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POSSIBLE ROLES OF CYSTEINE RESIDUES IN THE STRUCTURE AND FUNCTION OF EXOENZYME Y FROM *PSEUDOMONAS AERUGINOSA*

by

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A thesis

submitted in partial fulfillment

of the requirements for the degree of

Master of Science in Microbiology

from the Department of Biological Sciences

at Idaho State University, Pocatello, Idaho

Spring 2016

COMMITTEE APPROVAL

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Thesis Abstract – Idaho State University – 2016

Pseudomonas aeruginosa is an opportunistic human pathogen responsible for many nosocomial infections of immune-compromised patients and is often multi-drug resistant. During infection, ExoY has been associated with penetration of epithelial barriers and inhibition of phagocytosis. Unlike similar bacterial toxins with adenylyl cyclase activity, ExoY is not activated by calmodulin and contains 5 cysteine residues. Cysteines are highly conserved and may fill a variety of roles within a protein, such as structural disulfide formation, catalytic participation and/or metal ion coordination. Cysteines at positions 331 and 345 were determined to have significant roles in structure and function, along with position 100 to a lesser degree. Residue 279 appears necessary for function alone, and residue 205 does not have a role identified by this research. Finally, F-actin was validated as an activator of ExoY adenylyl cyclase activity. This research serves to broaden the understanding of ExoY's mechanism of action during infection.

HYPOTHESIS AND SPECIFIC AIMS

I hypothesized that the 5 cysteine residues in ExoY, a promiscuous adenylyl cyclase from *Pseudomonas aeruginosa*, are present in the protein's primary structure because they are required for cAMP production during infection of eukaryotic cells. Possible functions may include structural disulfide formation, catalytic participation or metal ion coordination. To test this hypothesis, I propose 4 aims: First, to generate cysteine-to-serine mutations for each of the 5 residues of interest from an existing ExoY clone. Second, I will evaluate the circular dichroism spectra of each mutant in an attempt to identify possible structural functions of each cysteine residue in the ExoY protein. Third, I aim to use an enzyme-linked immunoassay to determine the adenylyl cyclase activity of each mutated ExoY. Fourth and finally, recent research has identified F-actin as an activator or ExoY activity and I will use the same immunoassay to evaluate this possibility.

CHAPTER I: INTRODUCTION

General Characteristics

Pseudomonas aeruginosa is a ubiquitous, Gram-negative, motile bacillus and is the type species for its genus[1]. Originally identified by its blue-green growth due to production of the pigments pyocyanin[2] and pyoverdine[3], it successfully grows on Simmons citrate agar and tests positive in urea hydrolysis , catalase and oxidase tests. Production of 2-aminoacetophenone is responsible for a distinct grape-like scent that is still considered an identifying trait[4,5]. Found in soil[6], water[7] and often human body flora[8], the species is well equipped to survive in a wide variety of environments. It is a facultative anaerobe that can use nitrate as a terminal electron acceptor[9], and it can also ferment arginine[10] and pyruvate[11] in the absence of preferred nutrients. *P. aeruginosa* has even been shown to degrade crude oil and gasoline in hydrocarbon-polluted sites[12,13]. Indeed, *P. aeruginosa*'s production of rhamnolipids has been utilized in a variety of bioremediation projects where they are exploited as powerful biosurfactants[14–16]. To facilitate such metabolic versatility, *P. aeruginosa* harbors a relatively large genome (~6.3Mbp)[17] compared to other common bacteria such as *Bacillus subtilis* (~4.2Mbp)[18] and *Escherichia coli* (~5.5Mbp)[19]. In the *P. aeruginosa* lab strain, PAO1, this includes 5,572 annotated open reading frames that provide for a variety of metabolic pathways in addition to a plethora of virulence factors and antimicrobial resistance mechanisms[20,21].

Clinical Significance

P. aeruginosa is an opportunistic pathogen responsible for a significant portion of nosocomial infections. Able to colonize incredibly nutrient-poor environments, *P. aeruginosa* can be rapidly spread among patients by hospital staff from a variety of reservoirs[22]. For example, *P. aeruginosa* is capable of colonizing sink drains and is then aerosolized during hand-washing, recontaminating hospital personnel in spite of proper procedure[23]. Of particular concern for immune-compromised patients, this organism is responsible for infection of burn wounds[24–26] and colonization of medical equipment[27] such as urinary catheters[28] and the endotracheal tubes of mechanical ventilators[29,30]. Chronic infection by *P. aeruginosa* is very common in patients with cystic fibrosis and causes a significant increase in morbidity and mortality for those with this disease[31–33]. Hospital-acquired, systemic bacteremia is also a large concern[34,35]. Further complicating treatment of these infections, *P. aeruginosa* is often multidrug-resistant[36,37] and can also rapidly develop or acquire further resistances when exposed to antimicrobial treatment[38,39]. Study of the mechanisms through which *P. aeruginosa* establishes infection and so efficiently resists antimicrobial agents will hopefully allow discovery or creation of more effective methods to control its growth.

Antibiotics

Beta-lactams

Beta-lactam antibiotics are a large class of antimicrobial agents united by the inclusion of a beta-lactam ring within their molecular structure. Divided into four groups based on their superstructure and spectrum of action, they are as follows: penicillins, cephalosporins, monobactams and carbapenems. Each of these groups inhibits specific

penicillin-binding proteins (PBPs) present in the target cell. PBPs are generally enzymes involved in cell wall synthesis, such as transpeptidases, or are somehow involved in bacterial cell growth[40,41]. Their inhibition results in lysis of the affected cell making penicillins bactericidal to target organisms[42]. Most penicillins and cephalosporins, with notable exceptions, are specific to Gram-positive bacteria. The penicillins piperacillin[43] and carbenicillin[44], as well as some third- and fourth-generation cephalosporins[45,46], are considered "broad spectrum" and can act against Gram-negative bacteria, including *P. aeruginosa*. The monobactam aztreonam[47] and carbapenems like doripenem[48] and imipenem[49] are able to target PBPs and induce lysis in susceptible *P. aeruginosa* strains.

Aminoglycosides

Beginning with the discovery of streptomycin in 1944[50], aminoglycosides have been widely used to combat infections caused by aerobic, Gram-negative bacilli, including *P. aeruginosa*. These antimicrobials are divided into two general groups based on their origin. Drugs with a –mycin suffix are derived from the genus *Streptomyces*, while those with the –micin suffix are produced by *Micromonospora*. Most aminoglycosides are naturally occurring in their respective genera, though amikacin[51] and netilmicin[52] are examples of synthetically modified versions of their natural compatriots. Common structural features of all aminoglycosides include several modified sugars glycosidically linked to a dibasic cyclitol[53]. Unlike beta-lactams, aminoglycosides are generally too large to enter a target cell through porin channels and must be actively transported across the membrane[54]. Once in the cytoplasm, all aminoglycosides act by specifically and irreversibly binding various sites on the bacterial 16S rRNA of the 30S

ribosomal subunit. This inhibits proper assembly of the complete ribosome, interfering with translational proofreading and causing production of nonsense proteins[55,56]. The subsequent lack of proper protein synthesis leads to cell death, making this class of antibiotics bactericidal.

Quinolones

The original quinolone, 1,8-naphthyridine or nalidixic acid, was limited in use to Gram-negative infections of the urinary tract[57]. However, this class of antibiotics has been greatly expanded through modification and addition of various structural elements. Addition of a piperazine ring conferred activity against *P. aeruginosa*, while fluorination at the 6-position along with additional modifications allowed expansion of the quinolones (or fluoroquinolones) to affect Gram-positive bacteria[58]. The target of all quinolones is bacterial topoisomerases. Under normal conditions, topoisomerases relieve positive supercoiling that occurs ahead of DNA helicase during DNA replication and transcription and introduce or remove catenation (two interlocked circles of DNA)[59,60]. They first create a double-stranded break in the supercoiled DNA, pass another strand through the break, and then seal the gap. However upon introduction, a quinolone antibiotic will bind a target topoisomerase, inhibiting re-ligation of the cleaved strands and keeping the enzyme stuck in complex with its DNA substrate[61,62]. Cell death occurs due to fragmentation of the genome caused by this interruption of a topoisomerase halfway through its enzymatic action[63].

Antibiotic Resistance Mechanisms

Reduced Permeability – Efflux Pump and Porin Regulation

One of the most broadly effective mechanisms by which *P. aeruginosa* resists antibiotics is an array of efflux pumps. These transmembrane protein constructs efficiently extrude antibiotics that have entered the cell via a drug-proton antiporter mechanism, reducing the effective membrane permeability of affected molecules[64]. While multiple efflux systems have been identified in *P. aeruginosa*[65], the MexAB-OprM system is expressed constitutively in all lab and clinical strains[66] and is responsible for a broad range of intrinsic resistances. This range includes large groups of beta-lactams and quinolones, while aminoglycoside resistance is due to another efflux system, MexXY-OprM[67,68]. The MexAB-OprM system is expressed at basal levels under standard conditions due to a repressor, MexR[69]. However, upon exposure to an antibiotic agent, *P. aeruginosa* strains with elevated MexAB-OprM expression are rapidly selected for[39,70]. Naturally, *mexR*, is often the target of mutations that prevent MexR function and allow such overexpression of the efflux system[71].

