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Proteolytic regulation of alginate overproduction in *Pseudomonas aeruginosa*

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SUMMARY

Pseudomonas aeruginosa, a Gram-negative bacterium, is a significant opportunistic pathogen associated with skin and soft tissue infections, nosocomial pneumonia, and sepsis. In addition, it can chronically colonize the lungs of cystic fibrosis (CF) patients. Overproduction of the exopolysaccharide called alginate provides *P. aeruginosa* with a selective advantage and facilitates survival in the CF lung. The *in vitro* phenotype of alginate overproduction observed on solid culture media is referred to as mucoid. Expression of the alginate machinery and biosynthetic enzymes are controlled by the extracytoplasmic sigma factor, σ^{22} (AlgU/T). The key negative regulator of both σ^{22} activity and the mucoid phenotype is the cognate anti-sigma factor MucA. MucA sequesters σ^{22} to the inner membrane inhibiting the sigma factor's transcriptional activity. The well-studied mechanism for transition to the mucoid phenotype is mutation of *mucA*, leading to loss of MucA function and therefore activation of σ^{22}. Recently, regulated intramembrane proteolysis (RIP) has been recognized as a mechanism whereby proteolysis of the anti-sigma factor MucA leads to active σ^{22} allowing *P. aeruginosa* to respond to environmental stress conditions by overproduction of alginate. The goal of this review is to illuminate the pathways leading to RIP that have been identified and proposed.

Keywords

RIP; alginate; σ^{22}; AlgU/T; MucA; AlgW; MucP; Prc; ClpXP; Opr86; KinB

Introduction

The genetic disease cystic fibrosis (CF) affects multiple organ systems of the human body with one of the consequences being increased susceptibility to respiratory infections by opportunistic pathogens (Lyczak *et al.,* 2002). CF is caused by mutation of the CF transmembrane conductance regulator (CFTR) gene (Kerem *et al.*, 1989, Rommens *et al.*, 1989). Mutations in CFTR abrogate lung functions resulting in an impaired ability to eradicate inhaled microorganisms (Welsh *et al.,* 2001). One consequence of CF is the formation of thick mucus in the lungs, creating an environment suitable for microbial growth. With the advances in modern pharmacology, many of the once dominant respiratory pathogens that historically afflicted those with CF can now be controlled, leading to increased life expectancy. Even with such advancements in antibiotic therapies, the Gram-negative bacterium *Pseudomonas aeruginosa* remains the most common CF pathogen (Govan & Deretic, 1996). Given its inherent antibiotic resistance and plethora of virulence factors, *P. aeruginosa* is a direct cause of most of the morbidity and mortality in those with

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CF. The onset of chronic *P. aeruginosa* infection of the CF respiratory tract is marked by the emergence of the mucoid phenotype. Mucoid *P. aeruginosa* overproduce and secrete an exopolysaccaride known as alginate (Fig. 1). Alginate forms a capsule that protects *P. aeruginosa* from various host defenses (Leid *et al.,* 2005), antibiotics (Govan & Fyfe, 1978), and phagocytosis (Schwarzmann & Boring, 1971).

Several mechanisms exist which lead to the induction of the mucoid phenotype, but most mechanisms converge upon a common pathway through the extracytoplasmic sigma factor, σ^{22} (AlgU or AlgT). σ^{22} has 65% similarity to its *Escherichia coli* homologue σ^{E} (Hershberger *et al.,* 1995, Martin *et al.,* 1993a), otherwise known as RpoE. In Table 1, the *P. aeruginosa* proteins that will be discussed throughout this review are presented, along with their *E. coli* homologues. *P. aeruginosa* σ^{22} directs RNA polymerase to activate expression of the alginate biosynthetic genes, which are encoded in the *algD* alginate biosynthetic operon at loci PA3540 to PA3551. The functions of the proteins encoded by the alginate biosynthetic genes are well characterized and have recently been expertly reviewed (Franklin *et al.,* 2011, Rehm, 2009).

The principal regulator of σ^{22} is the cognate anti-sigma factor MucA. σ^{22} and MucA are encoded in an operon (σ^{22}*-mucA-mucB-mucC-mucD)* at loci PA0762-PA0766 (Table 1) on the PAO1 genome. MucA localizes to the inner membrane through a single transmembrane domain (Mathee *et al.,* 1997) with the amino- and carboxyl-terminal regions in the cytoplasm and periplasm, respectively. Typically, MucA sequesters σ^{22} to the inner membrane (Mathee *et al.,* 1997). When MucA is mutated, σ^{22} may not be sequestered (Martin *et al.,* 1993b). In the absence of MucA repression, σ^{22} is active and directs transcription with RNA polymerase at σ^{22}-dependent promoters throughout the genome (Firoved *et al.,* 2002). Since alginate genes are regulated by σ^{22}, this loss of MucA-repression results in the constitutive mucoid phenotype. Another negative regulator of σ^{22} was identified just downstream of *mucA* (Goldberg *et al.,* 1993, Martin *et al.,* 1993b) and is named *mucB* (Table 1). It was later shown that MucB is a periplasmic protein (Mathee *et al.,* 1997) that binds MucA (Cezairliyan & Sauer, 2009, Mathee *et al.,* 1997, Wood & Ohman, 2009). *mucC* is another putative regulatory gene (Table 1) (Boucher *et al.,* 1997). A function of MucC has not been established, but one study has clearly defined the promoter of the downstream gene *mucD*, to be within the *mucC* gene (Wood & Ohman, 2006). *mucD* is the final gene of the σ^{22} locus which encodes another negative regulator of alginate overproduction. MucD is a periplasmic chaperone protease homologous to *E. coli* DegP (Table 1) (Boucher *et al.,* 1996).

It is generally accepted the *P. aeruginosa* strains that initially infect CF patients are obtained directly from the environment, but some studies have documented patient-to-patient spread (Armstrong *et al.,* 2003, Jones *et al.,* 2002, McCallum *et al.,* 2001) and sharing of strains among CF siblings (Renders *et al.,* 1997). The *P. aeruginosa* strains that initially infect these patients are generally nonmucoid and sensitive to antibiotics (Burns *et al.,* 2001, Doggett *et al.,* 1966). Phenotypically nonmucoid *P. aeruginosa* strains, when cultured *in vitro,* produce low levels of alginate (Anastassiou *et al.,* 1987, Pier *et al.,* 1986). CF patients that only have nonmucoid strains infecting their lungs have antibody responses specific for alginate (Pedersen *et al.,* 1990). In fact, the ability to produce alginate is key to establishing chronic colonization of CF mice (Coleman *et al.,* 2003). When low levels of alginate are produced, but the mucoid phenotype is not evident, we will refer to this phenotype as “alginate production”. For clarity throughout this review, we will refer to the mucoid phenotype as “alginate overproduction”.

While the exact conditions to induce the mucoid phenotype are not completely understood, several key studies have recently been performed that show how cell wall stress (Wood *et*

al., 2006) or overexpression of an envelope protein (Qiu *et al.,* 2007) can activate proteolytic degradation of MucA by regulated intramembrane proteolysis (RIP). RIP is a mechanism that is conserved from bacteria to humans (Brown *et al.,* 2000). In the model detailed in this review, proteases respond to environmental conditions and cleave MucA allowing σ^{22} to initiate transcription of its regulon. It has been proposed that the most opportune time to block chronic infection of *P. aeruginosa* in CF is before the conversion to constitutively mucoid phenotype (Ramsey & Wozniak, 2005). Building upon this idea, we propose that the RIP proteases responsible for the mucoid phenotype may be potential drug targets for eradication of *P. aeruginosa* from the lungs of CF patients. The goal of this review is to summarize the key literature that formed the basis for the model of MucA RIP, identify remaining questions, and propose future directions for the field.

