AmrZ is a Postive Regulator of Swimming Motility in *Pseudomonas stutzeri*

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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 swimming motility in *P. stutzeri*. As in *P. syringae*, AmrZ functions as a positive regulator of motility within *P. stutzeri*, which suggests that the functions of this protein with regards to swimming motility have switched at least twice across Pseudomonads. We further show that divergence between *P. stutzeri* and *P. aeruginosa* alleles of AmrZ does not explain shifts in regulatory mode. 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 (Dekel & Alon, 2005; Stoebel, Dean & Dykhuizen, 2008; Price et al., 2016). 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 (McAdams, Srinivasan & Arkin, 2004; Perez & Groisman, 2009; Mitchell et al., 2009). However, although

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there exists great appreciation for the general plasticity of bacterial regulatory networks, our understanding of how particular pathways can be transcriptionally rewired 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* (Jones et al., 2014). 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 (Pryor et al., 2012; Xu et al., 2015). Moreover, although the mechanism of repression by AmrZ canonically involves binding to operator regions, how this protein specifically activates transcription remains unknown (Pryor et al., 2012; Xu et al., 2015). Regulatory pathways involving AmrZ are best described for *P. aeruginosa,* where expression from *amrZ* is directly promoted by AlgT (Tart, Blanks & Wozniak, 2006). Once expressed and translated, AmrZ has been shown to directly activate 9 genes and to directly repress repress 49 genes within *P. aeruginosa* (Jones et al., 2014). 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 (Jones et al., 2014). In contrast, AmrZ has been shown to promote regulation of the alginate operon and the type IV pilus (Baynham et al., 1999; 2005)}. In *P. fluorescens* F113, *amrZ* mutants are hypermotile and iron uptake genes are de-repressed (Martinez-Granero et al., 2014). While in *P. syringae* DC3000 *amrZ* is a positive regulator of motility as well as a variety of other other

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virulence genes (Prada-Ramírez et al., 2015). 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 (Lalucat et al., 2006). Phylogenies of Pseudomonads demonstrate that *P. stutzeri* branches off early within this genus, when *Cellvibrio japonicus* is used as an outgroup, and thus information about the role of AmrZ from this species could polarize our understanding of regulatory modes for this protein.

Here we demonstrate that AmrZ acts as a positive regulator of swimming motility in *P. stutzeri* and that this shift in activity compared to *P. aeruginosa* is likely due to changes in genomic context of regulation rather than alterations in the AmrZ protein itself. 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.

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 (Romanchuk et al., 2014). Strain propagation of *P. stutzeri* largely took place at 27^{o}C in King's B Media supplemented with rifampicin. Antibiotics were used in the following concentrations where appropriate: rifampicin 50 ug/mL, tetracycline 10 ug/mL, nitrofurantoin 40 ug/mL, gentamycin 10 ug/mL, kanamycin 20 ug/mL.

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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* (Romanchuk et al., 2014). 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 plated out 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

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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. 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 $MgCl_2$, the OD_{600} of each strain was standardized at 1.0 in 10Mm $MgCl_2$. 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 **https://dx.doi.org/10.6084/m9.figshare.3365299.v1**. 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 (R Core Team, 2013), 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

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independent variable did not affect overall statistical outcomes (data not shown). Tukey's HSD was then used to classify strain effects.

Results and Discussion

Survival of bacterial populations requires gene regulatory schemes that can respond to fine scale gradients and rapid shifts in environmental conditions ({Perez:2009ir). Over evolutionary time, data suggests that these regulatory schemes are optimized to appropriately respond to a variety of possible environments {Dekel:2005ey, Mitchell:2009el}. 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* (Jones et al., 2014; Martinez-Granero et al., 2014; Prada-Ramírez et al., 2015). 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 1). However, that these lines are not completely amotile demonstrates that *amrZ* is not the sole positive regulator for flagellar operons in *P. stutzeri*. Loss of motility can be

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complemented by expression of *amrZ* with its native promoter from the Tn7 site (Figure 2). Interestingly, integration of any cassette into the Tn7 site appears to increase motility (compare DBL332 and DBL390 in figure 1). This result directly contrasts with what has been reported across multiple other *Pseudomonas* species (Tart, Blanks & Wozniak, 2006; Martinez-Granero et al., 2014), but supports the role of AmrZ as a positive regulator of motility in *P. syringae (*Prada-Ramírez et al., 2015). Moreover, since *P. fluorescens* and *P. syringae* represent an independent clade than that of *P. aeruginosa* and *P. stutzeri*, both ancestral states (positive or negative regulator) are equally parsimonious at present (Gomila et al., 2015). In either case, this example demonstrates that modes of regulation for dual-function proteins like AmrZ can be quite plastic, changing relatively rapidly under appropriate evolutionary conditions.

