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The *Pseudomonas aeruginosa* AmrZ C-terminal domain mediates tetramerization and is required for its activator and repressor functions

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Summary

***Pseudomonas aeruginosa* is an important bacterial opportunistic pathogen, presenting a significant threat towards individuals with underlying diseases such as cystic fibrosis. The transcription factor AmrZ regulates expression of multiple *P. aeruginosa* virulence factors. AmrZ belongs to the ribbon–helix–helix protein superfamily, in which many members function as dimers, yet others form higher order oligomers. In this study, four independent approaches were undertaken and demonstrated that the primary AmrZ form in solution is tetrameric. Deletion of the AmrZ C-terminal domain leads to loss of tetramerization and reduced DNA binding to both activated and repressed target promoters. Additionally, the C-terminal domain is essential for efficient AmrZ-mediated activation and repression of its targets.**

Introduction

The Gram-negative bacterium *Pseudomonas aeruginosa* is ubiquitous. It can be widely found in freshwater and soil, and as an opportunistic pathogen also imposes a threat to immunocompromised humans. *Pseudomonas aeruginosa* versatility is achieved by rapidly adapting to new environments, in which multiple levels of regulation are involved. A large pool of transcription factors participate in this regulation, and one such factor AmrZ (alginate and motility regulator Z) functions as a global activator and repressor. Well-studied AmrZ targets include those involved in exopolysaccharide production (alginate, Psl and Pel), flagella, twitching motility and metabolism of the second messenger bis-(3′, 5′) cyclic di-guanylate (c-di-GMP) (Baynham and Wozniak, 1996; Ramsey *et al*., 2005a; Baynham *et al*., 2006; Tart *et al*., 2006; Jones *et al*., 2013; 2014; Martínez-Granero *et al*., 2014). For instance, AmrZ is necessary for transcription of the alginate biosynthesis operon, the promoter of which is designated as P*algD* because *algD* is the first gene of this operon. Alginate is responsible for the mucoid phenotype and contributes to increased resistance to antimicrobials and host immunity (Gacesa, 1998; Baynham *et al*., 1999; Ramsey and Wozniak, 2005b). AmrZ also represses transcription of *gcbA* (also referred to as *adcA* or PA4843), which encodes a diguanylate cyclase that synthesizes c-di-GMP (Jones *et al*., 2014; Petrova *et al*., 2014). Deletion of *gcbA* results in increased motility and reduced biofilm production (Jones *et al*., 2014; Petrova *et al*., 2014). The Δ*amrZ* mutant displays increased levels of Psl and c-di-GMP and therefore forms enhanced biofilms compared with the wild-type strain (Jones *et al*., 2013; 2014).

The 12.34 kDa protein AmrZ is composed of three segments: a flexible N terminus (residues 1–11), a ribbon–helix–helix domain (residues 12–66), and a C-terminal domain (CTD, residues 67–108) (Fig. 1A). The ribbon–helix–helix domain mediates DNA binding and its structure is highly similar to other members, such as Arc, Mnt and MetJ, in the Arc superfamily (Vershons *et al*., 1985; Knight *et al*., 1989; Schreiter and Drennan, 2007; Waligora *et al*., 2010). All Arc superfamily proteins are phage or bacterial transcription factors, which bind to DNA via N-terminal ribbon–helix–helix domains. C-terminal domains of different Arc proteins, however, mediate diverse functions including ligand binding and oligomerization (Schreiter and Drennan, 2007). Arc proteins form dimers or higher order oligomers, which are often necessary for their functions (Waldburger and Sauer, 1995; Schreiter and Drennan, 2007). In our

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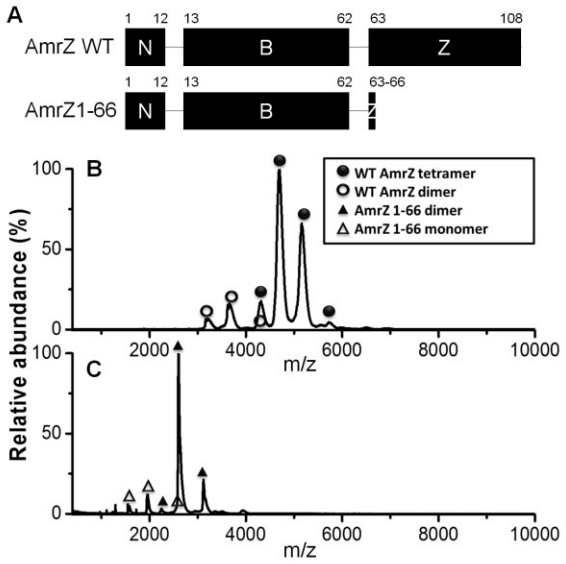