Membrane stress leads to expression of the σ^{22} regulon

Wood *et al*. hypothesized that the σ^{22}-MucA signal transduction pathways would respond to environmental stresses leading to expression of the σ^{22} regulon (Wood *et al.,* 2006). To test this hypothesis they utilized a reporter construct to identify stress agents that could induce σ^{22} activity. Many classes of stress compounds (50 total) were screened for their ability to activate a σ^{22}-dependent promoter fusion of P*algD* with a chloramphenicol resistance gene (P*algD-cat*). Of the ten compounds that activated P*algD*, most were inhibitors of peptidoglycan synthesis. In particular D-cycloserine induced very P*algD* high expression (Wood *et al.,* 2006), suggesting that inhibiting peptidoglycan synthesis caused membrane stress and affected the integrity of the cell. These authors also showed that the σ^{22} gene was required for P*algD* activity in the presence of D-cycloserine, suggesting that D-cycloserine induced loss of repression of σ^{22} by MucA. Transcriptional profiling was performed to determine the genes dysregulated during growth in the presence of D-cycloserine. In addition to the established σ^{22} regulon (Firoved *et al.,* 2002, Firoved & Deretic, 2003, Firoved *et al.,* 2004a, Firoved *et al.,* 2004b, Tart *et al.,* 2005), D-cycloserine also affected expression of hypothetical, efflux, pyoverdine, peptidoglycan, LPS, and intermediary metabolism genes (Wood *et al.,* 2006).

Activation of AlgW protease leads to degradation of MucA

Activation of σ^{22} by cell wall inhibitors was a pivotal observation that confirmed the hypothesis that alginate production was a stress response that could be turned on by environmental conditions. Based on this finding, Wood *et al*. (2006) hypothesized that proteolysis of MucA was a possible mechanism. Genes encoding two envelope proteases, MucD and AlgW were previously implicated as potential regulators of alginate biosynthesis (Table 1) (Boucher *et al.,* 1996). However, both of these proteases were considered negative regulators depending on the strain background. In context of nonmucoid PAO1 background, when the *mucD* gene was inactivated, alginate was overproduced, but inactivation of *algW,* did not cause the mucoid phenotype (Boucher *et al.,* 1996). To test the possibility that AlgW could be a positive regulator of alginate overproduction, an *algW* mutant was subjected to D-cycloserine treatment and, as expected, failed to induce the P*algD* activity (Wood *et al.,* 2006). This suggested that without *algW*, σ^{22} was not activated and that AlgW was likely a protease of MucA and a positive regulator of σ^{22} activity (Fig. 2). Furthermore, the overexpression of *algW* caused alginate overproduction (Wood *et al.,* 2006). From these data, it was concluded AlgW was the functional homologue (42% identical) of *E. coli* DegS. Figure 3 indicates the protein domains of the various regulatory proteases of *P. aeruginosa* including AlgW. *E. coli* DegS acts on the anti-sigma factor RseA (MucA homolog) in response to misfolded or accumulated proteins (such as OmpC) in the periplasm (Ades, 2008). The mechanism of DegS proteolysis of RseA allows stress conditions to activate σ^{E}-dependent gene expression in *E. coli*. Due to the homology of AlgW-DegS and the data that

AlgW was required for P*algD* expression, Wood *et al.* proposed a model where membrane stress agents activate AlgW and release σ^{22} (Wood *et al.,* 2006).

It seemed likely that misfolded proteins in the periplasm may activate degradation of MucA by a mechanism similar to that of *E. coli* DegS. Since DegS must be activated to degrade RseA, there are likely AlgW activators encoded in the *P. aeruginosa* genome. In a study by Qiu *et al.,* transposon mutagenesis of nonmucoid strain PAO1 was used to identify new alginate regulatory genes. A mucoid mutant was found with a transposon insertion into the promoter region of the gene encoded at PA4033 (Qiu *et al.,* 2007). Due to the orientation of the transposon and the lack of a transcriptional terminator in the gentamicin resistance gene (*aacC1*) within the transposon, it was hypothesized that overexpression of PA4033 caused alginate overproduction. To test this, PA4033 was overexpressed *in trans* from an arabinose inducible P_{BAD} promoter and alginate overproduction was observed (Qiu *et al.,* 2007). Bioinformatics suggested that PA4033 encodes an envelope protein and is now referred to as MucE (Table 1) (Qiu *et al.,* 2007). MucE has a C-terminus that is similar to proteins that activate DegS proteolysis of RseA in *E. coli*. In *P. aeruginosa,* when MucE is overexpressed AlgW cleaves MucA, which results in alginate overproduction (Qiu *et al.,* 2007) (Fig. 4). The importance of the C-terminus region of MucE in activating AlgW was investigated by mutating the three C-terminal amino acids (tryptophan, valine, phenylalanine, WVF) of MucE; these studies showed that the WVF motif was required to activate AlgW (Qiu *et al.,* 2007). Furthermore, it was also shown that other triplet C-terminal (YVF, LVF, WIF, and WVW) signatures can also activate AlgW (Qiu *et al.,* 2007). Numerous AlgW-activating sequences are encoded in the *P. aeruginosa* genome but not all outer membrane or envelope proteins contain these sequences (Qiu *et al.,* 2007). For example, the major outer membrane porin, OprF, lacks an AlgW activating sequence. It has been demonstrated that overexpression of OprF does not cause alginate overproduction, but addition of the AlgW activation signal (WVF) to the C-terminus of OprF caused alginate overproduction (Qiu *et al.,* 2008a).

Genetic evidence suggested a proteolysis-driven model with AlgW degrading MucA and thus releasing and activating σ^{22}. This model of AlgW proteolysis was confirmed in a separate study by *in vitro* biochemical analysis using MucE as the activator of AlgW (Cezairliyan & Sauer, 2009). In this study, a major cleavage site as well as three minor cleavage sites were identified in the C-terminus of MucA (Cezairliyan & Sauer, 2009) and are depicted in Figure 2A. The domain structure of AlgW is similar to DegS with a protease domain and one PDZ domain (Fig. 3). PDZ is an acronym derived from the three proteins in which the domain was first identified (Kennedy, 1995). The PDZ domain of *E. coli* DegS keeps the protease inactive until misfolded proteins bind, inducing a conformational change resulting in proteolytic activity (Hasselblatt *et al.,* 2007, Walsh *et al.,* 2003). Deletion of the PDZ domain of *E. coli* DegS causes constitutive protease activity (Walsh *et al.,* 2003). However, deletion of the PDZ domain of AlgW renders the protease active but less efficient than wild-type AlgW (Cezairliyan & Sauer, 2009). This data confirmed previous *in vivo* studies that had shown that the PDZ domain of AlgW was required for proteolysis of MucA (Damron *et al.,* 2009, Qiu *et al.,* 2007). Cezairliyan and Sauer also observed that the “LA loop” of AlgW inhibits MucA binding. LA loops of DegS-like serine proteases have been shown to play roles in hindering the active site (Cezairliyan & Sauer, 2009). Without the LA loop, AlgW could cleave MucA in the absence of MucE, but inefficiently. These experiments show that AlgW is regulated through protein-protein interactions and that the LA loop and the PDZ domain participate in controlling proteolytic activity. In addition to characterizing how AlgW is activated and cleaves MucA, Cezairliyan and Sauer also showed *in vitro* that MucB binds the C-terminus of MucA, which protects MucA from proteolysis by AlgW (Cezairliyan & Sauer, 2009).

