compared to empty control vector (pMQ72) in PA103 with the P_{lacUV5}-*exsCEBA'-'lacZ exsA* translational reporter. The results are representative of three independent experiments carried out in triplicate (n = 3) and error bars represent standard deviation. Error bars of pMQ72 sample in (B) are present but very small. *$p < 0.05$ compared to PA103 pMQ72.

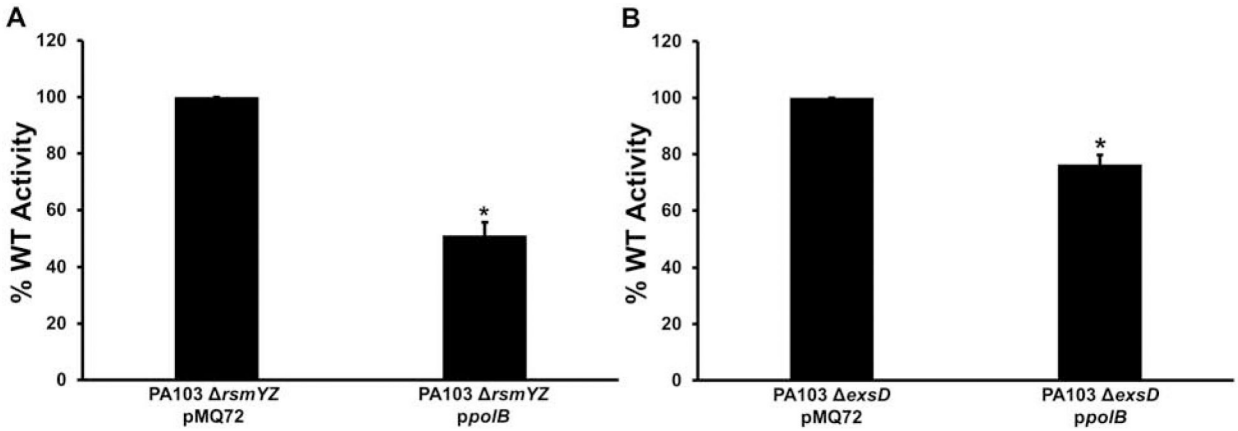

Figure 3. *polB* bypasses the *rsmYZ* and *exsD* mediated inhibition of T3SS.

Inhibition of 3SS activity is present in (**A**) PA103 Δ*rsmYZ* and (**B**) PA103 Δ*exsD* when *polB* is overexpressed compared to empty control vector (pMQ72). The results are representative of three independent experiments carried out in triplicate (n = 3) and error bars represent standard deviation. Error bars of pMQ72 samples are present but very small. *$p < 0.05$ compared to pMQ72.

Table 1.

Stains and plasmids used in this study

Strains	**Relevant Characteristics**	**Source**
PA14	Wild type	[8]
PA103	Wild type	[8]
PA14 P_{exsD}-*lacZ*	T3SS transcriptional reporter	[14]
PA103 P_{exsD}-*lacZ*	T3SS transcriptional reporter	[14]
PA103 P_{exsD}-*lacZ* Δ*rsmYZ*	Transcriptional reporter, isogenic deletion of *rsmYZ*	[8]
PA103 P_{exsD}-*lacZ* Δ*exsD*	Transcriptional reporter, isogenic deletion of *exsD*	This study
PA103 P_{lacUV5}-*exsCEBA'-'lacZ*	ExsA translational reporter	[14]
Plasmids		
pSMC296	*exsD* isogenic deletion	[16]
pMQ72	Empty control vector	[12]
p*polB*	*polB* expression plasmid	[10]