Regulation of the Type III Secretion System by PtsN in *Pseudomonas aeruginosa*

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Abstract:

There are many virulence factors in *Pseudomonas aeruginosa* that contribute to this opportunistic pathogen's ability to evade the immune system and persist during drug therapy. Previous studies recognized the protein PtsN of the nitrogen-related phosphotransferase system (Nitro-PTS) as having a connection to virulence in *P. aeruginosa* by impacting biofilm levels through an unknown mechanism. Preliminary transcriptomic sequencing data identified that PtsN differentially regulates another virulence factor: the Type III Secretion System (T3SS). The T3SS is a needle-like protein structure embedded in the membrane of many Gram-negative, pathogenic bacteria. This protein complex gives bacteria the ability to inject effector proteins directly into host cells' cytoplasm, increasing the pathogen's ability to colonize the host. My research utilizes luciferase reporter strains to measure the activity of promoters of T3SS genes identified in transcriptome data. These experiments will observe the effect of PtsN on T3SS gene expression, help determine if PtsN is a novel regulator of T3SS in *P. aeruginosa* and develop a hypothesis for how PtsN interacts with the regulatory proteins that control the expression levels of the T3SS.

Introduction:

Pseudomonas aeruginosa is an opportunistic pathogen that commonly causes nosocomial infections due to its antibiotic resistance, posing an ongoing medical threat. The *P. aeruginosa* strain studied here, PA14, is a highly virulent isolate that is frequently encountered in clinical settings (Mikkelsen, et al., 2011). Its virulence has been attributed to drug therapy resistance via virulence factors, including the production of biofilm—a self-secreted substance made of polysaccharides, DNA, and proteins that binds cells together, shielding colonies from their environment (Iglewski, 1996). Previous work identified PtsN, part of a phosphorelay called the Nitro-PTS, as a negative regulator of biofilm formation when PtsN is unphosphorylated (Cabeen, et al., 2016) (Lüttmann, et al., 2012). Wild-type (phosphorylated) PtsN displays high wrinkling (high biofilm production). In contrast, deleting an upstream kinase, PtsP, shows a smooth (low biofilm) morphology. Although PtsN shows striking phenotypic changes, few gene targets of PtsN regulation are known. Because PtsN does not contain a DNA binding domain, it is hypothesized that PtsN imposes its effect on gene expression and protein function on a post-translational level or post-transcriptionally.

Transcriptomic sequencing was utilized to find genes differentially expressed when comparing the absence of PtsN (PA14Δ*ptsP*Δ*ptsN*) versus an unphosphorylated version of PtsN (PA14Δ*ptsP*). Again, the unphosphorylated PtsN background was utilized due to its ability to be a negative regulator of biofilm formation. Additionally, this comparison exposes the impact of deletion of PtsN versus in reference to an unphosphorylated PtsN background. Due to the previous studies which observed PtsN having an impact on biofilm, it was expected to see biofilm related genes in the transcriptomic data. However, transcriptomic data did not suggest any known biofilm genes to be differentially regulated when PtsN is not phosphorylated versus when it is absent. Instead,

transcriptome sequencing analysis uncovered other virulence genes that were differentially regulated in the PA14Δ*ptsP*Δ*ptsN* versus PA14Δ*ptsP* comparison. Among these were genes constituting the Type III Secretion System (T3SS).

The T3SS is a virulence determinant of PA14 and other Gram-negative bacteria. The T3SS forms a needle complex that can inject, deliver proteins, and therefore manipulate host cells. The PA14 T3SS is under two levels of regulation: 1) signals which initiate T3SS endotoxin secretion and 2) gene transcription (Williams McMackin, 2019). The mechanism of the regulatory proteins of the T3SS is displayed by **Figure 1**. Gene transcription regulation is activated by a protein called ExsA. This protein is bound to an anti-activator called ExsD in a 1:1 complex. For ExsD to release ExsA, it will preferentially bind to ExsC which is in a 2:1 complex with a protein called ExsE. When environmental signals (such as low environmental Ca^{2+}) are sensed by the cell, ExsE disassociates from ExsC, allowing ExsD to preferentially bind to EsxC in a 2:2 complex (Williams McMackin, 2019). This releases ExsA to bind to the promoter of T3SS genes and activate transcription. Transcriptomic data showed that expression of *exsE*, *exsC*, and *exsD* is increased in PA14Δ*ptsP*Δ*ptsN* in the comparison to PA14Δ*ptsP*. Perhaps PtsN is interacting with one or more of these regulatory proteins to have an impact on T3SS expression. A sample of genes upregulated when PtsN is deleted is shown in Table 1.