Inner membrane protease MucP acts on MucA

Inspection of the locations that AlgW acts on MucA indicates that after AlgW proteolysis (Cezairliyan & Sauer, 2009) about half of MucA would still remain (Fig. 2A). In *E. coli*, the MucA homolog RseA requires further degradation by an inner membrane protease named RseP (YaeL) (Alba *et al.,* 2002), which cleaves the transmembrane domain of RseA (Akiyama *et al.,* 2004). In *P. aeruginosa* PAO1 the homologue of *E. coli* RseP is PA3649 and referred to as MucP or YaeL (Table 1). Both *algW* and *mucP* are required for the mucoid phenotype in a strain overexpressing *mucE* (Qiu *et al.,* 2007). In later studies, it was established that MucP plays a role in degradation of MucA and activation of σ^{22} when cells are cultured in the presence of D-cycloserine (Wood & Ohman, 2009). Furthermore, critical residues in the predicted protease domain of MucP are required for activation of σ^{22} (Damron & Yu, 2011). Unlike AlgW, MucP has two putative PDZ domains presumably for substrate recognition (Fig. 3). After proteolysis of MucA at the major AlgW cleavage site, the remainder of the protein would likely be membrane anchored. Based on this observation, it could be hypothesized that MucP may be required for degrading mutant MucA proteins; however, this has not been investigated.

Chaperone-protease MucD regulates pathways leading to MucA proteolysis

AlgW and MucP are proteases that are positive regulators of alginate production due to their direct action upon MucA. MucD is the only protease identified in *P. aeruginosa* that is a negative regulator of alginate overproduction (Boucher *et al.,* 1996). Based on its similarity to *E. coli* DegP and the presence of a leader peptide, MucD is likely localized to the periplasm (Boucher *et al.,* 1996). MucD is encoded in the σ^{22} regulator operon but unlike the situation in *E. coli* (Hiratsu *et al.,* 1995), *mucD* is expressed from both σ^{22}-dependent and -independent promoters (Wood & Ohman, 2006). Inactivation of *mucD* causes alginate overproduction (Boucher *et al.,* 1996). MucD is an HtrA-family protein and is similar to AlgW because they are both serine proteases (Boucher *et al.,* 1996). The protease domain of MucD is required for its ability to regulate alginate production (Wood & Ohman, 2006, Yorgey *et al.,* 2001). In addition to the protease domain, MucD also has two PDZ domains (Fig. 3). Since the protease domain is required for suppression of alginate overproduction, it is conceivable that MucD may intercept misfolded or accumulated proteins in the periplasm that could activate AlgW. Overexpression of MucD suppresses the MucE-induced mucoid phenotype, which suggests MucD may act on accumulated proteins in the periplasm (Qiu *et al.,* 2007). *E. coli* DegP recognizes misfolded substrates causing DegP monomers to assemble into a functional oligomer and then the oligomer degrades periplasmic proteins lacking appropriate conformation (Krojer *et al.,* 2008a, Krojer *et al.,* 2008b). It is possible the PDZ domains in MucD are responsible for substrate recognition as they are in *E. coli* (Ortega *et al.,* 2009). The presumptive chaperone function of MucD (i.e., when it lacks its protease activity) can decrease alginate overproduction (Yorgey *et al.,* 2001). This suggests MucD may not only degrade but may also chaperone misfolded proteins that leads to a slower rate of RIP of MucA and therefore decreased alginate overproduction. It could be suggested that while the direct molecular function of DegP/MucD proteases may be destructive, the goal is to maintain balance, homeostasis, and overall integrity of the cell in stress conditions.

In *E. coli,* sequential cleavages, first by DegS and then by RseP, are required to degrade RseA and activate σ^{22} (Ades, 2008). Adapting this model to *P. aeruginosa*, it would be predicted that AlgW would always be required for activation of σ^{22}. In the absence of MucD, AlgW would likely cleave MucA. However, a *mucD/algW* double mutant is mucoid on standard laboratory media such as L-agar (unpublished observations) or *Pseudomonas* isolation agar (PIA) (Damron & Yu, 2011), suggesting that MucA proteolysis can occur

even in the absence of AlgW. When *mucD* and *mucP* proteases are inactivated, alginate overproduction does not occur and further investigation revealed that MucA degradation is diminished by the inactivation of *mucP* (Damron & Yu, 2011). These data suggest that while AlgW-MucP sequential digestion of MucA is the normal mechanism, a second pathway, where only MucP proteolysis is required, exists. This discrepancy begs the question: how is MucP activated independent of AlgW cleavage of MucA? Acid stress activates σ^E in *Salmonella enterica* serovar Typhimurium (Muller *et al.,* 2009) independent of DegS but dependent upon RseP. In *Salmonella,* it seems the PDZ domain of RseP is dispensable for induction. From these data, Muller *et al.* proposed that disruption of the interaction between the RseP PDZ domain and RseA may be a novel signal to activate σ^{22} (Muller *et al.,* 2009). It would be interesting to see if acid stress can activate RIP of MucA in *P. aeruginosa.* One study, which was performed prior to the identification of the mechanism of MucA-RIP, does indicate regulated intramembrane proteolysis independent of AlgW; a PAO1 strain lacking *algW* became mucoid in the presence of paraquat (Boucher *et al.,* 1996). Paraquat is a redox cycling compound that causes superoxide production, which can be detrimental to bacteria. This data suggests that paraquat may activate MucP and that RIP of MucA can occur without AlgW.

ClpXP: positive regulator of σ^{22} and cytoplasmic protease of MucA

Regulated proteolysis of wild-type MucA begins with degradation by AlgW or MucP, as described above. Membrane stress and overexpression of envelope proteins are at least two mechanisms that can activate this cascade. Presumably, proteolysis of MucA at the transmembrane region (Fig. 2) would leave approximately half of the MucA protein still attached to σ^{22}. This suggests that another protease may participate in the final degradation of MucA from σ^{22}. The *muc-25* mutation (Fyfe & Govan, 1983) truncates MucA to 94 amino acids (Qiu *et al.,* 2008b). Due to this short length, it is likely that MucA25 is not localized to the inner membrane, since it lacks a complete transmembrane domain. The MucA25 protein has been used to address the question as to what other proteases in the cytoplasm may be responsible for degradation of MucA and activation of σ^{22}. Through transposon mutagenesis, Qiu *et al.* identified three proteases, *clpP*, *clpX,* and a *clpP* paralogue that were required for alginate overproduction in the *mucA25* strain (Table 1) (Qiu *et al.,* 2008b). ClpXP is a molecular machine that binds unstructured peptides, unfolds, and then degrades the protein (Baker & Sauer, 2011). Since MucA25 is stabilized in absence of the *clp* genes, it seems that cytoplasmic protease complex ClpXP (Fig. 2) is responsible for removal of residual MucA leading to active σ^{22} (Qiu *et al.,* 2008b). ClpX contains two PDZ-like domains (Levchenko *et al.,* 1997), which likely recognize unfolded or truncated MucA that is attached to σ^{22} and then facilitates degradation of MucA by ClpP. The two PDZ-like domains of ClpX are shown in Figure 3 as a single ClpB domain. While ClpXP protease was originally identified for its requirement in a *mucA* mutant, ClpX is also required for the mucoid phenotype in strains with wild-type *mucA* (unpublished observations).