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 (Jones et al., 2014). Furthermore, no AmrZ binding was observed upstream of *fleQ* in *P. fluorescens* F113*,* even though *amrZ* mutants are hypermotile (Martinez-Granero et al., 2014). 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.* To control for mRNA levels, we drove expression of this *P. aeruginosa* allele from a chromosomal site (Tn7) using the native *P. stutzeri* promoter. As shown in figure 2, the *P. aeruginosa* allele of *amrZ* is able to complement the motility defect 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

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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. We also note that the *P. aeruginosa* allele activates motility to a greater extent than the *P. stutzeri* allele (even though the promoter for each is identical), which suggests fine tuning of activation capabilities within each strain background.

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 (Jones et al., 2014; Martinez-Granero et al., 2014; Prada-Ramírez et al., 2015). We provide further evidence for the evolutionary plasticity of AmrZ, by demonstrating that this protein acts as a positive regulator of motility in *P. stutzeri*. Therefore, there have been at least two independent shifts in function for AmrZ for the regulation of swimming motility although we are unable to pinpoint the ancestral state at the current time.

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1 **Table 1. Strains and Plasmids Used in This Study**

Strain Number	Strain Description	Citation
DBL332	P. stutzeri DBL332, Rif^R	Romanchuk et al. 2014
DBL1052	P. stutzeri DBL332 with deletion of amrZ, Rif^R	This manuscript
DBL390	P. stutzeri DBL390, Rif^R $Gent^R$ LacZ+	Romanchuk et al. 2014
DBL1053	P. stutzeri DBL390 with deletion of *amrZ*, Rif^R $Gent^R$ LacZ+	This manuscript
DBL1058	P. stutzeri DBL1052 wth Tn7 tranposition from pME3280a, Rif^R $Gent^R$	This manuscript
DBL1059	P. stutzeri DBL1052 with Tn7 transposition from pDBL93, Rif^R $Gent^R$	This manuscript
DBL1060	P. stutzeri DBL1052 with Tn7 transposition from pDBL94, Rif^R $Gent^R$	This manuscript
DBL830	DBL332 with pDBL64 integrated, Rif^R Tet^R Suc^S	This manuscript
DBL831	DBL390 with pDBL64 integrated, Rif^R GentR LacZ+ Tet^R Suc^S	This manuscript

Plasmid Number	Plasmid Description	Citation
pME3280a	Tn7 empty vector	Zuber et al. 2003
Tn7-GW	prmoterless Gateway destination vector for Tn7 transposition	Jeff Chang, unpublished
pDBL91	*P. stutzeri amrZ* ORF with stop codon and *P. stutzrei* promoter in pDONR207	This manuscript
pDBL92	*P. aeruginosa amrZ* ORF with stop codon and *P. stutzeri* promoter in pDONR207	This manuscript
pDBL93	*P. stutzeri amrZ* ORF with stop codon and *P. stutzeri* promoter in Tn7-GW	This manuscript
pDBL94	*P. aeruginosa amrZ* ORF with stop codon and *P. stutzeri* promoter in Tn7-GW	This manuscript
pMTN1907	Gateway destination vector for generating deletions in *P. stutzeri*	Baltrus et al. 2012
pDBL63	*P. stutzeri amrZ* deletion construct in pDONR207	This manuscript
pDBL64	*P. stutzeri amrZ* deletion construct in pMTN1907	This manuscript

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Figure 1. ***AmrZ* is a Positive Regulator of Motility in *Pseudomonas stutzeri*.** Strains DBL830 and DBL831 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. Motility is 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.