Fig. 1. The C-terminal domain of AmrZ mediates tetramerization. (A) Proposed domains of full-length AmrZ and AmrZ1-66. N: N terminus; B: DNA binding domain; Z: oligomerization domain. Numbers above indicate residues flanking each domain. (B and C) Nano-ESI MS measurements of purified AmrZ (B) and AmrZ1-66 (C). Purified proteins were buffered exchanged into 500 mM ammonium acetate and analysed by nano-ESI MS. The X-axis represents mass/charge (m/z) ratios, whereas the Y-axis was calculated by normalizing to the highest signal intensity. A certain protein/complex may have different charges, resulting in a series of peaks in MS. This information was used to calculate molecular masses of different AmrZ oligomeric species. Specifically, electrospray ionization produces charged droplets, and desorption and/or desolvation helps formation of gas phase ions. Proteins have multiple protonation sites and will be protonated with different charges as solvent evaporates. Therefore, in ESI MS, protein analytes usually have a distribution of charge states. Molecular weights (MW) of proteins are calculated based on the equation: $m/z = (MW + nH^+)/n$, and the average of all charge states is considered the MW of a specific protein.

previous studies, glutaraldehyde cross-linking experiments suggested that cross-linked AmrZ forms oligomers independent of its N terminus (Waligora *et al*., 2010). However, it remains unknown which AmrZ domain(s) mediates oligomerization, or what roles this may play during AmrZ-mediated activation or repression.

In this report, results from multiple biochemical and genetic approaches reveal that AmrZ exists primarily as tetramers in solution. Oligomerization requires an intact AmrZ CTD, the loss of which leads to reduced DNA binding as well as a diminished ability of AmrZ to mediate activation and repression.

Results and discussion

AmrZ forms tetramers in solution via its CTD

In the Arc superfamily, the basic functional unit is dimeric; however, proteins such as Mnt and TrwA form tetramers and even higher order oligomers when bound to DNA (Schreiter and Drennan, 2007). In this study nano-electrospray ionization mass spectrometry (nano-ESI MS) was used to determine the AmrZ oligomeric state in solution. Mass spectrometry precisely measures the size of a molecule and nano-ESI MS allows the detection of proteins or protein complexes when they are still folded (Karas *et al*., 2000; Ma *et al*., 2014). As shown in Fig. 1B, most peaks correspond to an AmrZ complex with the size of approximately 51.0 kDa. Due to tag cleavage following purification, recombinant AmrZ contains three extra residues (Gly-Pro-His), resulting in its monomeric molecular weight as 12.63 kDa (Pryor *et al*., 2012). The AmrZ complex seen in MS therefore reflects an AmrZ tetramer. AmrZ tetramerization was confirmed by size exclusion chromatography (SEC) (Fig. S1), a dominant negative *in vivo* genetic test (Fig. S2), and a glutaraldehyde cross-linking experiment (Fig. S4).

We next determined which part of AmrZ mediates oligomerization. Previous work showed that removal of N-terminal residues 1–11 does not prevent AmrZ oligomerization, and the ribbon–helix–helix domain (residues 12–66) is responsible for DNA binding and dimerization (Fig. 1A) (Waligora *et al*., 2010; Pryor *et al*., 2012). Specifically, AmrZ1-66 was used in determining the crystal structure of DNA-bound AmrZ, and this truncation variant interacts with DNA as a dimer of dimers (Pryor *et al*., 2012). We therefore hypothesized that the AmrZ CTD mediates tetramerization. To test this, we determined the oligomeric state of purified AmrZ in the absence of the CTD (AmrZ1-66). Nano-ESI MS revealed that AmrZ1-66 exists as dimers and monomers, but no tetramer was observed (Fig. 1C). This is consistent with data obtained from protein cross-linking experiments (Fig. S4). These results collectively showed that the AmrZ CTD mediates tetramerization.