Prc: an envelope protease that acts on mutant MucA proteins

MucA22 is a common mutant MucA protein that arises in both CF isolates and laboratory strains (Mathee *et al.,* 1999). The *mucA22* mutation is due to loss of a guanine at base 430 resulting in a premature stop codon, truncating the resultant protein to 146 amino acids (Figure 2B). The location of the stop codon of MucA22 suggests that the C-terminus would reside in the periplasm (Figure 2B) and it was also shown that MucA22 remains sequestered σ^{22} to the inner membrane (Rowen & Deretic, 2000). Thus to release σ^{22} from a truncated MucA such as MucA22, proteolysis is a plausible mechanism. Using a mucoid *mucA22* mutant strain (PAO578), suppressor of mucoidy (SOM) mutants were isolated (Reiling *et*

al., 2005). Complementation with a cosmid library identified several of the SOM mutations were in a gene encoded at locus PA3257. PA3257 was identified as a homologue of the periplasmic protease Prc from *E. coli* (Table 1) (Reiling *et al.,* 2005). Inactivation of *P. aeruginosa prc* was shown to only affect strains with mutant and not wild-type MucA proteins, leading the authors to suggest a model where Prc degrades mutated MucA proteins to facilitate activation of σ^{22}; Prc was also shown to promote alginate overproduction in two additional mucoid *mucA* strains (CF20 and CF25), suggesting that it may have a broad substrate specificity for *mucA* mutant proteins (Reiling *et al.,* 2005). Based on genetic experiments, it seems clear that Prc has a role in degradation of mutant MucA proteins, but this has yet to be confirmed by Western blot analysis. In terms of structure, Prc has a signal peptide (for periplasmic localization), one PDZ domain, and a tail specific protease domain (TSPc) that cleaves substrates (Fig. 3). Prc also contains an uncharacterized domain that is conserved in other proteases. Since PDZ domains recognize specific sequences and bind the C-termini of proteins, it is possible that the PDZ of Prc interacts with mutant MucA and regulates proteolysis by the tail-specific protease domain.

As a result of degradation by proteases AlgW and MucP, it is possible that truncated MucA proteins exist which would be of similar size to the truncated MucA proteins such as MucA22 (Fig. 2). This begs the question of whether or not Prc has a role in degrading wild-type MucA. Loss of Prc seems to only affect strains with mutant MucA(Reiling *et al.,* 2005) and Prc was not required for the mucoid phenotype of PAO1 when RIP is activated (Damron *et al.,* 2011). Overexpression of Prc does not activate alginate overproduction in PAO1, but P*algD* activity suggests Prc may play a slight role in degradation of MucA (Wood *et al.,* 2006). However, stability of MucA was not affected by inactivation of *prc* (Wood *et al.,* 2006). These conflicting results suggest that Prc likely has a minor role in the degradation of wild-type MucA.

In vitro conditions can induce alginate overproduction

In a recent study, a medium has been formulated which causes wild-type nonmucoid strain PAO1 to overproduce alginate, independent of mutations (Damron *et al.,* 2011). The medium contained the standard components of PIA supplemented with ammonium metavanadate (PIA-AMV). In *E. coli,* growth on media containing AMV increased σ^{E} activity (Tam & Missiakas, 2005). In *P. aeruginosa*, PIA-AMV medium did not cause mutations in genes such as *mucA*, but rather resulted in inducible alginate overproduction. It was also shown that triclosan and magnesium chloride components of PIA were necessary for the mucoid phenotype of PAO1 on PIA-AMV (Damron *et al.,* 2011). One result of growing PAO1 on PIA-AMV was the palmitoylation of lipid A (Damron *et al.,* 2011), which has also been observed in chronic CF isolates (Ernst *et al.,* 1999). PhoP is a response regulator that is essential for palmitoylation of lipid A in *P. aeruginosa* (Ernst *et al.,* 1999) and interestingly, *phoP* was required for the mucoid phenotype of PAO1 on PIA-AMV. This indicated that growth of PAO1 on PIA-AMV results in remodeling of the outer-leaflet via modification to lipid A but other experiments showed O-antigen LPS chain length was not affected by growth on PIA-AMV (Damron *et al.,* 2011). Predictably, proteases AlgW and MucP were both required for the mucoid phenotype of PAO1 on PIA-AMV; furthermore, degradation of MucA was observed as a result of growth on PIA-AMV. Western blot analysis indicated that chaperone protease MucD was upregulated during growth on PIA-AMV, which suggested that the medium caused membrane stress. From these observations, a model was proposed where PIA-AMV medium affects membrane integrity that may cause misfolding or accumulation of envelope proteins (Damron *et al.,* 2011). Consequently, MucA degradation by AlgW/MucP and σ^{22} activation were necessary to compensate for the stress of the PIA-AMV medium environment (Damron *et al.,* 2011). However, the question remains: what are the direct molecular targets of AMV? A study has shown that vanadate

binds to siderophores inhibiting iron uptake (Baysse *et al.,* 2000) in *P. aeruginosa.* Also, since vanadate mimics phosphate there are many potential proteins and enzymes that may interact with and/or be inhibited by vanadate. It may be possible to use this PIA-AMV medium to further characterize the RIP pathways leading to induction of alginate overproduction.

Abrogated outer membrane protein processing results in the formation of membrane vesicles

Alginate overproduction on PIA-AMV medium suggested that stress conditions could cause misfolding of proteins in the envelope. Along these same lines, if protein processing is abrogated, then misfolded proteins would accumulate and activate RIP. Upstream of *mucP* (A3649) there is a gene *opr86* (PA3648) that encodes an Omp85/YaeT/BamA family outer membrane protein. In *E. coli,* YaeT is a part of a complex that directly plays a role in assembly of β-barrel outer membrane proteins (OMPs) (Ruiz *et al.,* 2006). Opr86 localizes to the outer membrane and has been shown to be essential in *P. aeruginosa* and depletion of Opr86 causes blebbing of the outer membrane (Tashiro *et al.,* 2008). These authors hypothesized that misfolded outer membrane proteins cause membrane vesicle (MV) formation. Since MucD likely acts on misfolded proteins in the periplasm, Tashiro *et al.,* examined the role of *mucD* on the formation of MV. In the absence of MucD, high amounts of MV were observed (Tashiro *et al.,* 2009). Furthermore, overexpression of *mucD* or *algW* caused decreased MV production. Since *algW* overexpression would activate proteolysis of MucA (Wood *et al.,* 2006) and subsequently σ^{22} transcriptional activity leading to more MucD expression, these data fit with other previous findings. The authors proposed a three-step model for dealing with accumulated OMPs. In step one, MucD directly acts upon misfolded OMPs. In step two, if misfolded OMP concentrations exceed the capabilities of MucD, then misfolded proteins will be released in MVs. In step three, if release of MV and misfolded proteins cannot lower the level of misfolded proteins to maintain homeostasis, then RIP will activate σ^{22} (Tashiro *et al.,* 2009). This model fits well into the RIP model and adds an interesting layer to how *P. aeruginosa* deals with stress conditions by modulating multiple systems.