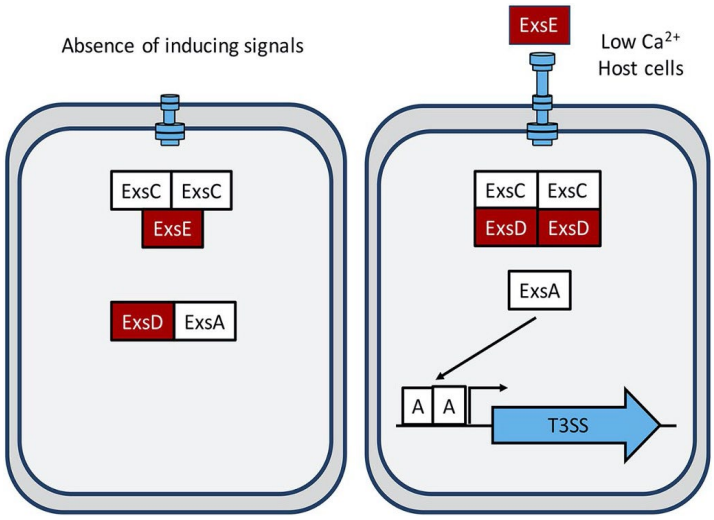

Figure 1: This image describing the regulatory protein mechanism of the T3SS is from the paper "Fitting Pieces into the Puzzle of *Pseudomonas aeruginosa* Type III Secretion System Gene Expression" by Williams McMackin, et.al. It shows ExsA as the T3SS activator, ExsD as the antiactivator, ExsC as the antiantiactivator, and ExsE as the secreted protein.

Table 1: Sample of Upregulated Genes from the PA14Δ*ptsP*Δ*ptsN* versus PA14Δ*ptsP* Transcriptomic Data Analysis

GeneID	Product	P value	logFC	Product ID
PA14_RS17140	SctL family type III secretion system stator protein PscL	2.66E-17	2.472381088	WP_003087735.1
PA14_RS17145	type III secretion system sorting platform protein PscK	7.66E-24	3.200645447	WP_003087734.1
PA14_RS17150	SctJ family type III secretion inner membrane ring lipoprotein Psc	5.98E-27	2.602528845	WP_003120329.1
PA14_RS17155	SctI family type III secretion system inner rod subunit PscI	7.40E-22	2.547524495	WP_003120330.1
PA14_RS17160	YopR family T3SS polymerization control protein PscH	1.86E-29	3.045112514	WP_003100725.1
PA14_RS17165	YscG family type III secretion system chaperone PscG	2.22E-18	2.852697727	WP_003140037.1
PA14_RS17170	type III secretion system needle filament protein PscF	1.79E-17	2.08267887	WP_003087729.1
PA14_RS17175	YscE family type III secretion system co-chaperone PscE	4.16E-15	2.017600326	WP_003100751.1
PA14_RS17180	SctD family type III secretion system inner membrane ring subunit PscD	4.81E-25	2.766198974	WP_003132859.1
PA14_RS17185	SctC family type III secretion system outer membrane ring subunit PscC	3.62E-30	2.377287965	WP_003140040.1
PA14_RS17190	YscB family type III secretion system chaperone PscB	3.70E-21	2.506663586	WP_003109510.1
PA14_RS17255	type III secretion system chaperone PscY	2.28E-05	2.188767872	WP_003113547.1
PA14_RS17260	type III secretion system protein PscX	2.31E-19	3.907969927	WP_003140044.1
PA14_RS17280	SctN family type III secretion system ATPase PscN	6.91E-25	3.473114914	WP_003100796.1
PA14_RS17285	type III secretion system central stalk protein PscO	8.13E-13	2.90035456	WP_003140046.1
PA14_RS17295	SctQ family type III secretion system cytoplasmic ring protein PscQ	2.86E-35	3.76232678	WP_003140061.1
PA14_RS17300	SctR family type III secretion system export apparatus subunit PscR	7.22E-21	3.520844904	WP_003087674.1
PA14_RS17305	SctS family type III secretion system export apparatus subunit PscS	6.17E-13	2.972934615	WP_003087672.1
PA14_RS17310	SctT family type III secretion system export apparatus subunit PscT	5.71E-17	2.822693488	WP_003132884.1
PA14_RS00230	T3SS effector bifunctional cytotoxin exoenzyme T	2.90E-27	2.811925579	WP_003136948.1

