Evolutionary Plasticity of AmrZ Regulation in *Pseudomonas*

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Abstract

amrZ, a master regulator protein conserved across Pseudomonads, can be either a positive or negative regulator of swimming motility depending on the species examined. To better understand plasticity in the regulatory function of AmrZ, we characterized the mode of regulation for this protein for two different motility related phenotypes in P. stutzeri. As in P. syringae, AmrZ functions as a positive regulator of swimming motility within P. stutzeri, which suggests that the functions of this protein with regards to swimming motility have switched at least twice across Pseudomonads. Shifts in mode of regulation cannot be explained by changes in AmrZ sequence alone. We further show that AmrZ acts as a positive regulator of colony spreading within this strain, and that this regulation is at least partially independent of swimming motility. Closer investigation of mechanistic shifts in dual function regulators like AmrZ could provide unique insights into how transcriptional pathways are rewired between closely related species.

Importance

Microbes often display finely tuned patterns of gene regulation across different environments, with major regulatory changes controlled by a small group of “master” regulators within each cell. AmrZ is a master regulator of gene expression across Pseudomonads, and can be be either a positive or negative regulator for a variety of pathways depending on the strain and genomic context. Here we demonstrate that the mode of regulation for AmrZ for swimming motility has switched at least twice independently in Pseudomonads, so that AmrZ promotes increased swimming motility in P. stutzeri and P. syringae but represses motility in P. fluorescens and P.
aeruginosa. Since such switches in regulatory mode are relatively rare, further investigation into the mechanisms underlying shifts in regulatory function for AmrZ could provide unique insights into the evolution of bacterial regulatory proteins.

**Introduction**

Transcriptional regulation of bacterial operons is often tightly balanced to enable rapid phenotypic changes while minimizing energetic costs associated with overproduction of mRNA and proteins (1-3). Dissection of the mechanisms of action and characterization of transcriptional responses for numerous repressors and activators across species has provided knowledge about the functions of these proteins while also creating a context for exploring how such pathways evolve (4-6). Although there exists great appreciation for the general plasticity of bacterial regulatory networks, our understanding of how particular pathways are transcriptionally rewired and how specific proteins change regulatory mode over evolutionary time remains far from complete.

**amrZ**, alginate and motility regulator Z, is a ribbon-helix-helix transcription factor that directly regulates a variety of pathways across Pseudomonas species and is a master regulator for numerous pathways associated with virulence in *P. aeruginosa* (7). Unlike the vast majority of characterized regulators, AmrZ can directly affect transcription both positively and negatively within the same cell, with the precise function depending on intrinsic structure of the protein and genomic context of the DNA binding site (8,9). Moreover, although the mechanism of repression by AmrZ canonically involves binding to operator regions, how this protein specifically activates transcription remains unknown (8,9). Regulatory pathways involving AmrZ are best described
for *P. aeruginosa*, where expression from *amrZ* is directly promoted by AlgT (10), directly activates 9 genes, and directly represses 49 genes including itself (7). Pathways regulated by AmrZ include swimming motility, and the operon controlling production the exopolysaccharide Psl, twitching motility, colony morphology, and biofilm formation, with a subset of these phenotypes influenced by AmrZ-dependent changes in cyclic di-GMP (7). In contrast, AmrZ has been shown to promote regulation of the alginate operon and the type IV pilus (11,12). In *P. fluorescens* F113, *amrZ* mutants are hypermotile and iron uptake genes are de-repressed (13). In *P. syringae* DC3000 *amrZ* is a positive regulator of motility as well as a variety of other other virulence genes (14). Given opposing information about the regulatory role of AmrZ for motility across Pseudomonads, we tested the function of AmrZ in a relatively divergent species than those previously screened. *Pseudomonas stutzeri* is an environmentally ubiquitous species known best as a denitrifier as well as for its diverse metabolic capabilities (15). Phylogenies of Pseudomonads demonstrate that *P. stutzeri* is placed uniquely in between other species where impacts of *amrZ* on swimming motility have been evaluated, and thus information about the role of AmrZ from this species could polarize our understanding of regulatory modes for this protein.