Unanswered questions

As detailed in this review, AlgW is positive regulator and key protease that acts on MucA to release σ^{22}. However, the study that originally identified *algW* classified it as a negative regulator of alginate production (Boucher *et al.,* 1996). Expression of plasmid-borne *algW* could turn off alginate production and P*algD* promoter activity in mucoid strain CF31 (Boucher *et al.,* 1996). It was apparent that *algW* encoded a serine protease due to its homology with HtrA-family proteins; however the mechanism of how AlgW suppressed alginate production was not clear. Here we have proposed that MucP RIP protease may act upon mutant MucA proteins such as MucA22. Previous work in *E. coli* has indicated that DegS inhibits RseP proteolysis of RseA (Grigorova *et al.,* 2004). Based on *algW* suppression of the mucoid phenotype in the *mucA* strain and the inhibition of RseP by DegS in *E. coli*, we speculate that AlgW may inhibit MucP proteolysis. Furthermore, the degradation profile of epitope-tagged MucA was notably different between the mucoid *mucD* and *mucD/algW* strains (Damron & Yu, 2011). Collectively these data suggest AlgW may have an inhibitory effect on MucP but a mechanism may also exist to activate MucP to act directly upon MucA. It is possible AlgW-MucP interactions may occur through one of MucP's two PDZ domains (Fig. 2 and 3), but this has not been investigated or experimentally determined.

AlgB-KinB is a two-component system (Table 1) that has been shown to regulate alginate overproduction (Goldberg & Ohman, 1984, Ma *et al.,* 1997). AlgB is a response regulator that has been extensively characterized and shown to activate P*algD* expression (Goldberg & Dahnke, 1992, Leech *et al.,* 2008, Wozniak & Ohman, 1991). Recently, KinB was characterized as a negative regulator of alginate overproduction in PAO1 (Damron *et al.,* 2009) (Fig. 4). In that study, it was observed that inactivation and deletion of *kinB* caused AlgW-dependent degradation of MucA. Additionally, the AlgB transcription factor and sigma factor RpoN (σ^{54}) were both required for P*algU* activity and complete degradation of MucA (Damron *et al.,* 2009). This suggested novel roles for RpoN and AlgB (Fig. 4) outside of the characterized roles at P*algD*. It is not clear why AlgW is degrading MucA in the absence of KinB (Damron *et al.,* 2009) and more studies are required to determine the pathway between KinB and AlgW-RIP. *kinB* has been shown to be required for virulence in a zebrafish model, biofilm formation and quorum sensing (Chand *et al.,* 2011), which suggested that *kinB* may control genes in addition to the alginate biosynthetic genes. Microarray analysis comparing a *kinB* mutant to a *kinB/rpoN* double mutant has revealed a large number of genes both up- and down-regulated (Damron *et al.,* 2012). Interestingly, it was observed that loss of *rpoN* decreased *algW* expression in the *kinB* mutant. This may explain why *rpoN* was required for high P*algU* and P*algD* promoter activities of this strain (Damron *et al.,* 2009). Due to the drastically altered transcriptome and the phenotypic changes that were observed, the *kinB* mutant and isogenic strains were used to challenge mice in an acute pneumonia model. Mucoid *mucA22* mutant (PDO300) was virulent in this model but the mucoid *kinB* mutant was not virulent (Damron *et al.,* 2012). Collectively these recent studies suggest that sensor kinase KinB, along with RpoN, control many genes related to multiple virulence phenotypes including alginate overproduction. KinB is clearly a pleiotropic regulator, but the environmental conditions controlling KinB and thus influencing gene expression are not known at this time.

MucA degradation by RIP has been observed by two *in vitro* conditions: membrane stress (growth in presence of D-cycloserine or on PIA-AMV) and mutations in regulators (MucE, MucD, MucB, KinB). How does each of these *in vitro* conditions relate to *in vivo* regulation of alginate overproduction? One study has shown that inducible alginate overproduction occurs during *P. aeruginosa* infection of murine lungs and as a result of anaerobic growth (Bragonzi *et al.,* 2005). This intriguing study suggests that alginate overproduction occurs in strains with wild-type MucA, which can be supported by other *in vitro* studies described in this review. Another study has indicated that inactivation of σ^{22} (*algU*) or *algW* caused attenuation of PAO1 in a rat chronic respiratory infection model (Potvin *et al.,* 2003). This further suggests MucA proteolysis is a key mechanism for infection. However, it is not clear how AlgW protease is activated *in vivo*. It can be hypothesized based on *in vitro* results that AlgW may be activated by misfolded proteins that accumulate during *in vivo* conditions. It is also possible that overall membrane integrity is compromised which leads to AlgW-mediated RIP. A third possible mechanism is that sensor kinase KinB may respond to *in vivo* conditions and activate RIP (Damron *et al.,* 2009).

Another currently unanswered question is how MucB is released from MucA to allow RIP via AlgW (Cezairliyan & Sauer, 2009) to proceed. A recent study in *E. coli* suggests RseB responds to lipid signals and that RseB is a “noise-filtering gatekeeper” which improves the quality of the response (Chaba *et al.,* 2011). Using this as a model, it seems possible in the context of *P. aeruginosa* that MucB signaling could be affected in situations where the mucoid phenotype is induced. Further research is necessary to understand MucB regulation of MucA and σ^{22}.

Directions towards therapeutics

Now that there is a basic understanding of the regulation of degradation of MucA, research towards impairing the functions of these proteases may be a key to deactivating alginate production. Such treatments may augment already available therapeutics to target this system and eradicate *P. aeruginosa* before it establishes a chronic infection in CF patients. Two themes can be drawn when thinking about the proteases that act on MucA. The first is that assuming MucP is required for degrading mutant MucA proteins: MucP and ClpXP are the only proteases that are currently recognized as required for degradation of both mutant MucA and wild-type MucA. Since MucP is a zinc metalloprotease, it may be possible to use inhibitors of metalloproteases to block alginate overproduction. *Pseudomonas* elastase (LasB) is a metalloprotease that plays an important role in virulence. Recently, novel inhibitors of LasB have shown promise as a therapeutic approach to eradicate *P. aeruginosa* biofilms (Cathcart *et al.,* 2011). Previous studies have shown that overexpression of LasB in nonmucoid strains resulted in alginate overproduction (Kamath *et al.,* 1998). If added to the current model of MucA-RIP, elastase accumulation in the periplasm (Kamath *et al.,* 1998) could cause misfolding in the envelope leading to activation of AlgW and MucP. It would be interesting to determine if zinc metalloprotease inhibitors can block alginate overproduction and increase the efficacy of current therapeutics. The second observation from the MucA-RIP model is that all of the MucA proteases contain PDZ domains (Fig. 3). PDZ domains may be a novel target to arrest RIP and block alginate production. PDZ domains exist in many human proteins, and it is possible that they would not be an advantageous target. However, inhibitors of PDZ domains have been shown to be promising pharmacotherapeutics in neuropathic pain and cocaine addiction (Thorsen *et al.,* 2010).