Table 1: This list highlights the *pscN-T* operon genes that were upregulated when comparing the transcriptome data of PA14Δ*ptsP*Δ*ptsN* versus PA14Δ*ptsP*. The *pscN-T* operon was utilized to construct luminescent reporters to track T3SS gene expression. The *pscN-T* operon comprises genes that are part of the T3SS machinery.

Methods:

Transcriptome sequencing data was analyzed by sorting through genes in a PA14Δ*ptsP*Δ*ptsN* versus PA14Δ*ptsP* comparison which had a $\log_2$ fold change ($\log_2$ FC) value larger than ± 2.00. A $\log_2$ fold change of ± 2.00 is a good statistical value to identify genes whose differential expression is due to the deletion of PtsN. Among these candidates were genes responsible for the T3SS. Among these T3SS genes, we selected the *pscN-T* operon—which encodes parts of the secretion machinery—to drive a luminescent reporter for T3SS expression detection. The luminescent reporter is encoded on a CTX-1 plasmid. To create an insert for this plasmid, the first gene in the *pscN-T* operon was determined and the 200 nucleotides before this position was used for the insert. Using these nucleotides for the insert would ensure that the promoter for the *pscN-T* operon was present, since promoters tend to be within 100 bp upstream of the first gene in an operon.

Luciferase luminescence assays ran for 24 hours with continual shaking. Cultures were placed into the 96 well plate around an OD of approximately 0.1. Readings for luminescence and OD_{600} were read every 10 minutes. For each strain we performed 3 biological replicates and 3 technical replicates for each condition. LB with 25 μg/ml of tetracycline was the media used for the luminescence assays because our strains have the CTX-1 plasmid with a tetracycline resistant gene cassette and our luminescent reporter integrated into the genome. Our LB with 25 μg/ml of tetracycline media has a low Ca^{2+} concentration, which is a favorable environment for inducing the T3SS and provides cells with enough nutrients to have strong growth rates (Williams McMackin, 2019). A BioTek Synergy H1 Multi-Mode Microplate Reader was used to measure OD_{600} and luminescence.

Luminescent reporters (CTX-1-*pscN-T-lux*) were constructed and put into *E. coli* strain SM10 for mating with PA14 strains. The following strains were made: PA14 *attB*::*CTX-1-pscN-T-lux*, PA14Δ*ptsP* *attB*::*CTX-1-pscN-T-lux*, PA14Δ*ptsN* *attB*::*CTX-1-pscN-T-lux*, and PA14Δ*ptsP*Δ*ptsN* *attB*::*CTX-1-pscN-T-lux*. A luminescence assay was conducted with these strains to confirm the transcriptomic data.

Deletions of *exsC* and *exsE* were made to create a strain with a constitutively off and a strain with a constitutively on T3SS, respectively. Luminescence assays were conducted with the PA14 PscN-T-lux, PA14Δ*ptsP* *attB*::*CTX-1-pscN-T-lux*, PA14Δ*ptsN* *attB*::*CTX-1-pscN-T-lux*, PA14Δ*ptsP*Δ*ptsN* *attB*::*CTX-1-pscN-T-lux*, PA14Δ*exsC* *attB*::*CTX-1-pscN-T-lux*, PA14Δ*exsE* *attB*::*CTX-1-pscN-T-lux*, PA14Δ*ptsN*Δ*exsC* *attB*::*CTX-1-pscN-T-lux*, and PA14Δ*ptsN*Δ*exsE* *attB*::*CTX-1-pscN-T-lux*.

