An Allele of an Ancestral Transcription Factor Dependent on a Horizontally Acquired Gene Product

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Abstract

Changes in gene regulatory circuits often give rise to phenotypic differences among closely related organisms. In bacteria, these changes can result from alterations in the ancestral genome and/or be brought about by genes acquired by horizontal transfer. Here, we identify an allele of the ancestral transcription factor PmrA that requires the horizontally acquired pmrD gene product to promote gene expression. We determined that a single amino acid difference between the PmrA proteins from the human adapted Salmonella enterica serovar Paratyphi B and the broad host range S. enterica serovar Typhimurium rendered transcription of PmrA-activated genes dependent on the PmrD protein in the former but not the latter serovar. Bacteria harboring the serovar Typhimurium allele exhibited polymyxin B resistance under PmrA- or under PmrA- and PmrD-inducing conditions. By contrast, isogenic strains with the serovar Paratyphi B allele displayed PmrA-regulated polymyxin B resistance only when experiencing activating conditions for both PmrA and PmrD. We establish that the two PmrA orthologs display quantitative differences in several biochemical properties. Strains harboring the serovar Paratyphi B allele showed enhanced biofilm formation, a property that might promote serovar Paratyphi B’s chronic infection of the gallbladder. Our findings illustrate how subtle differences in ancestral genes can impact the ability of horizontally acquired genes to confer new properties.

Introduction

The phenotypic properties that distinguish closely related bacterial species are often ascribed to differences in gene content [1,2]. These differences typically result from the acquisition of genetic material by horizontal gene transfer, a process that can lead to a bacterial species [2,3]. For instance, acquisition of the cholera toxin phage by Vibrio cholerae [4] or of the pathogenicity island LEE – for locus of enterocyte effacement – by enteropathogenic Escherichia coli (EPEC) [5] conferred virulence properties upon these bacteria. Indeed, these properties can be reconstructed in laboratory strains of E. coli by experimental introduction of the relevant DNA [6,7]. Likewise, the recovery of the same antibiotic resistance genes in unrelated bacterial species [8] indicates that horizontally acquired genes are capable of conferring new properties to organisms with significantly different genomes. However, this situation might be different if a horizontally acquired gene product targets ancestral proteins because allelic differences among ancestral orthologs might impact the ability of a horizontally acquired gene to function. Here, we address this issue by examining the molecular basis for the distinct abilities of Salmonella serovars to display resistance to the antibiotic polymyxin B under different environmental conditions.

Inducible resistance to polymyxin B in S. enterica serovar Typhimurium is controlled by the ancestral PmrA/PmrB two-component system, the major regulator of lipopolysaccharide (LPS) modification genes [9]. This system is directly activated by extracytoplasmic Fe3+ or Al3+ [10] or by low pH [11] that is detected by the sensor PmrB, which then promotes the phosphorylated state of the DNA binding protein PmrA (PmrA-P) [10,12], resulting in expression of PmrA-activated genes (Figure 1) [13]. Low Mg2+ indirectly activates the PmrA/PmrB system in a process that requires the horizontally acquired pmrD gene [14,15] (Figure 1). This is because low Mg2+ is an inducing signal for the PhoP/PhoQ two-component system [16], which governs pmrD transcription [14]. The PmrD protein protects PmrA-P from dephosphorylation by PmrB, thereby enhancing PmrA-P levels and promoting PmrA-dependent gene transcription [17]. Thus, S. typhimurium displays polymyxin B resistance when experiencing low Mg2+ and/or the presence of Fe3+.

We previously reported that natural isolates of S. enterica vary in the degree to which the horizontally acquired pmrD gene activates the PmrA/PmrB system [18]. This raised the possibility of genetic changes in the genome sequences common to the various S. enterica serovars accounting for the observed phenotypic diversity in polymyxin B resistance [19]. We now report that the
Author Summary

Horizontally acquired genes are typically viewed as independent units that confer new traits when introduced into different bacterial species. However, preexisting proteins in a bacterium can impact the ability of horizontally acquired gene products to bring about new functions when they target ancestral pathways. Here, we establish that a single amino acid difference in the ancestral transcription factor PmrA alters its dependence on the horizontally acquired gene product PmrD to promote gene expression within closely related Salmonella serovars. Consequently, S. enterica serovar Typhimurium, which infects a wide range of animals, expresses PmrA-dependent genes and displays antibiotic resistance in conditions that activate the PmrA and/or PmrD proteins. By contrast, the human-adapted S. enterica serovar Paratyphi B only does so in the presence of both PmrA- and PmrD-activating conditions. Bacteria harboring the Paratyphi B pmrA gene also exhibited enhanced biofilm formation, which may contribute to serovar Paratyphi B’s persistent infection of the gallbladder. Our findings demonstrate that the ability of horizontally acquired genes to confer new traits can be affected by ancestral proteins, even within one bacterial species. Therefore, a protein’s function in a given organism must be appreciated in the context of other proteins operating within the same genetic network.