Multiple pathways in *P. aeruginosa* converge at RIP of MucA (Fig. 4). From the studies reviewed here, it is clear that RIP occurs in strains with both wild-type and mutant *mucA*. While an abundance of research on this topic has focused on alginate overproduction, more research is still critical to understanding this stress response system of *P. aeruginosa*. We are hopeful that blocking this pathway could provide a much needed option for treating *P. aeruginosa* lung infections in CF.

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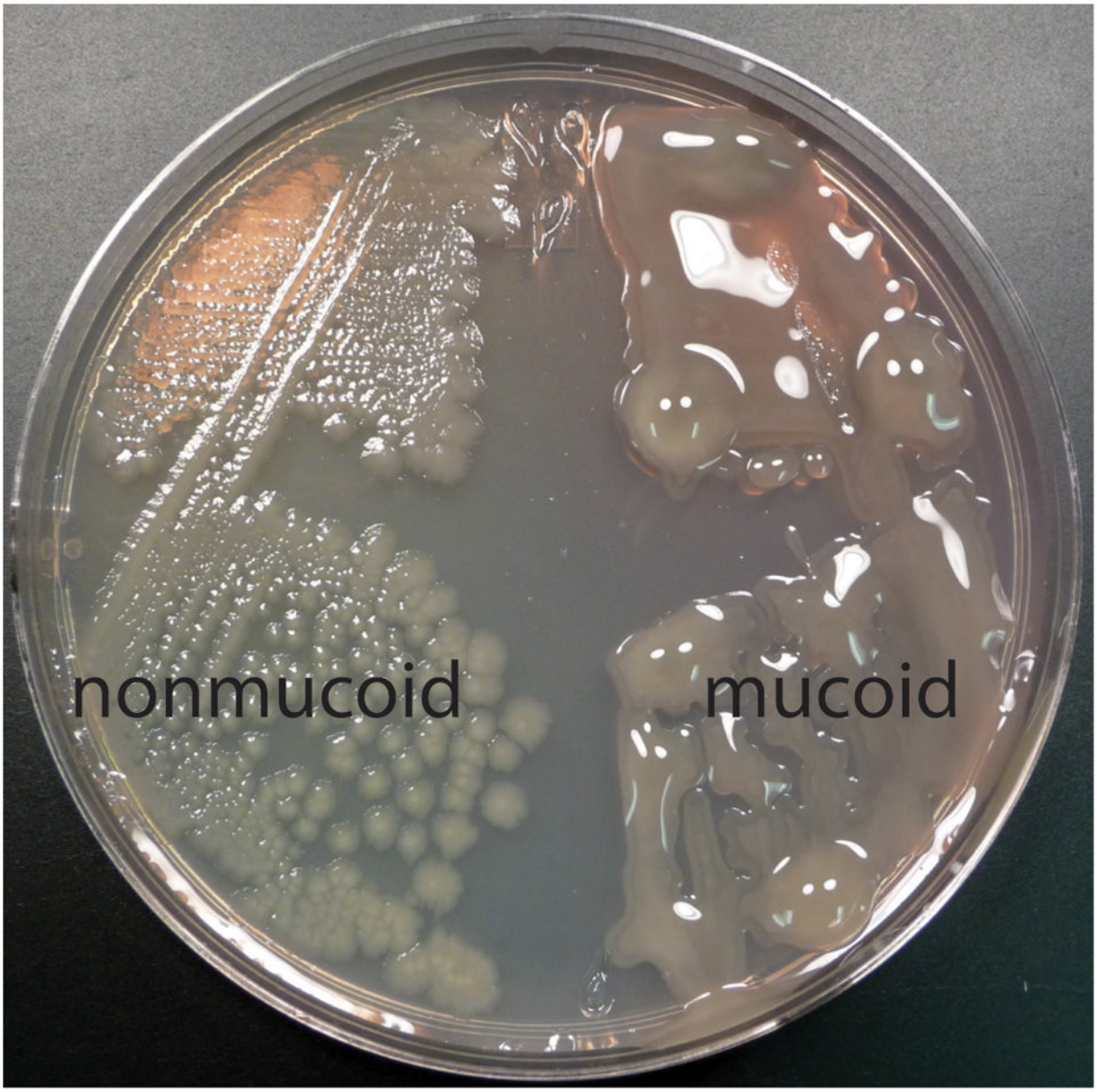

Figure 1. *P. aeruginosa* mucoid and nonmucoid phenotypes

Representative strains of the wild-type nonmucoid and mucoid phenotypes are shown. Mucoid strains overproduce the exopolysaccharide known as alginate. Strains were grown on *Pseudomonas* isolation agar (PIA) for 24 hours at 37°C, and then 24 hours at 25°C.

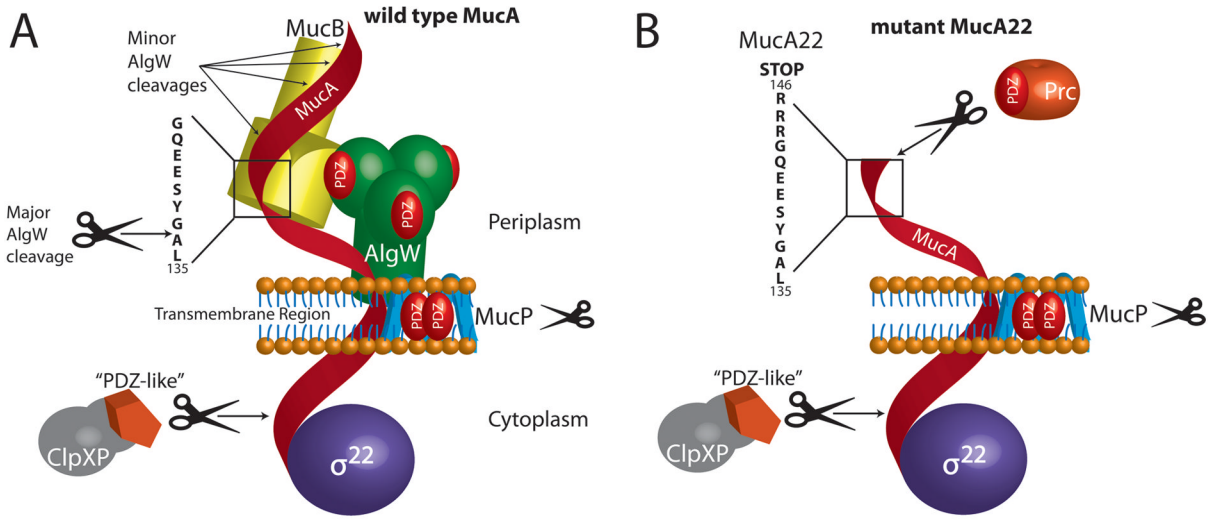

Figure 2. *P. aeruginosa* wild-type and mutant MucA and associated protease complexes

Proteolytic activities of proteins are indicated by scissors. A. Full length MucA protein sequestering σ^{22} is shown with MucB binding the C-termius of MucA. AlgW is indicated as a trimer as previously demonstrated (Cezairliyan & Sauer, 2009). The relative positions where AlgW cleaves are indicated with the major cleavage site. MucP is shown localized to the inner membrane. PDZ domains of each protease are indicated in red and it should be noted that all RIP proteases identified thus far harbor one or two of these domains. Cytoplasmic ClpXP cleaves residual MucA from σ^{22} in the final step of activation of the σ^{22} (Qiu *et al.,* 2008b). B. Mutant MucA22 is shown localized to the inner membrane. Prc is a protease that facilitates degradation of mutant MucA proteins. In this review, it is proposed that MucP may play a role in degradation of mutant MucA; however this has not been established. RIP of MucA leads to activation of σ^{22} and expression of the σ^{22} regulon. RIP of MucA is accomplished by the proteases AlgW, MucP, ClpXP, and Prc.