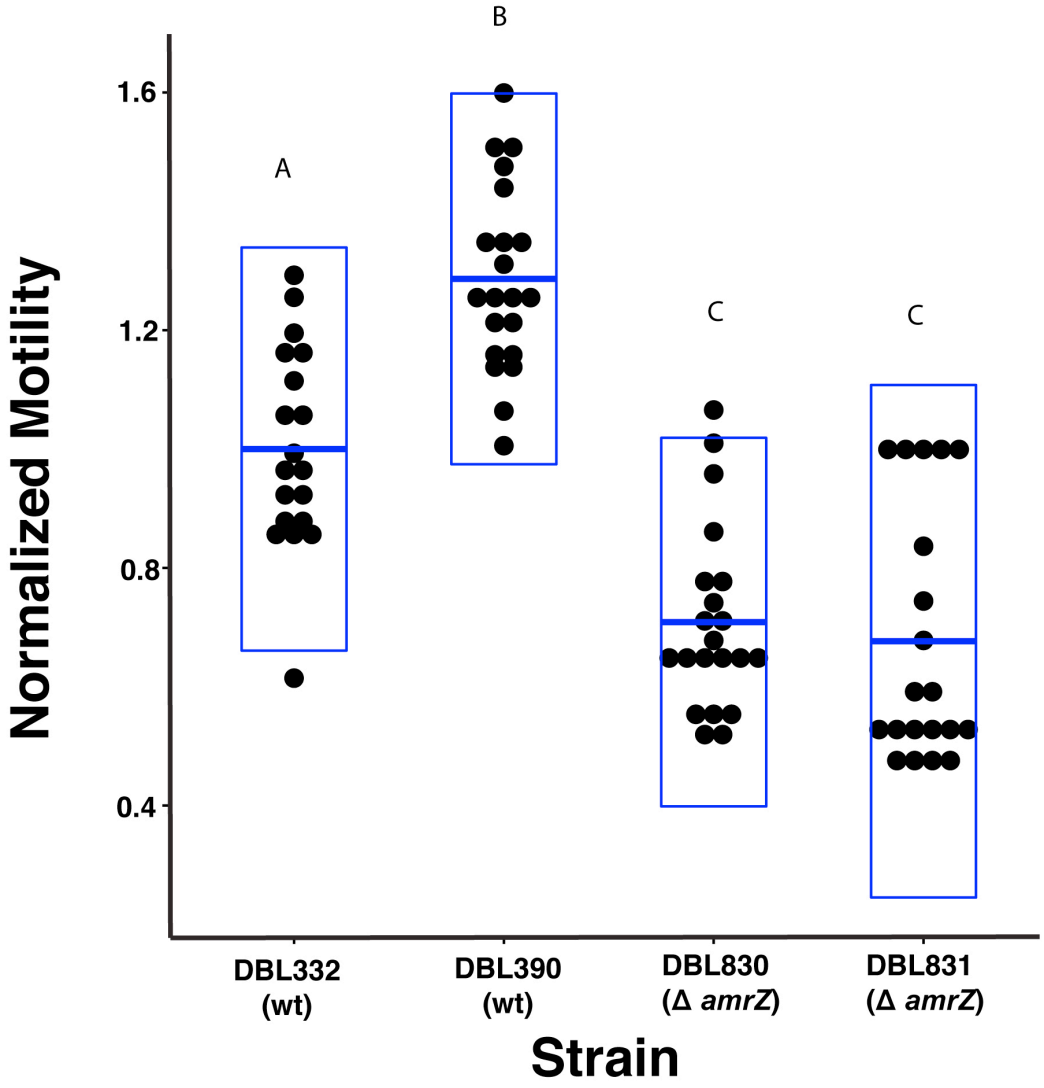

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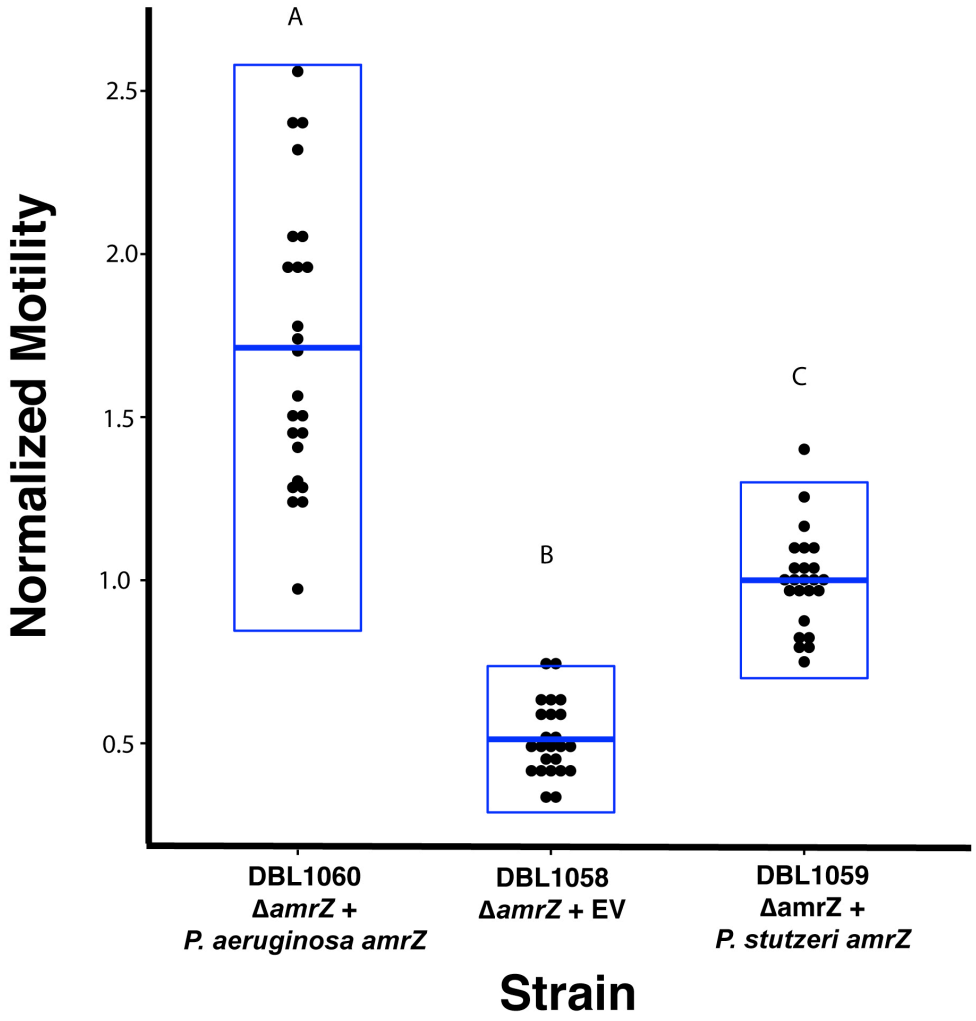
Figure 2. **Alleles of *amrZ* from Either *P. stutzeri* or *P. aeruginosa* can Phenotypically Complement an *amrZ* Deletion Strain *In Trans*.** Strains DBL1058, DBL1059 and DBL1056 are derived from strains DBL830 (Δ*amrZ)*. DBL1058 contains an empty vector gentamycin resistance cassette while DBL1059 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 is 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.



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Baltrus DA, Nishimura MT, Dougherty KM, Biswas S, Mukhtar M, Vicente J, Holub EB, Dangl JL 2012 The molecular basis of host specialization in bean pathovars of *Pseudomonas syringae* Mol Plant Microbe Interact 25(7):877-888

Baynham PJ, Brown AL, Hall LL, Wozniak DJ 1999. *Pseudomonas aeruginosa* AlgZ, a ribbon-helix-helix DNA-binding protein, is essential for alginate synthesis and *algD* transcriptional activation. *Mol Microbiol* 33:1069–1080.