Results:

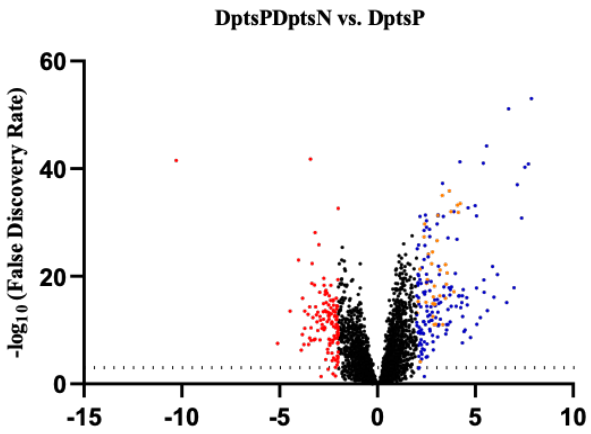

Figure 2: This volcano plot visualizes the genes upregulated and down regulated when comparing a *ptsN* deletion to an unphosphorylated PtsN form. Genes with a log fold change (logFC) value larger than ± 2.00 were considered differentially regulated.
Blue dots are upregulated by PtsN deletion, red dots are downregulated by PtsN deletion, and the orange dots are the genes of the T3SS. The black dots are genes that are not differentially regulated.

To discover genes that PtsN differentially regulates in its absence and presence and in its phosphorylated and nonphosphorylated states, transcriptomic sequencing data in the PA14 Δ*ptsP*Δ*ptsN* versus PA14 Δ*ptsP* comparison was analyzed. **Figure 2** is a volcano plot which displays genes that were upregulated and downegulated. These data suggest that the deletion of *ptsN* increases the expression of the T3SS genes, indicating that PstN might impact T3SS regulation as a repressor. To continue investigating how PtsN is differentially regulating these genes, we used luminescence reporters.

Initial luminescence assays were conducted to validate transcriptome sequencing data that suggested PtsN could be a regulator of T3SS genes. A luminescence reporter for the *pscN-T* operon—which codes for T3SS machinery—was constructed and mated with PA14, PA14Δ*ptsP*, PA14Δ*ptsN*, and PA14Δ*ptsP*Δ*ptsN*. To test the hypothesis that PtsN is a novel regulator of the

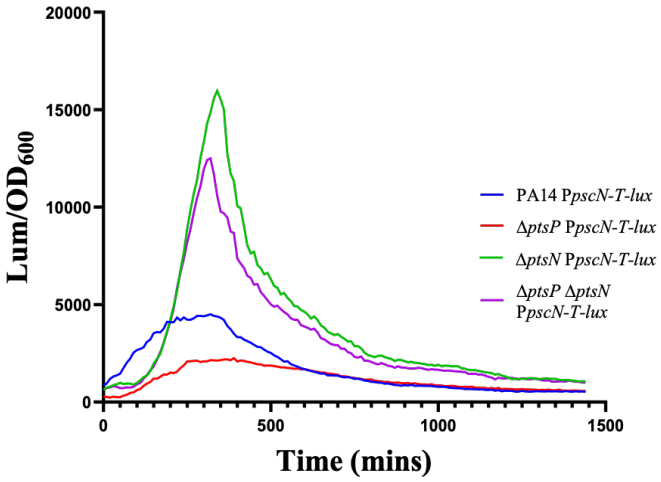

Figure 3: Luciferase assay displays the effect of *ptsN* deletion on the expression level on the T3SS operon PscN-T. We normalize the Luminescence data with OD to ensure that the data we see is not due to one strain having more cells than the other, but due to the level of T3SS expression.

T3SS, we measured T3SS expression in the presence and absence of PstN and when PtsN is phosphorylated versus unphosphorylated.

The data in **Figure 3** distinctly shows how the deletion of *ptsN* causes a significant increase in T3SS expression in comparison to the WT and the unphosphorylated-PtsN strain (PA14Δ*ptsP*). PtsN increases the maximum promoter signal activity by a factor of approximately 4. This observation confirms the transcriptome data we collected and further supports our hypothesis that PtsN can act as a repressor of the T3SS.