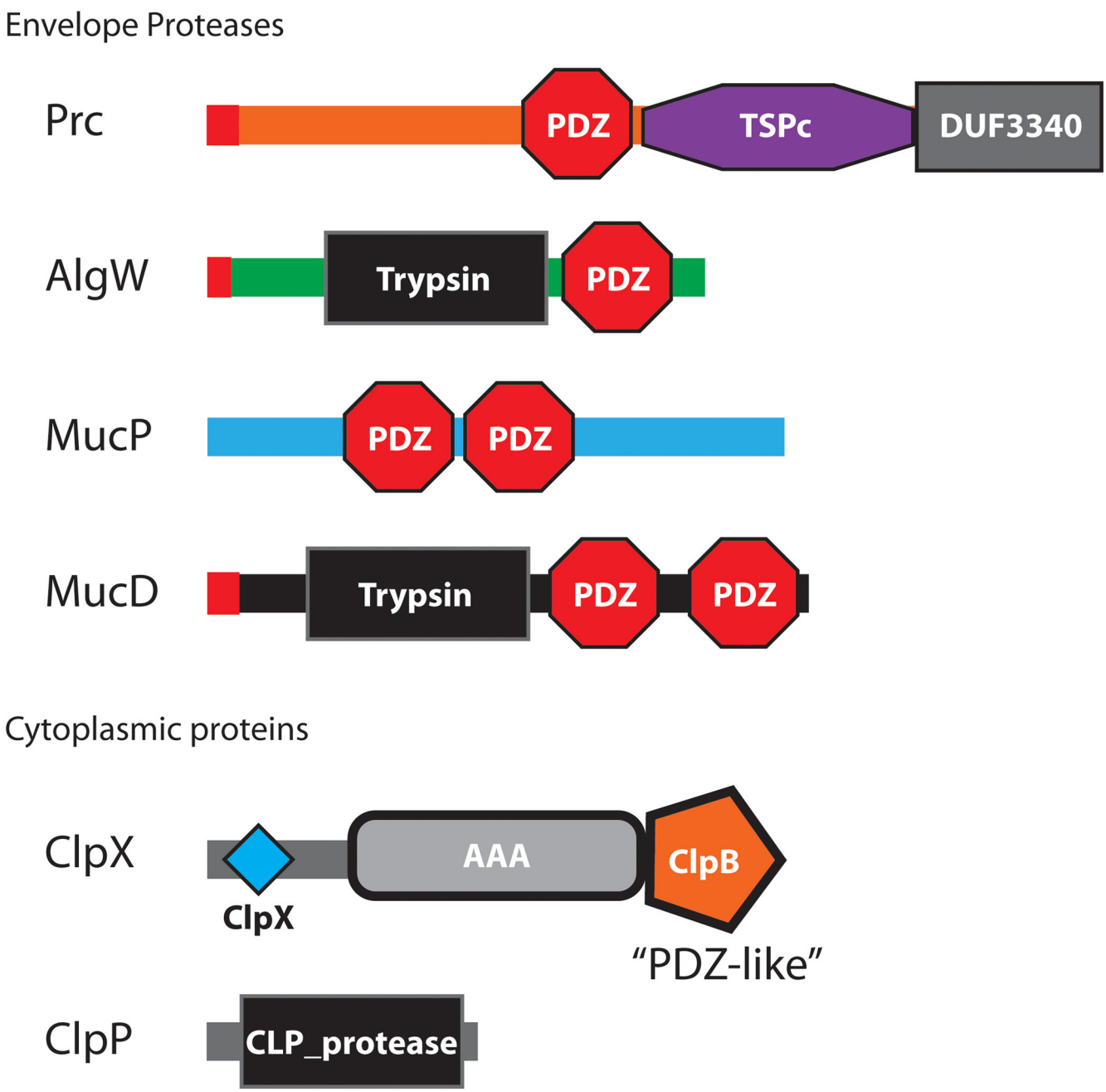

Figure 3. Domains of the proteases involved in regulation of alginate overproduction

The known proteases which are involved in regulating σ^{22} are shown with their respective domains as indicated by the SMART protein database (Letunic *et al.,* 2009). PDZ domains (red octagons) are protein-protein interaction domains and are found in all of the alginate regulatory envelope proteases. Envelope proteases AlgW and MucD both also have trypsin protease domains (black rectangle). TSPc (purple octagon) indicates the tail-specific protease domain on Prc. Prc also has a C-terminal domain of unknown function, which has yet to be characterized known as DUF3340. The ClpX protein has an AAA (ATPase Associated Activities) domain (gray oval). ClpX and ClpP are both required for the activation of σ^{22} (Qiu *et al.,* 2008b) which suggests they likely work in concert. ClpX also

has a ClpB domain that plays a role in protein recognition and is similar to a PDZ domain. Red blocks in the N-terminus indicate a signal sequence has been identified.