Baynham PJ, Ramsey DM, Gvozdyev BV, Cordonnier EM, Wozniak DJ 2005. The *Pseudomonas aeruginosa* Ribbon-Helix-Helix DNA-Binding Protein AlgZ (AmrZ) Controls Twitching Motility and Biogenesis of Type IV Pili. *J Bacteriol* 188:132–140. DOI: 10.1128/JB.188.1.132-140.2006.

Dekel E, Alon U 2005. Optimality and evolutionary tuning of the expression level of a protein. *Nature* 436:588–592. DOI: 10.1038/nature03842.

Gomila M, PeÃ a A, Mulet M, Lalucat J, GarcÃ a-ValdÃ s E 2015. Phylogenomics and systematics in *Pseudomonas*. *Front Microbiol* 6:3389. DOI: 10.1099/00207713-37-4-463.

Jones CJ, Newsom D, Kelly B, Irie Y, Jennings LK, Xu B, Limoli DH, Harrison JJ, Parsek MR, White P, Wozniak DJ 2014. ChIP-Seq and RNA-Seq Reveal an AmrZ-Mediated Mechanism for Cyclic di-GMP Synthesis and Biofilm Development by *Pseudomonas aeruginosa*. *PLoS Pathog* 10:e1003984. DOI: 10.1371/journal.ppat.1003984.s009.