**Materials and Methods**

**Bacterial strains, plasmids, and culture conditions:** All strains and plasmids used in the study are listed in Table 1. DBL332 was selected as a rifampicin resistant isolate of strain 23a24 (16). Strain propagation of *P. stutzeri* largely took place at 27°C in King’s B Media supplemented with rifampicin. Antibiotics were used in the following concentrations where appropriate: rifampicin
50 μg/mL, tetracycline 10 μg/mL, nitrofurantoin 40 μg/mL, gentamycin 10 μg/mL, kanamycin 20 μg/mL.

Creation of amrZ mutants. Creation of the construct for deleting amrZ is described in depth in https://dx.doi.org/10.6084/m9.figshare.3204178.v1. Briefly, regions upstream and downstream of amrZ in *P. stutzeri* DBL332 were amplified and spliced using overlap PCR. This fragment was recombined into pDONR207 using BP recombinase to create pDBL63. pDBL63 was then recombined with pMTN1907 to create a pDBL64, which can be used to cleanly delete gene regions within *P. stutzeri* (16,17). Once pDBL64 was created, it was mated into either DBL332 or DBL390 through tri-parental mating with the helper strain containing plasmid pRK2013 (DAB42). DBL390 is a gentamycin resistant version of DBL332 where lacZ has been integrated into the Tn7 site using pUC18-mini-Tn7T-Gm-LacZ prior to conjugation with pDBL64 and therefore represents independent deletion of amrZ. After mating, tetracycline resistant recombinants in both DBL332 or DBL390 backgrounds were selected on LB media. Each recombinant was grown overnight in LB media supplemented with rifampicin, and spread on KB plates containing 5% sucrose. Isolates of *P. stutzeri* DBL332 and DBL390 where amrZ has been deleted are distinguishable on when grown on KB media because they manifest as “rough” colonies. Clean deletion of amrZ was confirmed through PCR with primers DBL383 and DBL384.

Complementation of amrZ. Creation of complementation constructs is described in https://dx.doi.org/10.6084/m9.figshare.3365263.v1. Briefly, the amrZ ORF (including the stop codon) and native promoter for amrZ were amplified from strain DBL332. This fragment was
purified and recombined into pDONR207 using BP clonase to create pDBL91. This construct was then recombined from pDBL91 into a Tn7 transposon on the Gateway destination vector pTn7-GW (Jeff Chang, unpublished) to create pDBL93. Lastly, this construct was transposed onto the DBL1052 chromosome (a clean deletion of amrZ in the DBL332 background) through natural transformation after mixing cells with both pDBL93 and pTNS2. The complementation construct for the mutant allele of amrZ (DBL1074, AmrZV21L) was created using these same primer sets by amplifying this region from a strain naturally containing the allele and using BP clonase to create plasmid pDBL95. The resulting destination vector from this construct (pDBL96) was used for transposition in the same way as the wild type version described above. A complementation construct using the P. aeruginosa amrZ ORF was created in a similar way except that the BP reaction was carried out with a synthesized GBlock (Integrated DNA technologies, Coralville IA), to create pDBL92. Expression of the P. aeruginosa allele of amrZ in this construct is driven by the same promoter sequence (from P. stutzeri) as in pDBL93. pDBL94 was created through an LR reaction involving pDBL92 and pTn7-GW and transformed into DBL1052.

Motility Assays. Following overnight growth in LB media, and two washes with 1mL 10Mm MgCl₂, the OD₆₀₀ of each strain was standardized at 1.0 in 10Mm MgCl₂. A blunt ended toothpick was dipped into this inoculum and then dipped into the center of a 12 well tissue culture plate containing 1/2 strength LB media with 0.25% agar. Plates were parafilmed, and incubated for either 24 or 48 hours at room temperature (indicated in figure legend), after which point they were scanned at 600dpi. Images were imported into ImageJ, and the pixel area of each strain was quantified. Data for all assays can be found at
Motility of each strain for each assay was normalized to a control strain within the same experiment, with figure legends identifying the normalized strain, so that motility across experiments was comparable. Normalization did not affect overall statistical outcomes (data not shown). Each set of experiments was independently run twice, with at least 8 replicates per experiment. Statistical tests were carried out in R (18), where One Way ANOVAs with “Normalized motility” as the dependent variable and “Strain” as a fixed effect independent variable. Inclusion of “Assay” as a second independent variable did not affect overall statistical outcomes (data not shown). Tukey’s HSD was then used to classify strain effects.