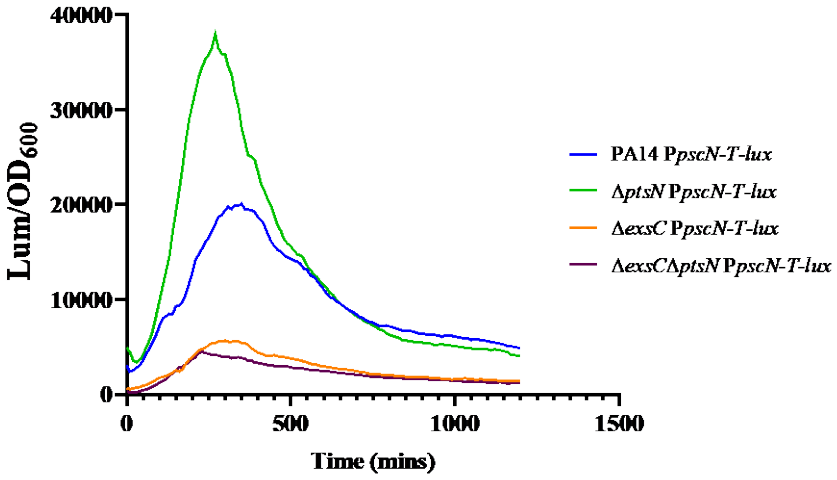

Figure 4: Luciferase assay for the effect of a *ptsN* deletion when the T3SS is constitutively inactive (due to a deletion of *exsC).*

To further understand how PtsN is impacting T3SS regulation, we wanted to observe PtsN's effect in the absence of known T3SS regulators. **Figure 4** shows that when PstN is deleted in a Δ*exsC* background (when the system is constitutively inactive) there is no substantial change in expression. This indicates that deletion of *ptsN* does not recover T3SS expression when ExsC is absent and T3SS is inactive. Because PtsN is known to increase T3SS, it is surprising that PtsN would not be able to recover an inactive T3SS due to a deletion of *exsC*. It is possible that ExsC must be present for PtsN to have its effect, but this hypothesis cannot be determined with the current collection of data. Details of future studies to further examine this observation are

discussed later in this paper. This observation piqued our interest for examining the effect of PtsN on a constitutively active T3SS and if it will show no significant impact on expression, as in **Figure 4** with the constitutively off system.

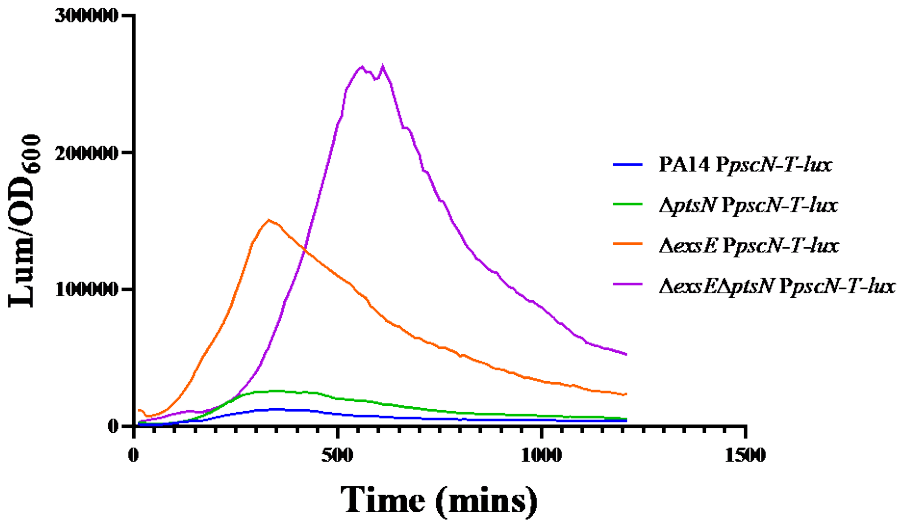

Figure 5: Luciferase assay shows the impact of a *ptsN* deletion when the T3SS is constitutively active due to deletion of *exsE*.