To unveil how the AmrZ CTD contributes to tetramerization, we used multiple tools to predict the secondary structure and properties of this domain. The National Center for Biotechnology Information (NCBI) conserved domain database failed to identify any conserved domain homologous to this CTD (Marchler-Bauer *et al*., 2014). The online prediction tools PSIPRED (Fig. S5A) and JPRED (B. Xu, unpublished) predicted that most residues in the AmrZ CTD form two α-helices (Jones, 1999; Drozdetskiy *et al*., 2015). The α-helix, which mediates DNA binding and is essential for transmembrane domain formation, is the most common secondary structure of proteins (Breitwieser, 2004). Many proteins, such as G-protein coupled receptors, form dimers or oligomers via helix–helix packing (Breitwieser, 2004). Therefore, these two α-helices are likely to be responsible for AmrZ oligomerization. Typically a small number of residues, called hot spot residues, are important for successful

helix–helix interactions (Bullock *et al*., 2011). Although hydrophobic and aromatic amino acids constitute the majority of hot spot residues, polar and charged residues such as arginine remain significant players at monomer–monomer interfaces (Bogan and Thorn, 1998; Bullock *et al*., 2011; Matthews *et al*., 2012). The ExPASy ProtScale tool (Gasteiger *et al*., 2005) predicted that most residues within the AmrZ CTD domain are polar or hydrophilic (Fig. S5B). In addition, overall, this domain is hydrophilic, with the grand average of hydropathicity at −0.512 (minus values suggest hydrophilic) (ProtParam; Kyte and Doolittle, 1982; Gasteiger *et al*., 2005). The AmrZ CTD contains five arginine residues, which, together with other polar residues, may contribute to oligomerization through hydrogen bonding and salt bridges (Ali and Imperiali, 2005; Matthews *et al*., 2012).

Taken together, we used four independent approaches (glutaraldehyde cross-linking, SEC, nano-ESI MS and genetics) to demonstrate the oligomeric state of native AmrZ as tetrameric, and identified that the CTD mediates tetramerization.

Impact of the AmrZ CTD on DNA binding, activation and repression

We next sought to determine the effects of removing the AmrZ CTD on DNA binding. Purified AmrZ and AmrZ1-66 were tested for DNA binding to promoters of AmrZ targets. A [6FAM]-labelled DNA fragment containing the AmrZ-binding site (ZBS) was amplified from its activated target P*algD* (Baynham and Wozniak, 1996), and AmrZ binding determined by EMSA (electrophoretic mobility shift assay) (Fig. 2A). Results showed that compared with full-length AmrZ, AmrZ1-66 has significantly reduced binding to P*algD* (Fig. 2B and C). However, the CTD is not absolutely necessary for DNA binding because DNA binding activities were seen when higher AmrZ1-66 concentrations were used (Fig. 2B and C). Both AmrZ and AmrZ1-66 recognize the DNA target specifically, as no binding was seen when a non-specific DNA was tested in this assay (Fig. 2D). Similar results were observed when the repressed target P*gcbA* was used (Fig. S6), indicating that this reduced DNA binding of AmrZ1-66 is likely to be conserved for different AmrZ targets.

Finally, we sought to investigate the significance of the AmrZ CTD *in vivo* in the type strain PAO1. In a previous study, we showed that AmrZ activates PA2146 and represses *gcbA* transcription (Jones *et al*., 2014). The hypothetical gene PA2146 is 92% similar to *Escherichia coli yciG*, which is associated with bacterial responses to glucose (Sunya *et al*., 2012). Moreover, PA2146 expression is repressed when *P. aeruginosa* is treated with inhibitory compounds such as protoanemonin and azithromycin (Kai *et al*., 2009; Bobadilla Fazzini *et al*., 2013). We compared the ability of AmrZ1-66 and AmrZ to regulate these two targets. In PAO1 Δ*amrZ* with plasmids containing AmrZ or AmrZ1-66, mRNA levels of PA2146 and *gcbA* were quantified at various arabinose concentrations. Consistent with findings in RNA-Seq (Jones *et al*., 2014), *gcbA* transcription remained de-repressed in the PAO1 Δ*amrZ* strain when the empty vector pHERD20T was introduced. However, even at 0% arabinose, pHERD20T-WT AmrZ (pAmrZWT) was sufficient to restore AmrZ-mediated repression of *gcbA*, whereas regulation by AmrZ1-66 was not observed until 0.1% arabinose was used (Fig. 3A). Similar results were