**Colony Spreading Assays.** Following overnight growth in KB media, and two washes with 1mL 10Mm MgCl$_2$, the OD$_{600}$ of each strain was standardized at 1.0 in 10Mm MgCl$_2$. 10µL of this suspension was pipetted onto King’s B (KB) media with 1.5% agar. Each experimental plate contained all strains within a given comparison (see Figure 1C). Plates were parafilmed, and incubated for either 72 hours at room temperature. At three time points (after 24, 48, and 72 hours of growth), plates were scanned at 600dpi. Images were imported into ImageJ, and the pixel area of each strain was quantified. Data for all assays can be found at https://figshare.com/s/bd8d3d0a12b8e9f36b5b. Amount of spreading was calculated by taking the difference in area between 24 and 72 hours for each strain on each plate except for one case where spreading was calculated between 48 and 72 hours. Spreading of each strain for each assay was normalized to a strain from the same plate within the same experiment, with figure legends identifying the normalized strain, so that motility across experiments was comparable. Normalization did not affect overall statistical outcomes (data not shown). Each set of
experiments was independently run twice, with at least 2 (but usually 4) replicated plates per experiment. Statistical tests were carried out in R (18), where One Way ANOVAs with “Normalized motility” as the dependent variable and “Strain” as a fixed effect independent variable. Inclusion of “Assay” as a second independent variable did not affect overall statistical outcomes (data not shown). Tukey’s HSD was then used to classify strain effects.

**Phylogenetic Comparisons.**

Bayesian phylogenies were created using protein sequences from conserved genes from each strain, obtained from the JGI Integrated Microbial Genomes (IMG) database (19). Strains used for this comparison were: *P. stutzeri* 23a24 (IMG ID 2565956579), *P. fluorescens* F113 (IMG ID 2511231156), *P. syringae* pv. *tomato* DC3000 (IMG ID 2508501074), *P. aeruginosa* PAO1 (IMG ID 637000218), and *Azotobacter vinelandii* CA (IMG ID 2541047084).

Protein sequences for GyrB and RpoD were used to infer phylogeny of “housekeeping” genes. GyrB and RpoD sequences were independently aligned using ClustalX (20) and then concatenated. MrBayes was used for Bayesian phylogenetic analysis on these sequences (21), using flat priors and a burn-in period of 25,000 generations. In each case, convergence of the run occurred before 100,000 total generations. Phylogenies built using whole genome sequences for these strains completely agree with the reported trees (data not shown). A phylogeny for AmrZ was built the same way as that of RpoD/GyrB. Alignments and output files from MrBayes can be found on Figshare at https://figshare.com/s/bd8d3d0a12b8c9f36b5b.

**Results and Discussion**
Survival of bacterial populations requires gene regulatory schemes that can respond to fine scale gradients and rapid shifts in environmental conditions (5). Over evolutionary time, data suggests that these regulatory schemes are optimized to appropriately respond to a variety of possible environments (1,6). Although much previous work has focused on defining how and when genes are regulated, there have been few examples that have pinpointed changes in the direction of regulation by the same protein across closely related lineages. Here we further document an example in Pseudomonads involving the ribbon-helix-helix transcriptional regulatory AmrZ.

AmrZ is a well-studied regulator of phenotypes important for environmental survival across Pseudomonads, including multiple virulence traits in P. aeruginosa (7,13,14). To better capture the diversity of AmrZ dependent regulation across this genus, we investigated regulation of swimming motility by amrZ in P. stutzeri using independent deletions followed by complementation in trans. In both deletion lines (DBL1052 and DBL1053), loss of amrZ leads to decreased flagellar motility, clearly demonstrating that AmrZ is a positive regulator for this phenotype within this strain (Figure 1A). However, that these lines are not completely amotile demonstrates that amrZ is not the sole positive regulator for flagellar operons in P. stutzeri (data not shown). Loss of motility can be complemented by expression of amrZ with its native promoter from the Tn7 site (Figure 2A). This result directly contrasts with what has been reported across multiple other Pseudomonas species (10,13), but supports the role of AmrZ as a positive regulator of motility in P. syringae (14). Using these same strain comparisons, we have also been able to show that AmrZ is a positive regulatory of colony spreading in P. stutzeri (Figures 1B, 1C, and 2B).
At the present time it is difficult to discern which molecular changes underlie shifts in amrZ function. In P. aeruginosa, AmrZ binds upstream of fleQ in order to directly repress expression of fleQ, although the specific binding sequence appears to be different than canonical AmrZ sites (7). Furthermore, no AmrZ binding was observed upstream of fleQ in P. fluorescens F113, even though amrZ mutants are hypermotile (13). As a first step to determine the molecular mechanism behind alterations in AmrZ-dependent regulation of motility, we tested whether the P. aeruginosa allele could complement the loss of motility in P. stutzeri. These alleles are slightly diverged from one another, but overall maintain relatively high sequence similarity (Figure 4C). As shown in figures 2A and B, the P. aeruginosa allele of amrZ is able to complement both the swimming motility and colony spreading defects in P. stutzeri, demonstrating that both versions of this protein regulate motility in a similar way. Therefore it does not appear that the phenotypic switch in regulation by AmrZ is due to amino acid changes in the protein itself, which strongly suggests that changes in genomic context or within interacting proteins mediate differential regulation of motility by AmrZ across these strains.