Figure 5 implies that deletion of *pstN* in a Δ*exsE* background (when the T3SS is constitutively active) causes the T3SS to be even more highly expressed. It is also seen that the Δ*exsE* and Δ*exsE*Δ*ptsN* strain have significantly higher expression levels than previously seen with the Δ*ptsN* strain. The delayed curve of the Δ*exsE*Δ*ptsN* strain is reproducible because the Δ*exsE*Δ*ptsN* strain is a slow grower.

Discussion:

Initially, luciferase assays seen in **Figure 3** determined that the deletion of *ptsN* causes an increase in T3SS expression. Before the results reported here, PtsN was not known to impact regulation of T3SS genes. Furthermore, constitutively inactive (Δ*exsC*) and active (Δ*exsE*) T3SS systems were

created to determine whether deleting *ptsN* continued to have an effect when the T3SS is "turned off" and "turned on." In other words, can PtsN expression recover an "off" system or further amplify expression in an active system? Deletion of *ptsN* could not recover a constitutively inactive T3SS (Δ*exsC*). However, a *ptsN* deletion in a constitutively active T3SS strain caused expression beyond the intrinsic limits of the T3SS. The intrinsic limit of the T3SS is seen by creating a system that is constitutively active in the wild-type presence of PtsN (deletion of *exsE*). The deletion of *ptsN* can bring T3SS expression to levels that are even higher than a wild-type condition expression limit. It is possible that there is no effect on T3SS expression when *ptsN* is deleted in a Δ*exsC* background because the protein that PtsN is interacting with is absent (ExsC). The phosphorylated PtsN form could be lowering the affinity of ExsC for ExsD, keeping ExsD bound to ExsA, or PtsN could play a role in keeping ExsE bound to ExsC until the cell receives signaling for T3SS gene expression. Future studies that delete *exsD* will provide more understanding on the intrinsic expression limits of the T3SS (ensuring that the *exsE* deletion is showing the maximum intrinsic expression for T3SS) and on which regulatory proteins PtsN is interacting with. Based on our current data, it is possible that PtsN could be interacting with ExsC, ExsD or ExsA to impact T3SS expression levels.

Future Work:

Future studies should aim to discover which T3SS regulatory protein has an interaction with PtsN. This can be deduced from further luciferase assays with an *exsD* and *exsA* deletions, which provide different versions of an active and inactive T3SS – deleting *exsD* would create an active system while deleting *exsA* would inactivate the system. Seeing the impact on expression when deleting *ptsN* in these strains with the presence of ExsC and ExsE will provide more evidence to suggest a regulatory protein that PtsN is regulating. Interaction studies such as co-immunoprecipitation or

fluorescence resonance energy transfer can be performed to see if PtsN is directly interacting with T3SS regulatory proteins on a post-translational level. Additionally, PtsN could be impacting the activity of these regulatory proteins via phosphorylation. Therefore, western blotting could visualize phosphorylation states of T3SS regulatory proteins in the absence and presence of PtsN. If a protein sample is phosphorylated, the protein band will "shift up," or have a higher band location on the gel, than a unphosphorylated control protein.

Additionally, since deletion of *ptsN* increases T3SS expression, thus identifying PtsN as a negative regulator of T3SS expression, future experiments can test the expectation that overexpressing *ptsN* will lower T3SS expression levels. To that end, an SM10 strain with pJN105-*ptsN*, a plasmid containing an arabinose-inducible promoter that will express *ptsN*, can be constructed and mated with experimental strains: PA14 *attB*::*CTX-1-pscN-T-lux*, PA14Δ*ptsN attB*::*CTX-1-pscN-T-lux*, PA14Δ*exsE attB*::*CTX-1-pscN-T-lux*, and PA14Δ*ptsN*Δ*exsE attB*::*CTX-1-pscN-T-lux*. The PA14Δ*exsC attB*::*CTX-1-pscN-T-lux* and PA14Δ*ptsN*Δ*exsC attB*::*CTX-1-pscN-T-lux* strains would not be used because it is expected that *ptsN* overexpression will drive down T3SS expression, and these strains already show minimal T3SS expression so overexpression of *ptsN* would likely have little effect. These overexpression experiments will further clarify how PtsN is regulating the T3SS.

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