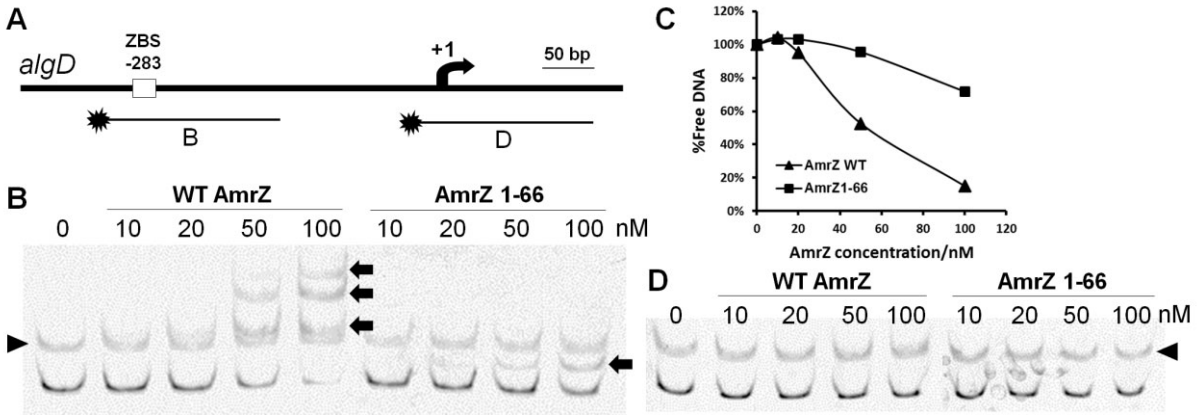

Fig. 2. Truncation of the C-terminal domain results in reduced binding affinity to P*algD*. [6FAM]-labelled DNA fragments (10 nM in each reaction) with (B) or without (D) the ZBS in P*algD* were incubated with various concentrations of AmrZ or AmrZ1-66 at room temperature for 20 min and resolved by 4% non-denaturing polyacrylamide gel electrophoresis on ice (B and D). Relative amounts of free DNA in (B) were quantified via densitometry of non-shift bands using ImageJ (v1.46r) and plotted against AmrZ concentrations (C). AmrZ monomeric concentrations ranged between 10 nM and 100 nM. The arrow in (A) represents the P*algD* transcription start site. Arrows in (B) indicate DNA mobility shifts induced by AmrZ or AmrZ1-66 binding. Triangles in (B and D) point to non-specific bands, which are also present in no protein lanes.