Interestingly, we observed that a mutant allele of amrZ (AmrZV21L) arose and swept to fixation during an ongoing evolutionary passage experiment carried out within our lab (data not shown, Figure 4C). This mutation occurs within a region of AmrZ known to be involved in homo-dimerization in P. aeruginosa (8), and therefore possibly affects interactions between independent copies of this protein in P. stutzeri. Surprisingly, we demonstrate here that the mutant AmrZV21L allele is able to complement the colony spreading phenotype but not the swimming motility phenotype within our amrZ knockout strains. This result suggests, even though colony spreading and swimming motility are both positively regulated by AmrZ within P. stutzeri, that the mechanisms of positive regulation for these two phenotypes are at least partially
We don’t yet know how regulation of these pathways mechanistically differ, but the position of the mutation suggests that homo-dimerization is only required for positive regulation of swimming motility. Given recent demonstrations that AmrZ can indirectly alter pools of cyclic di-GMP (7), and since cyclic di-GMP is a critical signaling molecule for some types motility across Pseudomonads (22), it is also possible that regulatory independence of these phenotypes reflects differential influence of cyclic di-GMP. In the very least, that these two phenotypes are independently regulated by AmrZ speaks to the evolutionary flexibility of positive regulation by AmrZ.

To demonstrate possible evolutionary scenarios explaining differential regulation of swimming motility by AmrZ across Pseudomonads, we built phylogenies of using critical strains for which swimming motility effects of AmrZ have been evaluated as well as an outgroup (A. vinelandii) that also contains a version of this regulator. As one can see in Figure 4B, there are three equally parsimonious scenarios for the evolution of positive regulation of swimming motility by AmrZ. Under the first scenario (labeled 1), AmrZ is a negative regulator of swimming motility in the ancestor of Pseudomonads and positive regulation has independently evolved twice. Under the second scenario (labeled 2), the ancestral version of AmrZ is a positive regulator of swimming motility, and negative regulation has independently evolved twice. Under the third scenario, AmrZ is a negative regulator of swimming motility in an ancestral strain, evolves to be a positive regulator before the split of P. stutzeri and P. syringae, and subsequently evolves as a negative regulator again in P. fluorescens. That the phylogeny of AmrZ matches that of RpoD/GyrB, coupled with the ability of the P. aeruginosa allele to complement P. stutzeri phenotypes, rules out scenarios implicating horizontal gene transfer of AmrZ itself in changes to the mode of regulation for swimming motility. In any case, this example definitively
demonstrates that the mode of regulation for AmrZ for swimming motility has changed at least twice independently.

Only a handful of bacterial transcriptional regulators are known to act as both activators and repressors of gene expression. One of these proteins, AmrZ, has been canonically considered a negative regulator of motility across Pseudomonads with one exception shown to date (7,13,14). We provide evidence for the evolutionary plasticity of AmrZ, by demonstrating that this protein acts independently as a positive regulator of swimming motility and colony spreading phenotypes in *P. stutzeri*. Therefore, we definitively show there have been at least two independent shifts in function for AmrZ in the context of swimming motility across Pseudomonads. Closer investigation of mechanistic shifts in dual function regulators like AmrZ could provide unique insights into how transcriptional pathways are rewired between closely related species.
## Table 1. Strains and Plasmids