While many antibiotics can be exported from the cell, some can simply be prevented from entering in the first place. OprD, previously known as Protein D2, is a porin that allows diffusion of basic amino acids and short peptides that contain them through *P. aeruginosa's* notoriously low-permeability outer membrane[72]. Imipinem is a carbapenem beta-lactam antibiotic commonly relied upon to treat *P. aeruginosa* infections and is able to diffuse through the OprD porin by utilizing basic amino acid binding sites within the channel[73,74]. However, strains containing mutations within the OprD coding or promoter regions are rapidly selected for upon antibiotic introduction,

reducing OprD expression[75,76]. Additionally, OprD appears to be coregulated with yet another efflux operon, *mexEF-oprN*, by MexT, a transcriptional regulator that represses OprD while activating expression of MexEF-OprN[77]. Such connectivity amongst resistance mechanisms highlights the intricacy of *P. aeruginosa*'s ability to mount significant defenses against single or multiple antibiotics.

Effector Modification - Beta-lactamases

P. aeruginosa resists a large number of antibiotics intrinsically through the inducible beta-lactamase, AmpC. A broad range of beta-lactamases exist that are categorized based on their target spectrum, but each acts by cleaving the beta-lactam ring that is common to all beta-lactam antibiotics. Under normal conditions, AmpC is produced at low levels and is regulated by an activator, *ampR*, as well as a repressor, *ampD*[78]. Spontaneous point mutations in one of both of these regulator genes can result in hyperexpression of the AmpC beta-lactamase[79,80]. Exposure to a susceptible antibiotic will select for hyperexpressive *P. aeruginosa* strains, and the infection will quickly become resistant to that drug[81]. Additionally, *P. aeruginosa* can obtain or develop resistances to antibiotics to which it is usually susceptible through several mechanisms. Biofilm formation, particularly during lung infections, can reduce the rate at which an agent reaches its target and induce beta-lactamase production[82]. Horizontal transfer of plasmids or transposons containing additional beta-lactamases can rapidly confer tolerance of a drug amongst the members of a multi-organism infection.

Substrate Modification – Target Site Mutation

In addition to exporting antibiotic molecules and simply degrading them, *P. aeruginosa* develops resistances through mutation of target molecules. For example, as previously discussed, quinolones act by targeting topoisomerases II (DNA gyrase) and IV and interrupting their function. DNA gyrase is composed of two subunits encoded by the genes *gyrA* and *gyrB,* while topoisomerase IV is produced from *parC*[59]. Most ciprofloxacin-resistant strains of *P. aeruginosa* have been shown to contain specific point mutations in the *gyrA* gene[83,84]. However, additional mutations in *gyrB* and *parC* confer further increases in resistance to the antibiotic[85]. Upon administration of a quinolone antibiotic, accumulation of mutations within one or more of these "quinolone resistance-determining regions" will be selected for, which removes the antibiotic's affinity for its target[63,86,87]. Clearly this mechanism of resistance acquisition extends to any antibiotic and its intended target molecule, and target modification is not limited to mutation. Mutation of PBPs may confer resistance to beta-lactam antibiotics[88], while methylation of 16S ribosomal RNA has shown to provide protection against aminoglycosides[89].

Virulence Factors

Quorum Sensing

While *P. aeruginosa* encodes a large number of antibiotic resistance and virulence factors, chronic infection by this organism involves coordinating masses of cells to facilitate establishment of a successful infection. Such coordination is possible due to chemical pathways that communicate between nearby cells and regulate gene expression in a concentration-dependent manner. However, a single cell cannot produce

concentrations sufficient to affect gene expression, and large numbers of adjacent cells must produce an effector molecule in concert to experience a regulatory effect. This cooperative regulatory behavior is termed quorum sensing (QS), and over 6% of the *P. aeruginosa* genome responds to QS signals[90–92]. Three QS pathways have so far been identified and heavily characterized, though it is important to note that additional pathways have been identified and are currently being investigated. Two QS pathways, Las and Rhl, are based on signaling by *N*-acyl-homoserine lactone (AHL) species, while the third utilizes 2-alkyl-4-quinolones (AQs). AHL systems are commonly found among Gram-negative species, and their frequency can even enable cross-species communication. Both Las and Rhl effectors belong to the LuxRI family of proteins, LasI and RhlI being the synthases of N-3-oxo-dodecanoyl homoserine lactone (3-oxo-C12-HSL) and *N*-butanoyl homoserine lactone *(*C4-HSL) respectively, while LasR and RhlR bind the AHLs and act as transcriptional regulators to affect gene expression[93–95]. A plethora of regulatory factors influence these two systems. Both AHL pathways are positively regulated by the global activator of antibiotic and cyanide synthesis (GAC) system, which is often credited with the switch from acute to chronic infection[96,97]. Consisting of a transmembrane sensor kinase (GacS) and a regulator protein (GacA), this system activates transcription of several small regulatory RNAs responsible for up-regulating genes for biofilm formation and repressing genes involved in motility and acute virulence, including the T3SS[98]. LadS (lost adherence sensor) is another sensor kinase that works in parallel with GacS, while RetS (regulator of exopolysaccharide and type III secretion) blocks GacS by binding to it and inhibiting phosphorylation of GacA. The Las system itself positively affects the Rhl pathway, as LasR complexed with 3-oxo-

C12-HSL increases expression of both RhlR and RhlI. However, LasR and RhlR differ in their influence when it comes to the third QS pathway often studied in *P. aeruginosa*. Known as the *Psuedomonas* quinolone signal (PQS), 2-heptyl-3-hydroxy-4-quinolone is synthesized by products of a gene quartet labeled *pqsABCD*[99]. PQS complexed with its regulator protein, PqsR, positively feeds back to the *pqsABCD* genes in addition to activating the Rhl system and production of pyocyanin, which is discussed below[100]. Conversely, the Rhl system down-regulates PQS, while Las has a positive effect on PQS expression. Additionally, several other factors have been shown to either positively or negatively regulate one or more of these pathways in an increasingly complex system that emphasizes the significance of a switch from acute to chronic infection by *P. aeruginosa*. Examination of individual virulence factors will illustrate how these pathways interact to regulate production of *P. aeruginosa*'s many effectors throughout establishment of an infection.

Alginate and Biofilm Formation

Overproduction of alginate is largely responsible for transformation of *P. aeruginosa* to a mucoid phenotype and establishment of chronic infection in the lungs of cystic fibrosis patients[101,102], although other contributing exopolysaccharides have also been identified[103,104]. Alginate is a viscous, anionic exopolysaccharide composed of mannuronic acid and guluronic acid monomers. It is polymerized by other members of the alginate biosynthesis operon[105–107]. Inactivating mutations in a regulator of this operon, mucA, enables over-expression of the pathway and conversion of *P. aeruginosa* to a mucoid phenotype[108]. Additionally, MucB, a second negative regulator of alginate

synthesis, is down-regulated by the AHL QS pathways to increase alginate synthesis[91]. Biofilm formation also appears to be regulated by iron availability[109,110]. Oxygen diffusion through an established polysaccharide matrix is reduced[111], and has been correlated to a reduction in antibiotic susceptibility[112]. This protective effect may even be extended to other species present within the biofilm matrix, compounding the deleterious effects of an infection[113]. Hypoxic conditions created by biofilm formation can aggravate respiratory difficulties in cystic fibrosis patients, and the protection from immune response afforded can allow rapid establishment of chronic infections[31–33,114].

Exotoxin A

Exotoxin A (ExoA) is a 66kDa, mono-ADP-ribosyltransferase that targets eukaryotic elongation factor 2 (eEF2) to cause inhibition of proper protein translation[115,116]. One of several type-II secreted toxins[117], ExoA enters a host cell by binding the α_2-macroglobulin receptor, which initiates receptor-mediated endocytosis[118,119]. Once internalized and acidified by an endosome, a local protease nicks and releases a 37kDa C-terminal fragment that is then translocated into the cytoplasm[120,121]. This C-terminal fragment is catalytically responsible for transfer of an ADP-ribosyl group from NAD^+ to eEF2[122,123]. Introduction of ExoA to human B-cells *in vitro* inhibited IgG and IgM production at 1ug/mL[124], while doses as low as 0.2ug have been shown to kill mice , making ExoA one of the most toxic effectors produced by *P. aeruginosa*[125].

Elastase

LasB, also known as pseudolysin or elastase, is a relatively non-specific zinc-metalloprotease that, like ExoA, is secreted by *P. aeruginosa*'s type-II secretion system[117]. Originally named for its ability to break down structural proteins like elastin and collagen[126], elastase has a very broad target range that enables *P. aeruginosa* to evade host immune response and penetrate epithelial and endothelial barriers. This spectrum includes such significant targets as IgA and IgG[127,128], multiple complement components[129], pulmonary surfactant proteins[130] and lysozyme[131]. Elastase has also been shown to degrade multiple proteins associated with the endothelial barrier, such as fibronectin and VE-cadherin, enabling passage of *P. aeruginosa* into the bloodstream and establishment of systemic bacteremia[132–134].

Pyocyanin

A number of pigments are produced by *Pseudomonas* species, but only *P. aeruginosa* secretes pyocyanin (PYO), a redox-active phenazine that gives its growth a characteristic, blue-ish hue. PYO is secreted during the stationary phase of growth, and its synthesis is regulated by the PQS pathway[100,135]. Phenazine-1-carboxylic acid (PCA), the precursor to PYO, is produced from two possible operons labeled *phzABCDEFG* 1 and 2 that are activated by the regulatory products of *phnA* and *phnB*. PCA is then converted to PYO through the actions of *phzM*, a methyltransferase, and *phzS*, a monooxygenase[136]. PYO confers a competitive advantage by acting as an antibiotic against a variety of organisms including bacteria[137] and fungi[138] based on their ability to withstand oxidative stress[139]. The reduced form of PYO is a free radical that rapidly

produces reactive oxygen species such as superoxide (O_2^-) and, with the help of an iron catalyst, hydroxyl radicals ($OH\cdot$)[140]. PYO is able to affect mammalian cells[141], causing apoptosis of neutrophils[142], inhibiting Ig secretion by B-lymphocytes[143] and disrupting epithelial barriers[144].