Lalucat J, Bennasar A, Bosch R, Garcia-Valdes E, Palleroni NJ 2006. Biology of *Pseudomonas stutzeri*. *Microbiol Mol Biol Rev* 70:510–547. DOI: 10.1128/MMBR.00047-05.

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McAdams HH, Srinivasan B, Arkin AP 2004. The evolution of genetic regulatory systems in bacteria. *Nat Rev Genet* 5:169–178. DOI: 10.1038/nrg1292.

Mitchell A, Romano GH, Groisman B, Yona A, Dekel E, Kupiec M, Dahan O, Pilpel Y 2009. Adaptive prediction of environmental changes by microorganisms. *Nature* 460:220–224. DOI: 10.1038/nature08112.

Martinez-Granero FM, Redondo-Nieto M, Vesga P, Martin M, Rivilla R 2014. AmrZ is a global transcriptional regulator implicated in iron uptake and environmental adaption in *P. fluorescens* F113. *BMC genomics* 15:1–10. DOI: 10.1186/1471-2164-15-237.

Perez JC, Groisman EA 2009. Evolution of Transcriptional Regulatory Circuits in Bacteria. *Cell* 138:233–244. DOI: 10.1016/j.cell.2009.07.002.

Prada-Ramírez HA, Pérez-Mendoza D, Felipe A, Martínez-Granero F, Rivilla R, Sanjuán J, Gallegos M-T 2015. AmrZ regulates cellulose production in *Pseudomonas syringae* pv. *tomato* DC3000. *Mol Microbiol* 99:960–977. DOI: 10.1111/mmi.13278.

Price M, Wetmore KM, Deutschbauer AM, Arkin AP 2016. *A Comparison of the Costs and Benefits of Bacterial Gene Expression*. *bioRxiv* preprint DOI: 10.1101/038851.

Pryor EE, Waligora EA, Xu B, Dellos-Nolan S, Wozniak DJ, Hollis T 2012. The Transcription Factor AmrZ Utilizes Multiple DNA Binding Modes to Recognize Activator and Repressor Sequences of *Pseudomonas aeruginosa* Virulence Genes. *PLoS Pathog* 8:e1002648. DOI: 10.1371/journal.ppat.1002648.s003.

R Core Team 2013. R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. URL http://www.R-project.org/

Romanchuk A, Jones CD, Karkare K, Moore A, Smith BA, Jones C, Dougherty K, Baltrus DA 2014. Bigger is not always better: Transmission and fitness burden of ~1MB

PeerJ Preprints | https://doi.org/10.7287/peerj.preprints.2041v1 | CC-BY 4.0 Open Access | rec: 11 May 2016, publ: 11 May 2016

Pseudomonas syringae megaplasmid pMPPla107. *Plasmid* 73:16–25. DOI: 10.1016/j.plasmid.2014.04.002.

Stoebel DM, Dean AM, Dykhuizen DE 2008. The Cost of Expression of *Escherichia coli lac* Operon Proteins Is in the Process, Not in the Products. *Genetics* 178:1653–1660. DOI: 10.1534/genetics.107.085399.

Tart AH, Blanks MJ, Wozniak DJ 2006. The AlgT-Dependent Transcriptional Regulator AmrZ (AlgZ) Inhibits Flagellum Biosynthesis in Mucoid, Nonmotile *Pseudomonas aeruginosa* Cystic Fibrosis Isolates. *J Bacteriol* 188:6483–6489. DOI: 10.1128/JB.00636-06.

Xu B, Ju Y, Soukup RJ, Ramsey DM, Fishel R, Wysocki VH, Wozniak DJ 2015. The *Pseudomonas aeruginosa* AmrZ C-terminal domain mediates tetramerization and is required for its activator and repressor functions. *Environ Microbiol Rep* 8:85–90. DOI: 10.1111/1758-2229.12354.

Zuber S, Carruthers F, Keel C, Mattart A, Blumer C, Pessi G, Gigot-Bonnefoy C, Schnider-Keel U, Heeb S, Reimmann C, Haas D 2003 GacS sensor domains pertinent to the regulation of exoproduct formation and to the biocontrol potential of *Pseudomonas fluorescens* CHA0 Mol Plant Microbe Interact 16(7):634-644

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