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Figure 1. AmrZ is a Positive Regulator of Swimming Motility and Colony Spreading in Pseudomonas stutzeri. Strains DBL1052 and DBL1053 are derived from strains DBL332 and DBL390, respectively, and contain independently created deletions in amrZ. Individual data points for each assay are plotted for each strain, with boxes representing two standard deviations and means plotted as horizontal blue lines at the center of the boxplots. Measurements within each assay have been normalized so that the value of DBL332 is 1. Letters above each boxplot indicate that mean values are significantly different at p<0.01 according to Tukey’s HSD. A) amrZ is a positive regulator of swimming motility, and loss of swimming motility is seen in independently created amrZ deletion lines. B) amrZ is a positive regulator of colony spreading, and loss of spreading is seen in independently created amrZ deletion lines. C) Representative example of colony spreading activity and positive regulation by AmrZ. The same plate is shown after being scanned after 1 day of growth (left) and after 3 days of growth (right). AmrZ+ strains spread outward on KB media over time, while AmrZ- fail to spread unless there are compensatory mutations.
**Figure 2. Alleles of amrZ from Either P. stutzeri or P. aeruginosa can Phenotypically Complement an amrZ Deletion Strain In Trans.** Strains DBL1058, DBL1059 and DBL1056 are derived from strains DBL830 (ΔamrZ). DBL1058 contains an empty vector gentamycin resistance cassette while DBL1059 contains amrZ (native promoter) integrated into the Tn7 site of the chromosome. DBL1060 contains the amrZ allele from P. aeruginosa (native promoter from P. stutzeri) integrated into the Tn7 site of the chromosome. Individual data points for each assay are plotted for each strain, with boxes representing two standard deviations and means plotted as horizontal blue lines at the center of the boxplots. Motility and colony spreading values are normalized so that the value of DBL1059 is 1. Letters above each boxplot indicate that mean values are significantly different at p<0.01 according to Tukey’s HSD. Swimming motility (A) and colony spreading (B) phenotypes in an amrZ deletion strain can be complemented by alleles of AmrZ from either P. stutzeri or P. aeruginosa B) amrZ is a positive regulator of colony spreading, and loss of spreading is seen in independently created amrZ deletion lines.
Figure 3. AmrZ Independently Regulates Swimming Motility and Colony Spreading.

Strains DBL1058, DBL1059 and DBL1074 are derived from strains DBL830 (ΔamrZ).

DBL1058 contains an empty vector gentamycin resistance cassette while DBL1059 contains amrZ (native promoter) integrated into the Tn7 site of the chromosome. DBL1074 contains a mutant version of amrZ (AmrZL23V) integrated into the Tn7 site of the chromosome. Individual data points for each assay are plotted for each strain, with boxes representing two standard deviations and means plotted as horizontal blue lines at the center of the boxplots. Motility and colony spreading values are normalized so that the value of DBL1059 is 1. Letters above each boxplot indicate that mean values are significantly different at p<0.01 according to Tukey’s HSD. A) The wild type version of AmrZ can complement the swimming motility defect of DBL830, but the AmrZL23V mutant version cannot. B) Both the wild type version of AmrZ and the AmrZL23V mutant version can complement a colony spreading deficiency of DBL830.
Figure 4. The Mode of Regulation of AmrZ in Swimming Motility Has Shifted at Least Twice Across Pseudomonas. Bayesian phylogenies were built using either RpoD/GyrB (A) or AmrZ (B) for four strains of Pseudomonas where the role of AmrZ in swimming motility has been evaluated, and using Azotobacter vinelandii as an outgroup. Support for all nodes on each phylogeny is >0.95 posterior probability, and the phylogeny of RpoD/GyrB matches that built from whole genome information (data not shown). B) Three equally parsimonious scenarios, labeled 1-3, for the evolution of mode of regulation of AmrZ for swimming motility are overlayed onto the phylogeny of AmrZ. “+” indicates that positive regulation arose during each of the three scenarios, while “-“ indicates that negative regulation arose. C) Protein alignments of AmrZ for the strains used in phylogenetic comparisons are shown. Red lines on top of this alignment indicate that amino acids have been shown to be involved in dimerization of AmrZ (following Pryor et al., 2012). We also highlight, in light blue, the amino acid that has changed in the AmrZL23V allele.