Type III Secretion System

The type III secretion system (T3SS) is commonly expressed among Gram-negative bacteria as a means of delivering virulence factors directly into the cytoplasm of a host cell[145]. In *P. aeruginosa*, multiple genes grouped mostly into five operons have been identified as affiliated with the T3SS including structural components, translocation machinery, regulatory proteins, effector chaperones and the secreted effectors themselves[146]. Four virulence effectors, ExoS, T, U and Y, are secreted through the T3SS. They vary in relative cytotoxicity and mechanism of action, but each contributes to the pathogenicity of a *P. aeruginosa* strain that expresses it[147,148]. The T3SS structure itself is composed of a ringed base and a filamentous needle that extends through the inner membrane, peptidoglycan layer and outer membrane and into the cytoplasm of the target host cell[149]. Regulation of the T3SS primarily involves the action of four proteins, ExsA, C, D and E[150,151]. The T3SS and ExsE are expressed at low levels under normal conditions, and ExsE is exported through the T3SS when secretion is activated by contact with a host cell or low Ca^{2+} conditions. However, under non-secretory conditions, ExsE accumulates in the cytoplasm and binds ExsC. ExsC is an inhibitor of ExsD, which in turn inhibits ExsA, the transcriptional activator of both the T3SS and its own gene. Under non-secretory conditions, ExsC is bound by accumulated ExsE, preventing it from

inhibiting ExsD. ExsD is then free to inhibit ExsA and, therefore, prevent T3SS transcription. Only upon activation of secretion is ExsE exported from the cytoplasm, freeing ExsC to bind ExsD so that ExsA is able to activate T3SS transcription. T3SS expression is also negatively regulated by the GAC system described previously, which is responsible for upregulating QS pathways[152]. It is possible that the T3SS is mostly involved in the initial establishment of acute infections by planktonic *P. aeruginosa*. It then becomes less useful (but not irrelevant) once bacterial populations have reached significant levels to coordinate expression of other virulence factors via the QS pathways[153,154]. If so, focus on study of the T3SS and its associated effectors may contribute to efforts to prevent establishment of chronic infections by providing methods to inhibit the activity of these early virulence factors.

ExoS and ExoT

Exotoxin S (ExoS) is a 49-kDa effector secreted by *P. aeruginosa* through the T3SS and has dual functionality during eukaryotic cell infection. The C-terminal domain of ExoS possesses ADP-ribosyltransferase activity with a broad set of targets including the Ras family of GTPases[155], vimentin[156], IgG and IgA[157]. The N-terminal domain of ExoS has GTPase-stimulating activity that targets Rho GTPases[158]. Sensitivity to ExoS depends on the cell line infected, but a common mechanism is interference with proper cytoskeletal function[159] causing loss of function in epithelial cells[160,161] and induction of apoptosis in neutrophils[162,163]. ExoS is activated by a member of the 14-3-3 protein family signaling molecules termed FAS (factor-activating ExoS)[164], which are conserved among eukaryotic cells[165]. Additionally, ExoS ADP-ribosyltransferase activity is most

efficient when the effector is localized to intracellular membranes by a leucine-rich motif[166–168].

Originally detected as a 53-kDa member of a protein aggregate with ExoS[169], it was determined that ExoT shares 76% sequence homology with ExoS but is expressed from a separate gene. Like its counterpart, ExoT is a bifunctional effector with N-terminal Rho GTPase-stimulating activity[170,171] that interferes with cytoskeletal function[172]. Also similar to ExoS, ExoT has C-terminal ADP-ribosyltransferase activity, but it targets Crk adaptor proteins that are involved in the process of phagocytosis[173,174].

ExoU

Exotoxin U (ExoU) is the most toxic effector introduced by the T3SS of *P. aeruginosa*[175]. Presence of secreted ExoU is a strong indicator of poor clinical outcome[148,176] as strains encoding ExoU are significantly more virulent than strains without and are correlated with severe cytotoxicity. Expression of ExoU facilitates systemic spread of *P. aeruginosa* in mice[177], outright kills the yeast *Saccharomyces cerevisiae*[178] and is required for infection of protozoa[179], indicating that this toxin plays a large role in most if not all eukaryotic infections in which it is involved. Efficient secretion of ExoU requires concomitant expression of a chaperone, SpcU, encoded just downstream of its position[180]. Weighing in at 74kDa, ExoU has been characterized as a potent phospholipase A_2 due to its shared homology with several known lipases and ability to increase the membrane permeability of affected cells[181,182]. Like ExoS and ExoT, ExoU is also activated by a eukaryotic effector, which in this case is ubiquitin[183]. Conserved within the eukaryotic domain, the ubiquitin signaling system is often hijacked

by bacterial effectors[184] allowing a broad spectrum of possible hosts. The C-terminal domain of ExoU is necessary for its toxicity, and it has been suggested that this region is responsible for ubiquitin association and subsequent localization to the host cytoplasmic membrane[185].

ExoY

Exotoxin Y (ExoY) is a promiscuous cyclase that that produces cyclic adenosine-monophosphate (cAMP) as well as other cyclic nucleotides[186,187]. ExoY is often compared to two other bacterial adenylyl cyclase toxins that share a homologous ATP-binding region, CyaA from *Bordetella pertussis* and EF from *Bacillus anthracis*. CyaA and EF are activated by Ca^{+}-calmodulin, but ExoY is not[186]. During infection, ExoY is secreted through the T3SS directly into the cytoplasm of the target cell where, according to unpublished research, it is activated by interaction with F-actin[188]. Once activated, ExoY increases the concentration of cytoplasmic cAMP, an important second-messenger molecule in a wide variety of cellular signaling pathways[189,190].

Introduction of ExoY to an infected cell has been shown to cause cell "rounding" in CHO cells and an inability of epithelial cells to repair gaps[191–193]. This may be due to increased Tau phosphorylation caused by increased cytosolic cAMP concentrations, which leads to inhibition of microtubule formation and function[194]. While the various effects of ExoY on eukaryotic cells have been investigated, the mechanism and kinetics of ExoY's cyclase activity have yet to be elucidated. Unlike similar adenylyl cyclases, ExoY contains five cysteine residues. This is unusual for a protein that functions within the eukaryotic cytoplasm due to the presence of endogenous reductases, which would

presumably prevent any stable disulfides from forming. If these cysteine residues do not have structural roles, it is possible that they are catalytically involved in ExoY's cyclase activity.

While cysteine is noted as one of the least abundant amino acids, it has been shown to perform a wide variety of diverse functions[195] and is highly conserved in protein sequences[196]. Proteins often rely on disulfide bridges formed from two cysteine residues by specialized cellular machinery to stabilize their structure[197,198]. However, cysteines are also common metal-binding residues, and such interactions can function similarly to a structural disulfide[199]. Additionally, cysteine residues often function as versatile nucleophiles in catalytic sites, as they may participate in either redox or non-redox reactions depending on whether or not the residue's redox state changes[200,201]. Analysis of cysteine function in ExoY could lead to elucidation of the toxin's mechanism of action, which may then be targeted for inhibition to affect pathogenesis.

CHAPTER II: METHODS

Site-Directed Mutagenesis

pET28b plasmid containing *exoY* with an N-terminal 6x His-tag and *kanR2* to confer kanamycin resistance was previously stored in XL1-Blue *E. coli* cells kept frozen at -80^{0}C. Plasmid isolation was performed using Wizard Plus SV DNA Purification minipreps (Promega, Madison, WI) according to the manufacturer's instructions. Site-directed mutagenesis was performed using the Change-IT Multiple Mutation kit (Affymetrix, Santa Clara, CA) with custom primers according to the manufacturer's protocol. This produced 5 mutated plasmids, each containing single cysteine-to-serine mutations at positions 100, 205, 279, 331 or 345, corresponding to *exoY*'s five cysteine residues. Following mutagenesis, each plasmid was then transformed into chemically competent JM109 *E. coli* cells (Fisher Scientific, Pittsburgh, PA) and plated on LB plates containing kanamycin (50 µg/mL) to select for successful transformants. Plasmid containing each mutation was then reisolated and sequenced to confirm presence of desired mutations and absence of unwanted modifications. Upon mutation confirmation, plasmids were transformed into chemically competent BL21 *E. coli* cells in preparation for protein expression.

Protein Expression and Purification

Individual colonies with each mutation were selected, cultured in LB broth with kanamycin (50 µg/mL) and induced to express ExoY with 0.5 mM isopropyl ß-D-1-

thiogalactopyranoside (IPTG). Cells were collected by centrifugation (10,000 x*g* for 10 mins at 4°C), resuspended and lysed using a French Press (Thermo Spectronic) set to 100 psi. Mutated ExoY was purified from the cell lysates via nickel-affinity chromatography and dialyzed in buffer (10 mM Tris pH 8.6, 250 mM NaCl, 6 mM $MgCl_2$, 2 mM dithiothreitol) overnight to remove excess imidazole. After removal from dialysis, samples were aliquoted and frozen at -20°C.