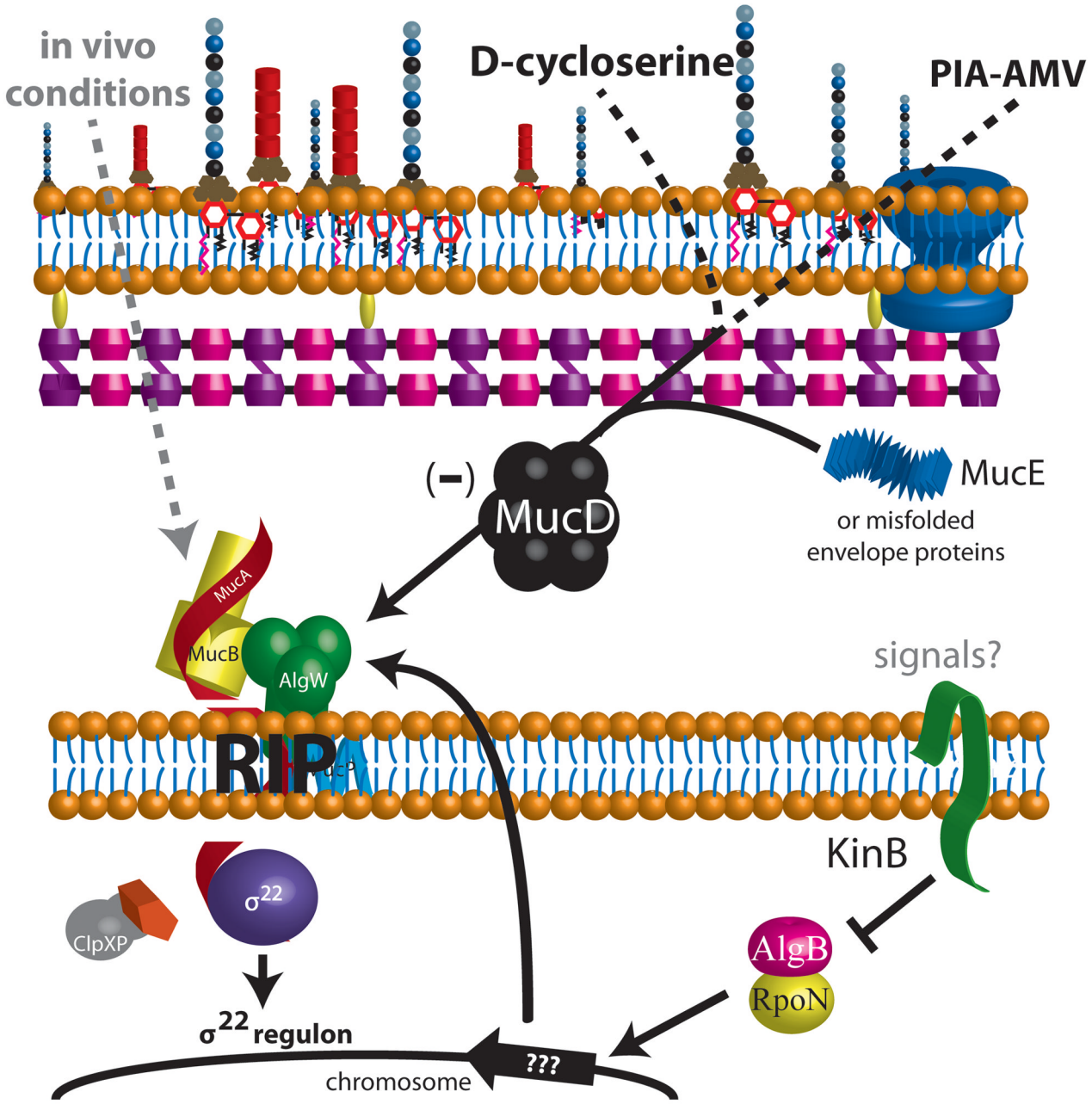

Figure 4. Pathways leading to regulated intramembrane proteolysis (RIP) of MucA

A composite model of the various pathways in *P. aeruginosa,* that lead to RIP of MucA and activation of σ^{22} are shown. Cell wall stress agents such as D-cycloserine can inhibit peptidoglycan synthesis and activate RIP of MucA (Wood *et al.,* 2006, Wood & Ohman, 2009). When the envelope protein MucE is overexpressed, RIP of MucA occurs due to activation of AlgW and MucP proteases (Qiu *et al.,* 2007). Growth on PIA-AMV medium causes RIP of MucA by AlgW and MucP protease, presumably due to misfolded proteins in the envelope (Damron *et al.,* 2011). MucD is a chaperone-protease that is in the periplasm. The protease activity of MucD is required for repression of alginate overproduction. Likely, MucD degrades proteins that accumulate in the periplasm. If the function of MucD is lost then RIP of MucA will occur. MucD overexpression can suppress the MucE signal (Qiu *et*

al., 2007) and is upregulated during growth on PIA-AMV (Damron *et al.,* 2011). However, in the absence of MucD, RIP of MucA and activation of σ^{22} only requires MucP and not AlgW(Damron & Yu, 2011). When the histidine kinase KinB is inactivated or deleted, AlgW-RIP of MucA occurs and is dependent upon response regulator AlgB and sigma factor RpoN (σ^{54}) (Damron *et al.,* 2009). It is hypothesized that AlgB/RpoN controls expression of genes that influence RIP. It is also not known what signals activate KinB. *In vivo* conditions are indicated in gray dashed line since a study does indicate alginate production can occur during infection (Bragonzi *et al.,* 2005), but the specifics of this pathway have not been elucidated. Based on the convergence to RIP by multiple pathways, future therapeutics inhibiting the RIP proteases may provide novel treatment options against *P. aeruginosa.*

Table 1

σ^{22} and proteins which play roles in regulated intramembrane proteolysis (RIP) of MucA in *P. aeruginosa*.

PA Locus #	Protein(s)	Alt. names	Homology (*E. coli*)	Type of regulator	Localization	Function(s) [a]	Key Reference(s) [b]
PA0762	σ^{22}	AlgU/T	RpoE	Alternative sigma factor	Cytoplasmic	Activates transcription	(DeVries & Ohman, 1994, Martin *et al.,* 1993a)
PA0763	MucA		RseA	Anti-sigma factor	Inner membrane	Sequesters and inhibit sσ^{22}	(Martin *et al.,* 1993b, Mathee *et al.,* 1997)
PA0764	MucB	AlgN	RseB		Periplasmic	Protects C-terminus of MucA from proteolysis	(Cezairliyan & Sauer, 2009, Goldberg *et al.,* 1993, Wood & Ohman, 2009)
PA0765	MucC	AlgM	none	Unknown	Unknown	Unknown	(Boucher *et al.,* 1997)
PA0766	MucD	AlgY	DegP	Chaperone/Protease	Periplasmic	Degrades proteins that activate AlgW and or MucP	(Boucher *et al.,* 1996, Damron & Yu, 2011, Qiu *et al.,* 2007, Wood & Ohman, 2009)
PA4446	AlgW		DegS	Protease	Periplasmc/Inner membrane	Cleaves C-terminus of MucA	(Boucher *et al.,* 1996, Damron & Yu, 2011, Qiu *et al.,* 2007, Wood & Ohman, 2009)
PA3649	MucP	YaeL	RseP	Protease	Inner membrane	Cleaves MucA near transmembrane domain	(Damron & Yu, 2011, Qiu *et al.,* 2007, Wood & Ohman, 2009)
PA4033	MucE		none	Envelope Protein	Periplasmic	Activates AlgW cleavage of MucA	(Cezairliyan & Sauer, 2009, Qiu *et al.,* 2007)
PA3257	Prc		Prc	Periplasmic Protease	Periplasmic	Facilitates degradation of mutant MucA	(Reiling *et al.,* 2005)
PA1802 PA1801	ClpX/P		ClpX/P	Cytoplasmic Proteases	Cytoplasmic	Facilitates degradation of N-termius of MucA	(Qiu *et al.,* 2008b)
PA3648	Opr86		YaeT/BamA	Outer Membrane Biogenesis	Outer membrane	Depletion of Opr86 upregulates MucD	(Tashiro *et al.,* 2009)
PA5484	KinB		PhoR/NtrB	Sensor Kinase	Inner membrane	Inactivation causes AlgW-cleavage of MucA	(Damron *et al.,* 2009)
PA5483	AlgB		NtrC	Response Regulator	Cytoplasmic	Required for σ^{22} activity in *kinB* null mutant	(Damron *et al.,* 2009)
PA4462	RpoN	σ^{54}	RpoN	Alternative Sigma Factor	Cytoplasmic	Required for σ^{22} activity in *kinB* null mutant	(Damron *et al.,* 2009)

[a] Protein may have more functions than indicated. The indicated function is that most relevant to regulated proteolysis of MucA.

[b] Additional references to the function of the proteins listed exist; here only key references relevant to the topic of this review are given.