human-adapted S. enterica serovar Paratyphi B does not activate the PmrA/PmrB system in response to low Mg$^{2+}$ and that activation of PmrA/PmrB in response to Fe$^{3+}$ requires the horizontally acquired pmrD gene product. We establish that this disparity from S. typhimurium is due to a single amino acid difference between the PmrA proteins, which dramatically alters PmrA’s affinity for its target promoters and the levels of PmrA-P in vivo. The Paratyphi B PmrA allele confers enhanced biofilm formation, which may aid survival of this human-adapted serovar in its particular habitat. Our work provides a singular example whereby quantitative differences in the biochemical properties of an ancestral transcription factor dictate the ability of a horizontally acquired gene product to confer new traits.

Results

S. paratyphi B Does Not Display Polymyxin B Resistance and PmrA-Dependent Gene Expression in Low Mg$^{2+}$

SARA46 is an S. enterica isolate belonging to the Paratyphi B serovar and is classified as a member of the systemic pathovar (SPV) that causes paratyphoid fever in humans [20,21]. This isolate could not grow on N-minimal media agarose plates containing polymyxin B and low Mg$^{2+}$ (Figure 2A) but grew when Fe$^{3+}$ was present (Figure 2A). This is in contrast to S. typhimurium, which grew on both media (Figure 2A). We determined that this behavior reflects expression of the PmrA-activated pmrD operon, which is required for polymyxin B resistance [22-24] (note that pmrD is often referred to as pmrHFIJKLM [23] or arn [25]). SARA46 failed to transcribe pmrD when grown in low Mg$^{2+}$ but could do so in the presence of Fe$^{3+}$ whereas S. typhimurium expressed pmrD under both conditions (Figure 2B). The behavior of SARA46 is exhibited by other S. paratyphi B (SPV) isolates (Figure 2B). This behavior cannot be ascribed to these isolates being human-adapted or part of the serovar Paratyphi B because the human-adapted serovar Typhi as well as S. paratyphi B strains belonging to the enteric pathovar (EPV), which cause local enteric infections [20], transcribed pmrD in low Mg$^{2+}$ regardless of the presence/absence of Fe$^{3+}$ (Figure 2B), like S. typhimurium. None of the investigated strains transcribed pmrD during growth in high Mg$^{2+}$, which is a non-inducing condition for the PmrA/PmrB system (Figure 2B).

The S. paratyphi B pmrD Gene Is Required to Express PmrA-Dependent Genes during Growth in Fe$^{3+}$

The inability of S. paratyphi B isolates to transcribe pmrD in low Mg$^{2+}$ resembles the behavior of an S. typhimurium pmrD null mutant [14]. This raised the possibility of S. paratyphi B (SPV) isolates harboring mutations in pmrD, like other natural Salmonella isolates [10]. However, DNA sequence analysis revealed that S. paratyphi B and S. typhimurium specify identical PmrD proteins. Moreover, pmrD transcription in S. paratyphi B was stimulated in low Mg$^{2+}$ (Figure 2C) as in S. typhimurium [14]. Then, why do serovars Paratyphi B and Typhimurium differ in the expression of PmrA-dependent genes when experiencing low Mg$^{2+}$ even though they specify identical PmrD proteins that are expressed under like conditions?

A Single Amino Acid Difference between the PmrA Proteins from S. paratyphi B and S. typhimurium Is Responsible for Their Distinct Dependence on PmrD

The results described above indicate that the inability of S. paratyphi B to transcribe the pmrD gene in low Mg$^{2+}$ is due to a difference from S. typhimurium in a gene(s) other than pmrD. Because PmrA-P constitutes the only known target of the PmrD protein [17], we explored whether the S. paratyphi B PmrA protein differs from the S. typhimurium homolog. Thus, we sequenced the pmrA gene from 32 natural isolates originating from the Salmonella reference collections A [21], B [26] and C [27]. An alignment of their deduced amino acid sequences demonstrated that the PmrA protein from S. paratyphi B (SPV) strains has a glutamate residue at position 211 (PmrA E211) whereas most other analyzed S. enterica isolates, including S. typhimurium and S. paratyphi B (EPV) strains, bear a glycine residue at that position (PmrA G211) (Figure S1A).
S. typhi s3333, with an arginine residue at position 211 (Figure S1A), constitutes a third allele of PmrA identified in Salmonella. These data suggested that the presence of a glutamate at position 211 of PmrA prevents expression of pbgP in low Mg\(^{2+}\); whereas isolates with glycine or arginine at that position are competent for pbgP transcription under these conditions (Figure 2B).