Protein Quantification

Sample protein concentrations were determined by running serial dilutions in parallel with bovine serum albumin (BSA) standards of known concentrations on 11% SDS PAGE gels. BSA standards containing 2.0, 1.5, 1.0, 0.5 and 0.25 µg of protein were prepared by diluting appropriate volumes of 2 mg/mL stock in nanopure water. Samples were prepared by performing alternating 5-fold and 2-fold serial dilutions of protein isolates to produce 1/5, 1/10, 1/50, 1/100 and 1/500 diluted samples. Standards and samples were loaded into an SDS PAGE gel and run at 75 volts through the stacking portion of the gel and then at 90 volts for 2 hours. The separated proteins were fixed, stained with Coomassie Brilliant Blue (Fisher Scientific, Pittsburgh, PA) and analyzed with a Chemidoc MP Imaging System (Bio-Rad, Hercules, CA). Volume analysis was acquired by measuring pixel density of the BSA standard bands and generating a standard curve, which was then used to calculate the original concentrations of each protein sample.

Circular Dichroism

The circular dichroism (CD) spectra of ExoY mutants were evaluated using a J-815 combination circular dichroism-fluorescence instrument (JASCO, Easton, MD) along with the corresponding Spectra Manager software. ExoY samples were diluted to 4 µM in dialysis buffer (components above), and measurements were collected at 30°C every 1 nm between 200 nm and 240 nm. Each sample measurement consisted of 4 repeated collections through the range of wavelengths that were then averaged to generate a single spectrum. Rotation of polarized light through the sample was measured in molar ellipticity (θ) with the units $deg \cdot cm^2 \cdot dmol^{-1}$. Investigation of possible association with calcium was attempted by combining WT or mutated ExoY with 400 µM $CaCl_2$ and collecting CD spectra as described.

Human Cell Culture and Lysis

A549 human lung epithelial carcinoma cells were previously frozen at -80°C in Dulbecco's modified eagle medium (DMEM) with 5% DMSO. Cells were gently thawed and inoculated into pre-warmed DMEM containing 10% fetal bovine serum and 1:100 streptomycin/penicillin G (Fisher Scientific, Pittsburgh, PA). Cells were incubated in 75 cm^2 flasks at 37°C and 5% CO_2 and reached confluence after about 48 hours. To harvest, cells were washed with phosphate-buffered saline (PBS) and detached from the flask surface with a 0.25% trypsin solution. Cells were then pelleted by centrifugation at 200 x*g* for 5 minutes and resuspended in a lysis buffer (20 mM Tris pH 8.6, 20 mM NaCl, 10 µg/mL RNase A, 10 µg/mL DNase I and 1:100 Halt protease inhibitor cocktail). Lysis was achieved by passage between two syringes through a double-ended

22-gauge needle at 4°C. Whole lysate was centrifuged at 10,000 x*g* for 10 minutes and centrifuged at 100,000 x*g* for 1 hour to sediment cell debris. The soluble lysate was collected, aliquoted and stored at -20°C. Total protein concentration within the soluble lysate was determined with a BCA assay (Qiagen, Hilden, Germany) according to the manufacturer's instructions.

Calcium Quantification

The concentration of calcium in soluble lysate of A549 lung epithelial carcinoma cells was determined via flame emission atomic absorption spectroscopy with a SpectraAA 220 instrument (Varian, Palo Alto, CA). Cell lysate was incubated at 37°C with proteinase K for several hours to remove any protein-metal associations. A standard addition method was utilized in which identical samples of cell lysate were "spiked" with known concentrations (0, 0.5, 1.0 or 1.5 mg/L) of calcium along with 10,000 ppm lanthanum chloride ($LaCl_3$) and 2% nitric acid (HNO_3). Each sample was aerosolized into an acetylene flame atomizer and excited by 422.7 nM emission from a calcium cathode lamp. Absorbance at that wavelength was measured for five 6-second intervals following a 3-second pre-read delay. A linear curve was constructed with the average absorbance for each calcium concentration and then used to calculate a calcium concentration of the original cell lysate.

F-actin Polymerization

Unpolymerized rabbit skeletal muscle actin (Cytoskeleton, Denver, CO) was obtained as a lyophilized powder and polymerized according to the manufacturer's

instructions to produce the F-actin utilized in ExoY cAMP immunoassays. Briefly, the lyophilized protein was reconstituted to 10 mg/mL in General Actin Buffer (5 mM Tris-HCl pH 8.0, 0.2 mM $CaCl_2$, 0.2 mM ATP) supplemented with 5% (w/v) sucrose and 1% (w/v) dextran. Aliquots were flash-frozen in liquid nitrogen and stored at -80°C until thawed on ice for experimental use. Before addition to an assay, an aliquot was diluted to 0.4 mg/mL in General Actin Buffer with 0.5 mM DTT. Addition of Polymerization Buffer (500 mM KCl, 20 mM $MgCl_2$, 10 mM ATP) followed by incubation for 1 hr at room temperature allowed the actin to polymerize for assay use.

cAMP Immunoassay

The DetectX Direct cyclic AMP Enzyme Immunoassay Kit (Arbor Assays, Ann Arbor, MI) was used to measure adenylyl cyclase activity of each mutated ExoY isolate. cAMP production reactions were prepared in ExoY activity buffer (40 mM Tris, 12 mM $MgCl_2$, 0.4 mM DTT, 4 mM ATP) with 15 nM ExoY activated by 4 µg A549 cell lysate protein or F-actin. Reactions were incubated for 30 minutes at 30°C and then quenched by adding EDTA (4 mM) and boiling for 5 minutes. Reaction mixtures were then centrifuged at 13,400 x*g* for 5 min to sediment cell debris and the supernatant was used in the assay as per the manufacturer's protocol. Briefly, standard cAMP solutions (150, 50, 16.67, 5.56, 1.85, and 0.617 pmol/mL) were prepared and the supernatants from cAMP production reaction mixtures were diluted to concentrations experimentally determined to be within this range (1:10, 1:80, 1:160 and 1:320). cAMP standards and samples were added to wells along with a cAMP-peroxidase conjugate with which they competed for anti-cAMP sheep IgG in wells coated with anti-sheep IgG antibodies. After a 2-hour

incubation at room temperature, the wells were washed and then further incubated for 30 minutes at room temperature with a peroxidase substrate, 3,3',5,5'-tetramethylbenzidine (TMB), to produce a color change. After quenching the reaction with hydrochloric acid, the absorbance of each well at 450 nm wavelength was recorded.

This assay was also used to evaluate possible effects of calcium on WT ExoY adenylyl cyclase activity. The assay procedure and cAMP standards were as described above, however sample cAMP production reactions contained either 15nM WT ExoY alone, with 10 µg A549 lysate only, with 15 µM Ca^{+2} only, or both lysate and calcium. K81M ExoY alone or with lysate and calcium was included as a negative control.

cAMP concentrations in sample wells were calculated using GraphPad Prism software. First, the absorbance value of a non-specific binding well that contained no anti-cAMP antibody was subtracted from all other wells. Each standard and sample value (B) was then divided by the absorbance of a maximum binding well (Bo) that contained no unconjugated cAMP. This value (B/Bo) for the standards was plotted versus the log of their known unconjugated cAMP concentration to form a sigmoidal standard curve. Finally, this function was used to calculate the concentration of unconjugated cAMP in each sample.

Statistical Analysis

Tests for significant difference between specific activities of the ExoY proteins were conducted using two-sample t-tests with assumed unequal variance in Microsoft Excel software. Significant differences between groups when $\alpha = 0.05$ are illustrated in figures by differing letter assignments.

CHAPTER III: RESULTS

Mutated ExoY Creation and Expression

Each single cysteine-to-serine mutation was generated in recombinant *exoY* in a pET28b plasmid vector using custom primers designed to introduce a single nucleotide change (Figure 1). This produced five individual mutations at positions 100, 205, 279, 331 and 345 in the *exoY* gene sequence. After the mutagenesis PCR reaction, plasmids were transformed into JM109 *E. coli* cells and then plated with kanamycin to confirm plasmid presence. Successful transformants were then selected from the plates for plasmid DNA isolation, and each plasmid was sequenced to confirm the desired mutation was definitely present. Successfully mutated plasmids were transformed into BL21 *E. coli* and production of recombinant, N-terminal 6x His-tagged ExoY was induced.

Harvest of the ExoY protein was accomplished via French pressure cell lysis followed immediately by nickel-affinity chromatography to specifically purify ExoY from the resulting *E. coli* cell lysate. Elutions were evaluated for ExoY content and relative purity (Figure 2), and those containing significant protein at the 42 kDa position were pooled, dialyzed and stored. Before any experimentation, though, each sample of ExoY protein was quantified via densitometric comparison to BSA samples of known concentration run simultaneously through an SDS PAGE gel (Figure 3). Visualization and software analysis allowed calculation of ExoY concentrations for samples used in the following experiments.

Figure 1. Locations and Identities of ExoY Mutations. Custom primers were used to introduce point mutations that altered codons for cysteine residues to codons for serine. Relative positions of each target codon are illustrated by positioning between the start and stop codons, and the actual mutations are shown below the wild-type sequence.