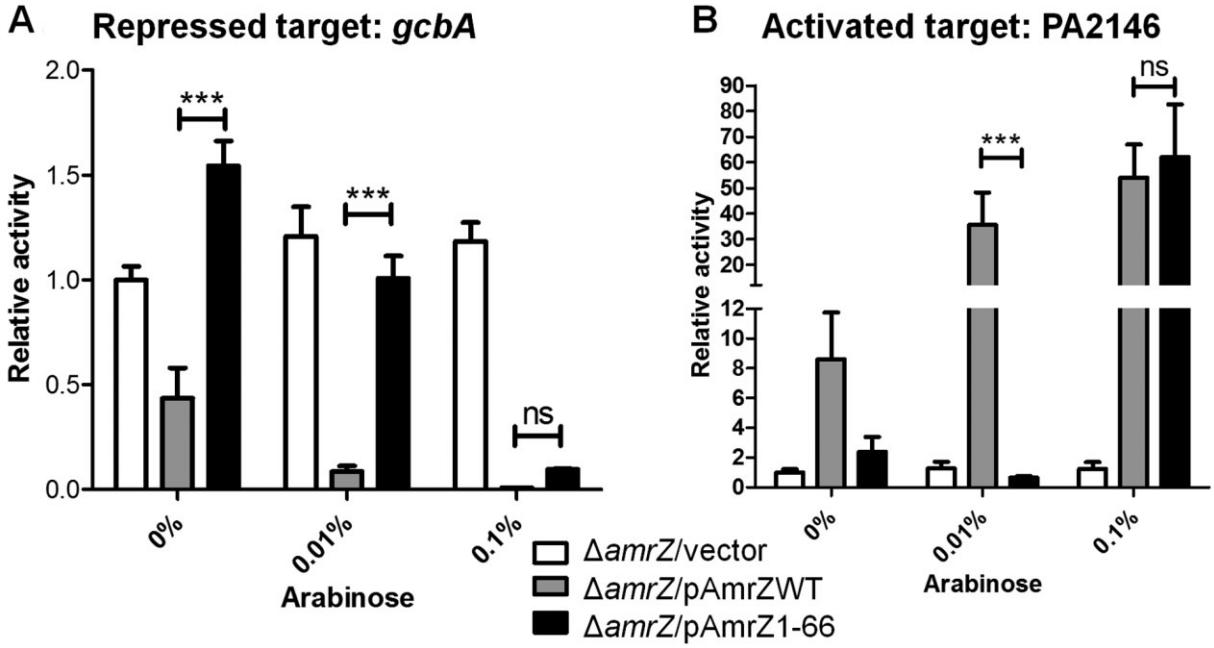

Fig. 3. The AmrZ C-terminal domain is required for efficient repression (A) and activation (B) of its targets. Arabinose-inducible plasmids encoding AmrZ or AmrZ1-66 were transferred into PAO1 *ΔamrZ*. After induction by various arabinose concentrations, cells were harvested and mRNA levels were quantified by quantitative reverse transcription polymerase chain reaction. Gene expression was normalized to the reference gene *rpoD*, and relative gene expression was compared with the *ΔamrZ* strain with the empty vector pHERD20T in the absence of arabinose. Unpaired two-tailed Student's *t*-tests were used for statistical analyses of three independent experiments. ***$P < 0.001$; ns: not significant.

observed with the activated target PA2146, as it required a much higher amount of AmrZ1-66 for transcription compared with full-length AmrZ (Fig. 3B). Reduced efficiency by AmrZ1-66 was also observed during AmrZ-mediated activation of P*algD* (Fig. S8) as well as twitching motility (Table S2).

Oligomerization not only provides an economic way to form large complexes, but also enables proteins, such as transcription factors, to flexibly modulate their DNA-binding specificity and affinity. Differential sequence specificity can be achieved through oligomerization of different combinations of transcription factors (Matthews *et al*., 2012). For some transcription factors such as the λ repressor, oligomerization via its CTD significantly enhances binding affinity to its operator sites within the phage genome (Bell *et al*., 2000). In the present study, we provide evidence that after removing the CTD, AmrZ loses the ability to form tetramers and exhibits reduced DNA binding to both activated and repressed targets, suggesting the significance of the CTD during AmrZ-mediated regulation.

Members in the Arc protein family represent diverse DNA-binding proteins in various phage and bacterial species, and some also contain CTDs. CTDs in proteins such as Mnt and TrwA mediate tetramerization (Waldburger and Sauer, 1995; Moncalián and De La Cruz, 2004; Madl *et al*., 2006), whereas at least two members (NikR and MetJ) exhibit significantly higher DNA-binding activities when their CTDs are bound by ligands (nickel and S-adenosylmethionine, respectively) (Rafferty *et al*., 1989; Chivers and Sauer, 2002). Sequence alignment using PROMALS3D was performed between AmrZ and other Arc family members including Mnt, TrwA, NikR and MetJ (Pei *et al*., 2008). However, significant similarity was observed only in their ribbon–helix–helix domains (Fig. S9). Although our current data support the requirement of the AmrZ CTD for tetramerization, with effects on DNA binding and subsequent gene expression, we cannot exclude a role of the CTD in other functions, such as ligand binding, which requires further investigations.

Conclusions

Overall, we have investigated the significance of the AmrZ CTD for oligomerization, DNA-binding affinity, as well as AmrZ-mediated activation and repression. Tetramers composed of four DNA-binding-proficient monomers are critical for AmrZ-mediated regulation. Further work is necessary to understand which residues are at the interface between monomers to mediate inter-monomer interactions. Because this CTD does not appear to be present in

other proteins, more understanding of this domain may provide insights into a novel oligomerization mechanism and potentially other unknown functions of this domain.

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Supporting information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Fig. S1. The primary form of AmrZ in solution is tetrameric.
Fig. S2. Dominant negative effect of AmrZR22A.
Fig. S3. AmrZR22A formed tetramers in solution.
Fig. S4. The C-terminal domain of AmrZ is required for tetramerization.
Fig. S5. Predicted AmrZ secondary structure and polarity of the AmrZ CTD.
Fig. S6. AmrZ1-66 displayed reduced binding affinity to the *gcbA* promoter.
Fig. S7. Western blot analyses of AmrZ1-66 after arabinose induction.
Fig. S8. Efficient activation of P*algD* requires the AmrZ C-terminal domain.
Fig. S9. Alignment of AmrZ with other Arc proteins.
Table S1. Strains, plasmids, and oligonucleotides used in this study.
Table S2. The AmrZ CTD is required for efficient activation of twitching motility.