If a difference in PmrA is solely responsible for S. paratyphi B’s inability to transcribe pbgP in low Mg\(^{2+}\), then replacing its pmrA

Figure 2. S. paratyphi B is susceptible to polymyxin B and does not transcribe pbgP during growth in low Mg\(^{2+}\). (A) Growth of S. typhimurium (14028s) and S. paratyphi B (SARA46) on plates containing polymyxin B (5 μg/ml) and low Mg\(^{2+}\) (i.e., 10 μM), or polymyxin B (5 μg/ml), low Mg\(^{2+}\) (i.e., 10 μM) and high Fe\(^{3+}\) (i.e., 100 μM). (B) β-galactosidase activity (Miller units) produced from a pbgP-lac transcriptional fusion in S. paratyphi B (SPV) (SARA42, SARA43, SARA45 and SARA46), S. typhimurium (14028s, SARA10, SARA15, SARA18), S. paratyphi B (EPV) (SARA52 and SARA56) and S. typhi (s3333) strains. The inset shows β-galactosidase activity (Miller units) produced from a pbgP-lac transcriptional fusion in S. paratyphi B SARA41. Bacteria were grown for 4 h in N-minimal medium at pH 7.7 with low Mg\(^{2+}\) (i.e., 10 μM), high Mg\(^{2+}\) (i.e., 10 mM) or low Mg\(^{2+}\) (i.e., 10 μM) and high Fe\(^{3+}\) (i.e., 100 μM). Data correspond to the mean values of three independent experiments performed in duplicate, and error bars show standard deviation. (C) mRNA levels of the PhoP-activated pmrD gene from S. paratyphi B SARA46 grown as described in (B) were determined by reverse-transcription-qPCR analysis. Expression levels were normalized to those of the 16S ribosomal RNA gene. Data correspond to the mean values of three independent experiments and error bars show standard deviation.

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(E211) by the pmrA (G211) allele should restore expression. To test this notion, we engineered isogenic S. paratyphi B SARA46 strains bearing a phgP-lac transcriptional fusion and either the pmrA (G211) or pmrA (E211) alleles under the control of the S. paratyphi B pmrCAB promoter at its normal chromosomal location. When grown in low Mg\textsuperscript{2+}, the S. paratyphi B strain (pmrA G211) produced 10 times more β-galactosidase activity than the isogenic pmrA (E211) strain (Figure 3A). As expected, deletion of pmrD eliminated phgP expression in both S. paratyphi B strains when grown in media containing low Mg\textsuperscript{2+} (Figure 3A), as described in S. typhimurium [14]; and no β-galactosidase activity was detected in a pmrA mutant under any growth conditions (Figure 3A).

Deleting the pmrD gene prevented S. paratyphi B (pmrA E211) from expressing phgP during growth in low Mg\textsuperscript{2+} + high Fe\textsuperscript{3+} (Figure 3A). This was surprising because Fe\textsuperscript{3+} is detected directly by the PmrB sensor [10], which activates the PmrA protein in a process that does not require PmrD in S. typhimurium [14]. By contrast, S. paratyphi B (pmrA G211) supported phgP transcription in a pmrD mutant incubated in low Mg\textsuperscript{2+} + high Fe\textsuperscript{3+} (Figure 3A). That a single amino acid difference between the PmrA orthologs can have such dramatic effects was reinforced by the phenotypes displayed by S. typhimurium strains with either one of the two pmrA alleles (Figure 3B), as they recapitulated the behavior of the S. paratyphi B strains (Figure 3A).

We then analyzed the ability of isogenic S. paratyphi B and S. typhimurium strains harboring the pmrA (G211) or pmrA (E211) alleles to survive killing by polymyxin B when grown on N-minimal media agarose plates containing low Mg\textsuperscript{2+} (Figure 3A). This was surprising because Fe\textsuperscript{3+} is detected directly by the PmrB sensor [10], which activates the PmrA protein in a process that does not require PmrD in S. typhimurium [14]. By contrast, S. paratyphi B (pmrA G211) supported phgP transcription in a pmrD mutant incubated in low Mg\textsuperscript{2+} + high Fe\textsuperscript{3+} (Figure 3A). That a single amino acid difference between the PmrA orthologs can have such dramatic effects was reinforced by the phenotypes displayed by S. typhimurium strains with either one of the two pmrA alleles (Figure 3B), as they recapitulated the behavior of the S. paratyphi B strains (Figure 3A).