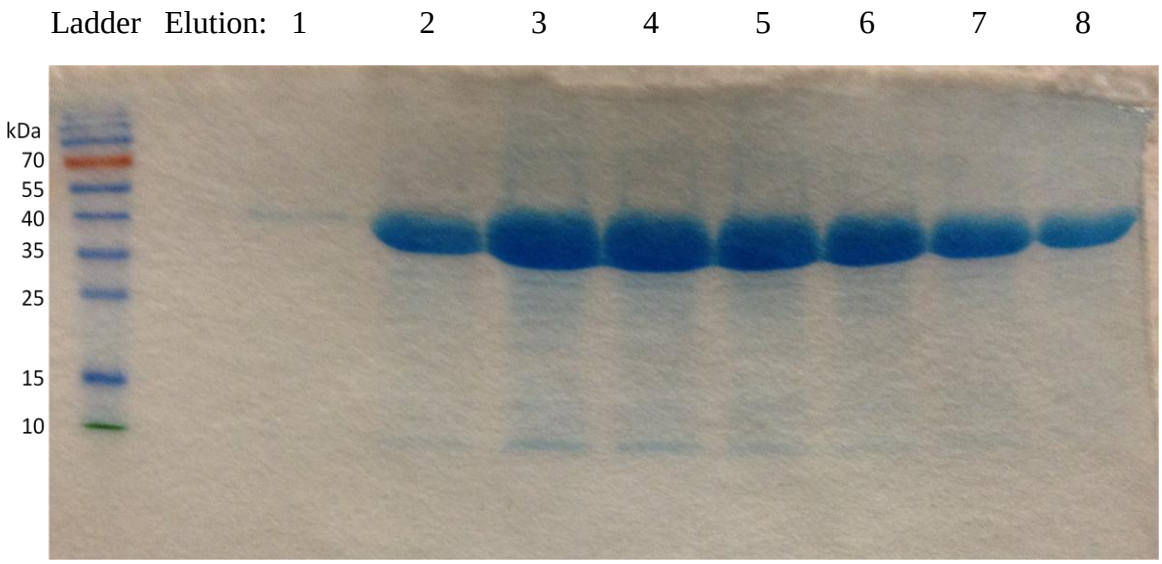

Figure 2. Nickel-Affinity Purification Elutions. Following sample binding and several washes, eight 250µL elutions were collected from each nickel-affinity column and run on an 11% SDS-PAGE gel. The first elution never contained significant protein of any size, but each following elution always contained dominant bands corresponding to the 42kDa size of ExoY. These seven elutions were pooled and dialyzed for analysis.

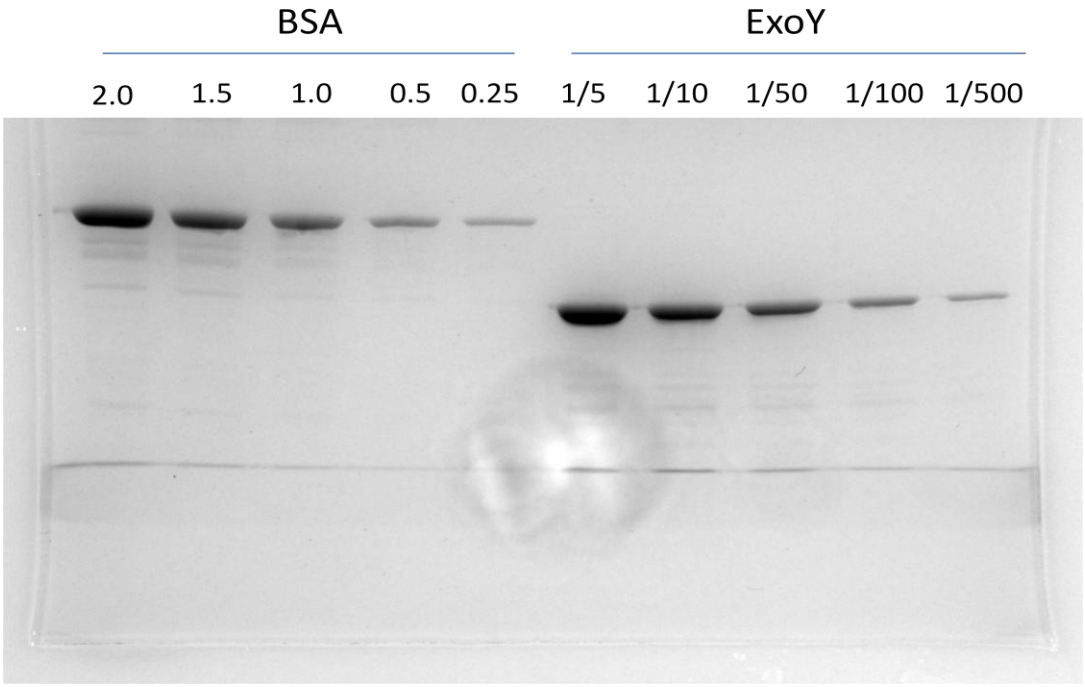

Figure 3. Example of SDS PAGE Densitometry. BSA standards (2.0, 1.5, 1.0, 0.5 and 0.25 ug) were run on the left and compared to serial dilutions of ExoY C205S samples on the right. Concentrations of ExoY dilutions are expressed as fractions of the stock concentration. Pixel density of the BSA bands was used to construct a standard curve that could be used to calculate concentrations of the ExoY bands.

Structural Evaluation of Mutated ExoY

To evaluate any effects of cysteine-to-serine mutations on the 3D structure of ExoY, circular dichroism (CD) spectra were obtained for WT ExoY and each mutated protein (Figure 4). WT ExoY exhibited a "double-dip" pattern with negative molar ellipticity (θ) peaks near 207 nm and 220 nm. This pattern is highly consistent with primarily α-helical secondary structure[202]. Each of the mutated samples exhibited the same double-dip pattern, but with varying magnitudes. WT ExoY displayed the greatest θ at both 207 nm and 220 nm, indicating a more structured conformation. All mutant samples appeared less structured than the WT; the C205S and C279S mutations displayed relatively similar structure to the WT, while the C100S, C331S and C345S mutations caused a clear loss of secondary structure.

Cysteine residues are known to sometimes associate with biologically active metal ions in a structural or catalytic capacity[199]. Mg^{+2} was present in all ExoY storage and assay buffers, so ExoY could not be analyzed in its absence. Zn^{+2} was of interest due to its common association with cysteine[203], but even concentrations in the nano-molar range caused ExoY to precipitate from solution. Ca^{+2} was investigated since similar adenylyl cyclase toxins are activated by calmodulin[186], a ubiquitous calcium-binding protein involved in many signal-transduction pathways[204]. CD spectra of each ExoY sample were acquired as before, but in the presence of 400 μM $CaCl_2$ (Figures 5). Comparison of θ in the presence and absence of calcium at 207 nm and 220 nm showed little difference in ExoY structure (Figure 6).

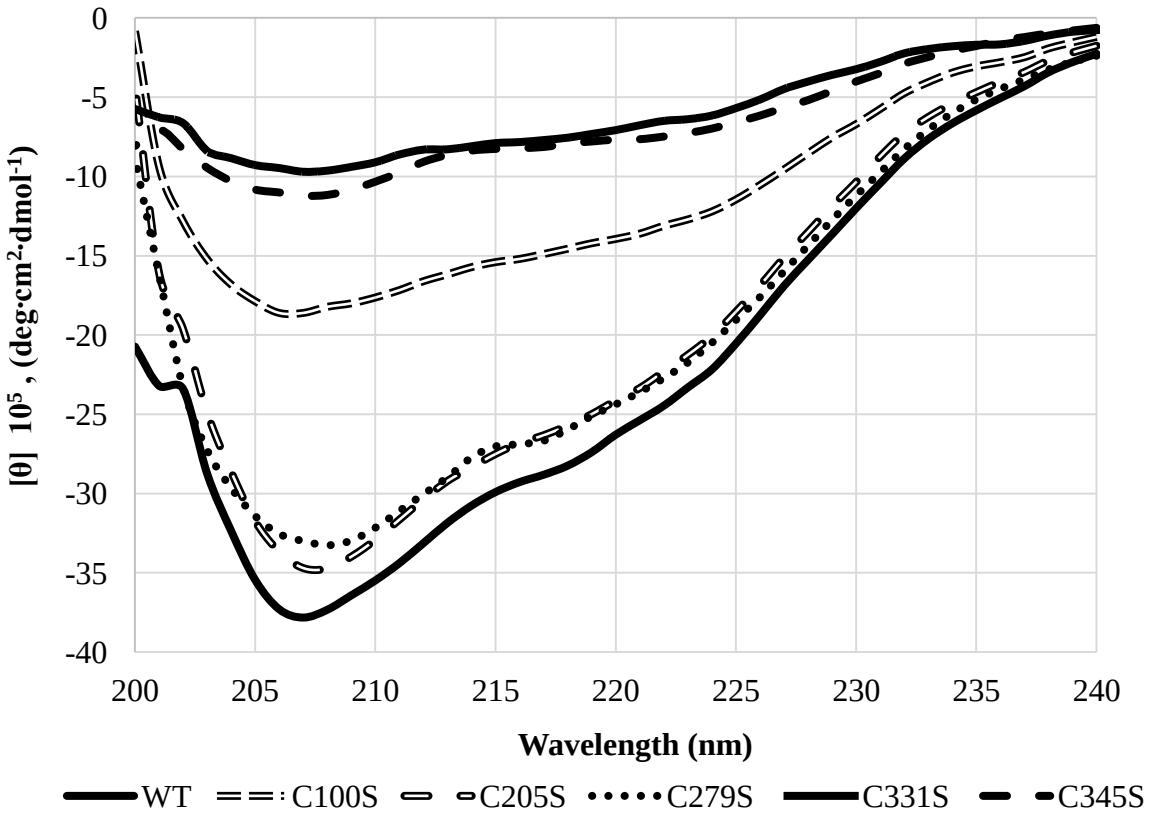

Figure 4. CD Spectra of ExoY Proteins. Each sample was diluted to 4.0 µM and circular dichroism spectra were collected between 200 nm and 240 nm. Rotation of polarized light was measured as molar ellipticity (θ) with the units deg·cm^2·dmol^{-1}. A larger magnitude θ indicates greater rotation of polarized light by the sample at the particular wavelength of interest. Each curve is an average of 4 measurements at each wavelength.

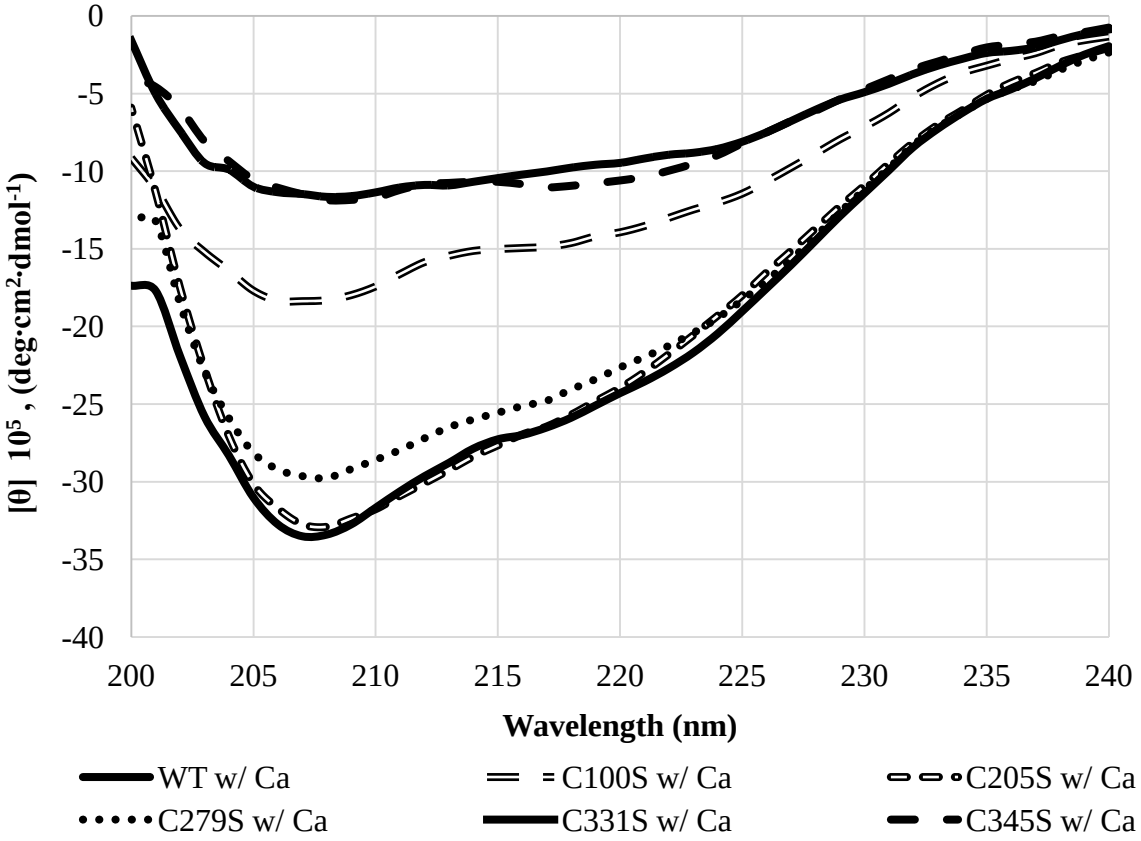

Figure 5. CD Spectra of ExoY Proteins with Calcium. Each sample was diluted to 4.0 µM with 400 µM $CaCl_2$ and circular dichroism spectra were collected between 200 nm and 240 nm. Rotation of polarized light was measured as molar ellipticity (θ) with the units deg·cm^2·$dmol^{-1}$. A larger magnitude θ indicates greater rotation of polarized light by the sample at the particular wavelength of interest. Each curve is an average of 4 measurements at each wavelength.

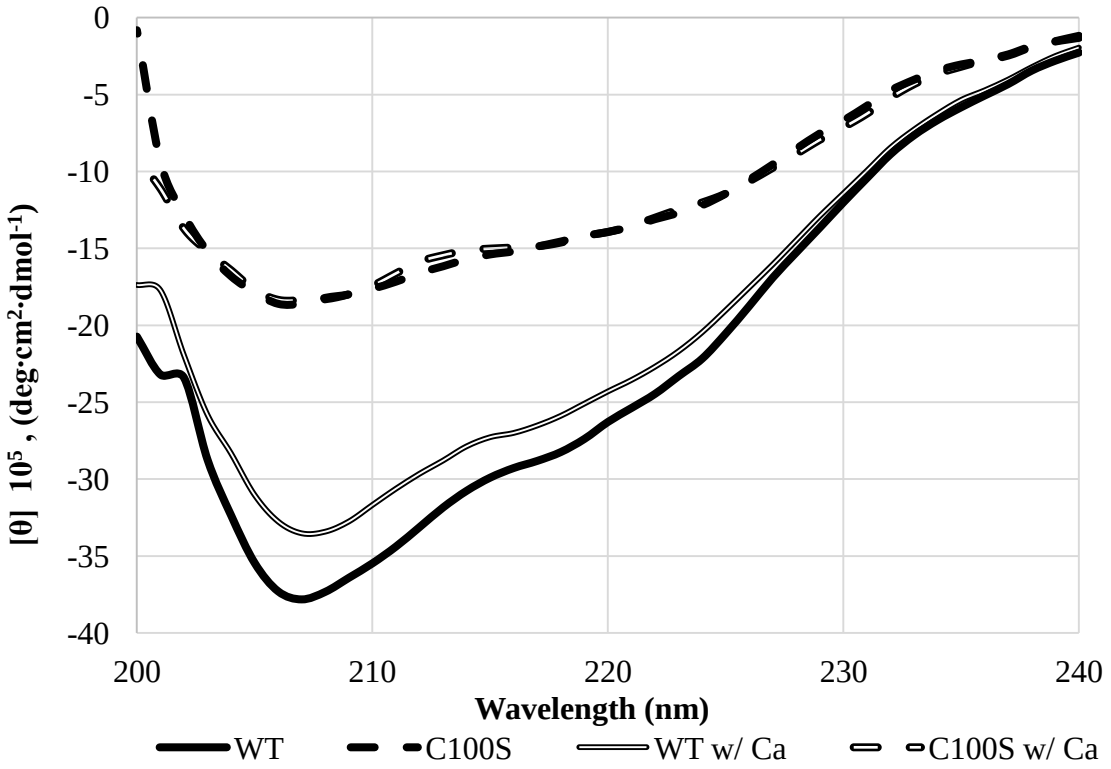

Figure 6. WT and C100S Spectra With and Without Calcium. Curves are transposed from Figures 4 and 5 to illustrate difference between CD spectra gathered in the absence and presence of Ca^{+2}. C100S protein clearly differs little in the presence or absence of metal ion. The WT spectra do display some apparent difference, but comparison of individual wavelength measurements yielded no significant differences at any point. Curves for each other mutant protein were also not significantly different.

Evaluation of Mutated ExoY Function

When initial activity assays were performed, the intracellular interaction required to activate ExoY adenylyl cyclase activity after secretion into a eukaryotic cell was unknown. To induce ExoY activity, whole cell lysate harvested from A549 human lung epithelial carcinoma cells was used. This cell line is commonly used to evaluate *P. aeruginosa* T3S[205,206,187], and previous research determined that introduction of this cell lysate induces ExoY adenylyl cyclase activity[208].

Using A549 lysate to activate ExoY, the adenylyl cyclase activity of each mutated protein was evaluated along with the known catalytic null, K81M, with respect to wild type ExoY. All activity assays were performed in triplicate and were interpreted with a set of cAMP standards that fit a 4 parameter logistic nonlinear regression equation. The results of these preliminary assays using A549 lysate (Figure 7) illustrated decreases in the activity of all mutant proteins except the C205S ExoY. Interesting pairings emerged between the cysteine residues at positions 100 and 209 (10-20% of WT activity), and those at positions 331 and 345 (40-50% WT activity).

To see if calcium affects the adenylyl cyclase activity of ExoY, WT protein was evaluated using the cAMP detection kit as before. Activity assay reactions included combinations of WT ExoY with A549 cell lysate and/or 15 µM Ca^{+2}, which was the concentration of calcium determined to be in the cell lysate. Specific activity of each sample was calculated as before (Figure 8), but showed that calcium did not significantly alter WT protein activity.

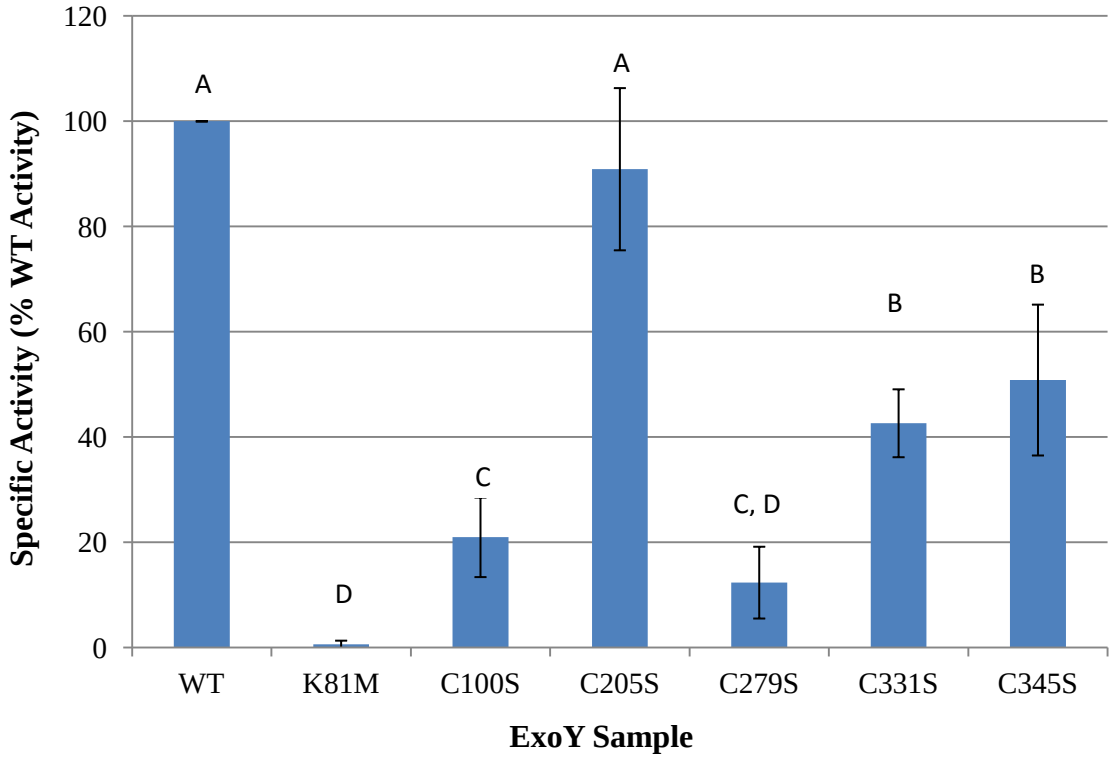

Figure 7. Normalized Specific Activity of ExoY Proteins. ExoY samples were activated by A549 cell lysate and allowed to produce cAMP for 30 minutes at 30°C. Concentration of cAMP (nM) was determined with the cAMP EIA Kit from Arbor Assays and then used to determine specific activity ([cAMP]/[ExoY]) of each sample. Due to variability in the assay, samples were normalized to the WT specific activity value for each replicate experiment. Values shown are averages of 3 replicates. Samples with the same letters are not significantly different according to a Two-Sample T-Test with $\alpha = 0.5$.

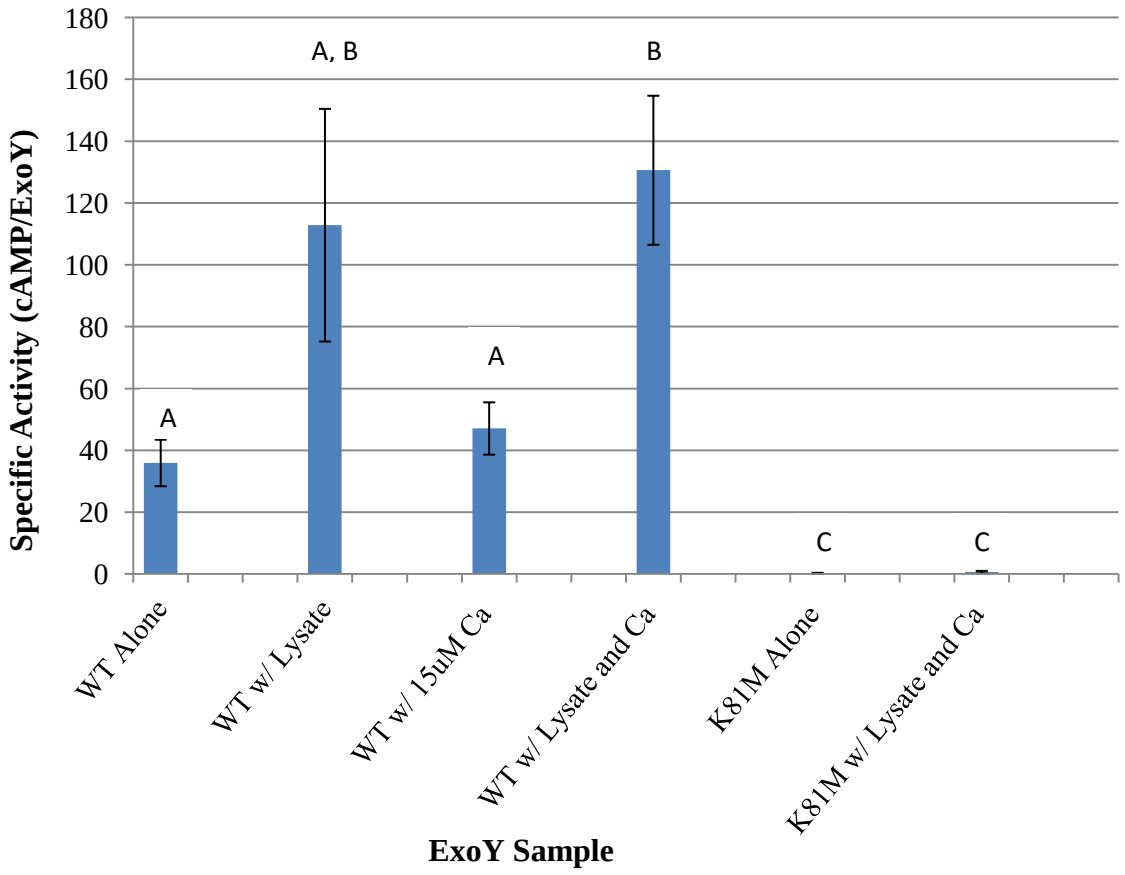

Figure 8. Comparison of WT With A549 Cell Lysate and/or Calcium. Activity assays were performed in triplicate with WT or K81M ExoY alone, with 10 µM A549 cell lysate, 15 µM Ca^{+2}, or both lysate and Ca^{+2}. Specific activity of each sample was calculated and used to determine if ExoY adenylyl cyclase activity is affected by the presence of calcium. Samples with the same letters are not significantly different according to a Two-Sample T-Test with $\alpha = 0.5$.

Confirmation of F-Actin as an Activator of ExoY

Shortly after the data described above were acquired, an unpublished manuscript was made publicly available with evidence that the previously unidentified activator of ExoY is actually F-actin[188]. Validation of this finding was attempted by performing the same activity assay described above, but using F-actin to activate ExoY rather than A549 cell lysate (Figure 9). Using F-actin increased the specific activity of the WT and C205S mutated proteins to levels well beyond the cAMP standard curve utilized. This was observed even when both the enzyme and the activator in activity reactions were significantly reduced, making it impossible to determine the specific activity of those samples without further optimization. However, the C100S, C279S, C331S and C345S mutant proteins remained within the standard curve, indicating significantly reduced specific activity consistent with the previous findings when A549 cell lysate was used as an activator.

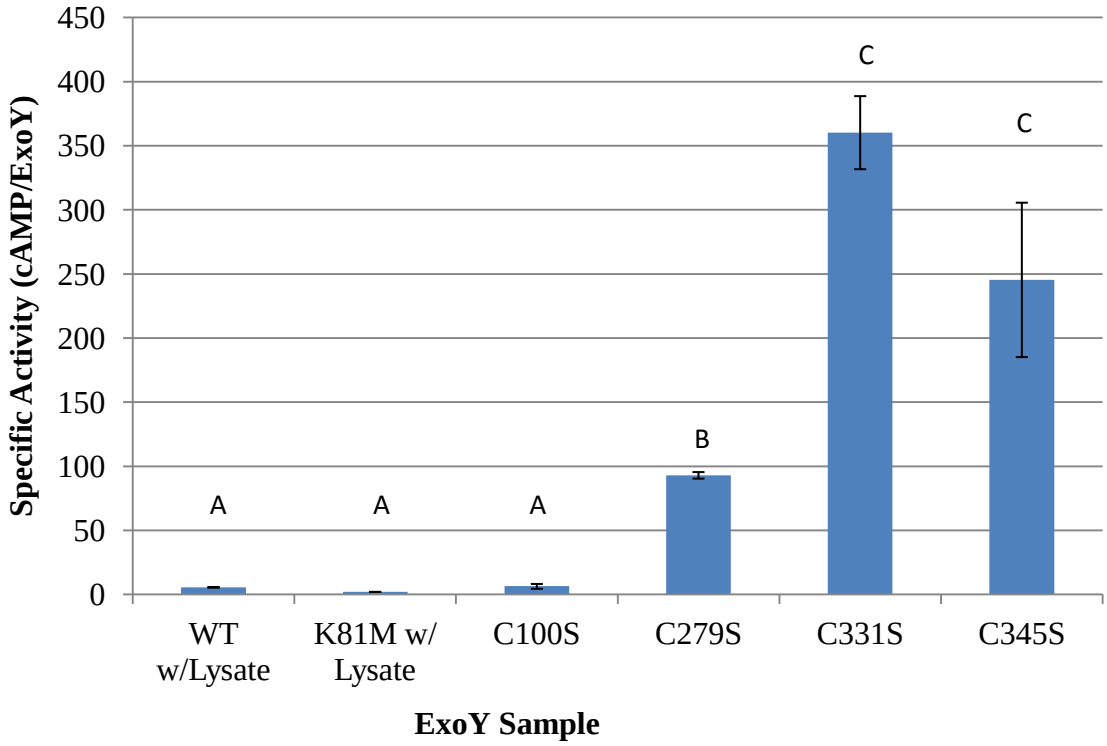

Figure 9. Specific Activity of ExoY Activated by F-Actin. ExoY samples were activated by F-actin and allowed to produce cAMP for 30 minutes at 30°C. Concentration of cAMP (nM) was determined with the cAMP EIA Kit from Arbor Assays and then used to determine specific activity ([cAMP]/[ExoY]) of each sample. Increased variability is due to the lack of normalization to a WT value, since WT was outside the standard curve and could not be quantified. Samples with the same letters are not significantly different according to a Two-Sample T-Test with $\alpha = 0.5$.

CHAPTER IV: DISCUSSION

ExoY is a promiscuous nucleotide cyclase toxin secreted by *Pseudomonas aeruginosa* during infection of eukaryotic cells[186]. The cAMP produced by this enzyme causes various negative effects in the infected cell. This includes, but is certainly not limited to, loss of microtubule stability due to increased Tau phosphorylation leading to impairment of cytoskeletal-mediated functions like endothelial barrier maintenance and phagocytosis[192–194,205]. Such effects enable *P. aeruginosa* to penetrate tissue barriers and evade host immune responses during infection. ExoY is similar in structure to the bacterial adenylyl cyclase toxins CyaA and EF, but does not share their common mechanism of activation. Additionally, ExoY's primary structure contains 5 cysteine residues, which is odd for an intracellularly active protein. Cysteine residues are among the rarest of amino acids, are often highly conserved, and often play essential roles in the proteins that contain them[195,196,200]. The purpose of this research was to investigate the possible roles these cysteine residues may have in the structure or function of the ExoY toxin.

Mutated ExoY proteins were generated that each lacked a particular cysteine residue at position 100, 205, 279, 331 or 345 in its primary structure. These were replaced via a point mutation with a serine residue that mostly maintains the polar and steric properties of cysteine. However, this substitution removes the thiol R-group that is usually responsible for cysteine's catalytic properties as well as its ability to form structural disulfide bonds. Evaluating the structure and function of these mutated ExoY

proteins could provide insight into the purpose of each cysteine residue and possibly the overall mechanism of ExoY.

Circular dichroism is a spectroscopic method that can be used to evaluate the secondary structure of a protein sample in solution[207]. When circularly polarized light of a particular wavelength is incident on an optically active molecule, it is rotated to a degree dependent on the identity of the active group or groups present. Rotation of the light can be measured as molar ellipticity (θ), which takes into account the sample concentration as well as the pathlength of the cuvette utilized. In this manner, protein secondary structures will exhibit characteristic CD spectra and can be identified if present in a sample. If any of ExoY's cysteine residues are responsible for forming structural disulfide bonds, their absence could inhibit formation of secondary motifs in ExoY's overall structure. This would manifest as a change in θ of the sample.

To examine any structural changes, CD spectra were obtained for the wild-type and mutated ExoY proteins, which revealed a consistent "double-dip" pattern with two distinctly negative peaks near 207 nm and 220 nm (Figure 4). This pattern is indicative of α-helices, which appeared to be the predominant secondary structure in WT ExoY[202]. Each of the mutated ExoY proteins also displayed similarly patterned spectra, but they showed widely varying θ magnitudes (Figure 6). ExoY with C205S or C279S mutations retained spectra similar to the WT, so these residues likely do not participate in a structural capacity and may have catalytic roles instead. C331S and C345S mutations, however, caused almost complete loss of CD signal, indicating a strong structural role for those cysteines. They may indeed form a disulfide bond that is essential for overall ExoY

structure. The C100S mutation hovered between these two extremes, so the 100 position may have structural significance, but not as much as the 331 or 345 positions.

Another common role for cysteine residues is the coordination of metal ions, which can simply provide structure or participate in a catalytic site[196,203]. Several biologically active metals can be associated with cysteine residues, including Mg^{+2}, Zn^{+2} and Ca^{+2}. As an adenylyl cyclase, ExoY already requires Mg^{+2} associated with substrate ATP for activity. Analysis with Zn^{+2} was attempted, but was thwarted by almost immediate precipitation of ExoY protein even at nanomolar metal concentrations. This left Ca^{+2}, which was also of interest because other adenylyl cyclase toxins to which ExoY is often compared are activated by calmodulin. ExoY is not, but may still require Ca^{+2} to be present for activity.

The possibility of calcium participation was also investigated with CD analysis. $CaCl_2$ was added to the same protein samples used for initial CD characterization up to a concentration 100 times greater than ExoY. Spectra were acquired and analyzed as before (Figure 5). The same pattern of structural roles was apparent in these spectra, with the 331 and 345 cysteines appearing necessary for structure while C205S and C279S showed little loss of structure and C100S sat in-between. Interestingly, the C331S and C345S mutants did exhibit a slight increase in θ after exposure to Ca^{+2}, while the other mutants showed no change or a slight decrease. While this could indicate that C331 and C345 associate with Ca^{+2}, the change was not significant and the lack of change otherwise suggests that Ca^{+2} is more likely not involved in the structure of ExoY.

With indications of structural significance established, the focus of investigation became the adenylyl cyclase activity of the WT and mutated ExoY proteins. This was

evaluated by combining ExoY samples with all the necessities for cAMP production and evaluating the amount of cAMP produced with an enzyme-linked, competitive-binding immunoassay. At the time these assays were performed the activator of ExoY was unidentified, so soluble lysate of A549 lung epithelial cells was used because it presumably must contain the activator and did cause an increase in cAMP production. While ExoY is known to produce cyclic nucleotides besides cAMP, particularly cGMP and cUMP, its production of cAMP has been most extensively studied and established as a pathogenic mechanism. If removed, cysteine residues that are required for adenylyl cyclase activity should reduce the amount of cAMP produced during an activity assay.

cAMP production by each mutated ExoY protein was evaluated alongside the WT as well as a K81M mutated ExoY that has been previously established as a catalytic null. Concentration of cAMP produced was calculated against the concentration of ExoY present to yield a specific activity value. These data were then normalized by setting the WT value to 1 and calculating the rest of the specific activities as percent of WT value (Figure 7).

The C205S mutation caused no loss of adenylyl cyclase activity. Taken with its CD data, this seems to show that the cysteine residue at position 205 is not significantly necessary for the structure or function of ExoY in an infected cell. This does not mean it lacks any role, but that it may be involved in processes prior to the introduction of ExoY into a host cell. Like other Type-III secreted toxins, ExoY requires a chaperone protein that guides it to the secretion apparatus within bacterial cells, and the cysteine at position 205 could be involved in that process. The C279S mutant, however, showed a large decrease in cAMP production. Like C205S, this mutant's CD spectra was similar to the

WT, so it may participate in the active site of ExoY, but isn't responsible for overall ExoY structure. The C100S also caused a significant decrease in cAMP production, but may also be involved structurally.

The C331S and C345S mutations caused a significant and identical loss of cAMP production, suggesting that the structural role these residues play is also necessary for ExoY activity. ExoY has yet to be successfully crystallized, possibly due to a rather "loose" conformation. Perhaps these two residues form a disulfide bond that is necessary for active site formation, but is rather unstable in the cytoplasm of eukaryotic cells where it would be rapidly reduced. However, contact with the ExoY "activator" could allow this disulfide to become stable, solidifying the active site. Further research using X-ray crystallography and cryo-electron microscopy would lend credence to this theory, but have yet to be successfully performed.

Further investigation of possible association with calcium was pursued by performing activity assays with the metal ion alone or alongside A549 cell lysate. Specific activities were calculated as before (Figure 8). The results were fairly clear, showing that WT ExoY has basal activity in the absence of any activator and it is not increased by the presence of Ca^{+2}. This further supported the CD data indicating that calcium is not significantly involved in ExoY adenylyl cyclase structure or activity.

Near the end of this project, an unpublished manuscript was made publicly available that identified F-actin as the activator of ExoY[188]. Validation of this finding was attempted by performing additional activity assays as before; with pure F-actin present in place of A549 cell lysate. Clearly, F-actin is indeed the activator of ExoY because it increased the activity of WT and C205S samples well beyond the assay's standard curve

even after reduction of both enzyme and F-actin concentrations (data not shown). Further optimization would be necessary to determine proper concentrations to evaluate such high cAMP production. However, activity of the other mutated ExoY samples did not exceed the standard curve, highlighting the effect of removing the cysteine residues at positions 100, 279, 331 or 345. They are certainly necessary for cAMP production in a structural and/or catalytic capacity as described above.

CONCLUSIONS

The ExoY toxin secreted by *Pseudomonas aeruginosa* contains 5 cysteine residues that appear necessary for structural integrity and/or catalytic activity based on CD spectra and cAMP detection assays. The C331 and C345 residues are necessary for overall ExoY structure and active site formation, possibly due to formation of a disulfide bond. The C279 residue is not involved in secondary structure formation, but is necessary for catalytic activity. The C100 residue is involved in catalytic activity, but also plays a role in ExoY secondary structure to some degree. Residue C205 doesn't appear necessary for secondary structure formation or cAMP production, but may be involved in chaperone association or some other process that occurs prior to secretion.

Despite the association of calcium-binding calmodulin with other adenylyl cyclase toxins, Ca^{+2} does not seem to be directly involved in ExoY structure or function to any significant degree. Finally, in accordance with other unpublished research, F-actin does appear to be the previously unidentified activator of ExoY. These findings, while seemingly varied, provide a clear contribution to the understanding of a toxin secreted by a commonly multi-drug resistant, opportunistic pathogen.

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