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The PmrA (E211) Protein Has Lower Affinity for the phgP Promoter Than the PmrA (G211) Protein

Why does the single amino acid difference between the PmrA proteins from S. paratyphi B and S. typhimurium render transcription of PmrA-activated genes dependent on PmrD in the former but not in the latter serovar when Fe\textsuperscript{3+} is present? And how does S. paratyphi B overcome the lower affinity of its PmrA protein for target promoters in order to stimulate PmrA-dependent expression under such conditions? When bacteria experience inducing conditions for the PmrA/PmrB system, the sensor PmrB phosphorylates the DNA binding protein PmrA, increasing PmrA’s affinity for target promoters and resulting in transcription of PmrA-activated genes [13,34]. The PmrD protein, which is produced in low Mg\textsuperscript{2+}, promotes the phosphorylated state of PmrA by protecting it from dephosphorylation by PmrB, an activity primarily present under PmrA non-inducing conditions [17]. Therefore, we hypothesized that the PmrA (G211) and PmrA (E211) proteins might differ in one or more of these biochemical properties, which, in turn, might impact the levels of phosphorylated PmrA in vivo.

First, we analyzed phosphotransfer from PmrB to each of the two purified PmrA proteins and determined that the identity of the amino acid residue at position 211 does not impact PmrA’s ability to accept a phosphoryl group from PmrB (Figure S3A and S3B). These experiments were performed with the purified cytoplasmic domain of the PmrB protein (PmrB\textsubscript{C}), because it retains all the known enzymatic activities of the full-length PmrB protein [17]. The phosphorylated PmrA (G211) and PmrA (E211) proteins also displayed comparable rates of PmrB\textsubscript{C}-mediated dephosphorylation in the absence of PmrD (Figure 5A and 5B, Figure S3C and S3D). However, we determined that PmrA (E211)-P is better protected
Figure 3. *S. paratyphi* B requires PmrD to express PmrA-dependent genes and to resist polymyxin B during growth in Fe³⁺. (A) β-galactosidase activity (Miller units) produced from a pbgP-lac transcripational fusion in the following strains: *S. typhimurium* (EG9241), *S. paratyphi* B (EG16652), *S. paratyphi* B (pmrA G211) (EG16275), *S. paratyphi* B ΔpmrD (EG16277), *S. paratyphi* B pmrA (G211) ΔpmrD (EG16274), and *S. paratyphi* B ΔpmrA (DC167). Bacteria were grown in N-minimal medium at pH 7.7 with low Mg²⁺, high Mg²⁺ or low Mg²⁺ and high Fe³⁺. Data correspond to the mean values of three independent experiments performed in duplicate, and error bars show standard deviation. Asterisks indicate statistically significant differences based on a two-tailed Student t-test (p < 0.05). (B) β-galactosidase activity (Miller units) produced from a pbgP-lac transcripational fusion in isogenic pmrD⁺ or pmrDΔ strains harboring either the pmrA (E211) or (G211) allele (EG9241, EG11775, EG14331 and DC300) were determined as described in (A). (C–D) Growth of *S. enterica* strains harboring either the pmrA (E211) or (G211) allele in the presence of the antibiotic polymyxin B (2.5 μg/ml) on plates containing 10 μM Mg²⁺ and 100 μM Fe³⁺. *S. paratyphi* B growth was determined in the wild-type (SARA46), in a strain harboring either the pmrA (E211) (DC282) or pmrA (G211) (DC280) gene, in a ΔpmrD mutant expressing the pmrA (E211)
by PmrD from PmrB’s phosphatase activity than PmrA (G211)-P (Figure 5A and 5B, Figure S3C and S3D).

Next, we examined whether the heightened protection of PmrA (E211)-P by PmrD led to higher levels of phosphorylated PmrA protein in vivo when bacteria were incubated with low Mg\(^{2+}\) and high Fe\(^{3+}\). Cell lysates from *S. typhimurium* strains expressing HA-tagged versions of PmrA (G211) or PmrA (E211) from the normal chromosomal location were separated on a Phos-tag gel, which retards phosphorylated proteins more than their unmodified forms and has been used to examine phosphorylated response regulators in vivo [35,36]. Western blotting with anti-HA antibodies revealed that the *S. typhimurium* (pmrA E211) strain had a higher proportion of PmrA-P compared to the isogenic *pmrA* (G211) strain (Figure 5C and 5D), despite both strains having similar levels of total PmrA protein (Figure 5C).

Cumulatively, these findings indicate that PmrD is more efficient in protecting PmrA (E211)-P than PmrA (G211)-P from PmrBc-mediated dephosphorylation, leading to increased amounts of phosphorylated PmrA (E211) in bacteria experiencing PmrD- and PmrA-inducing conditions. Because PmrA-P constitutes the active form of PmrA that binds target promoters in vivo [34], such an increase appears sufficient to compensate for the PmrA (E211) protein’s lower affinity for DNA, resulting in PmrA-dependent gene expression.

Figure 4. The affinity of PmrA-P for its target promoters controls PmrA levels in vivo. (A) Electrophoretic mobility shift assays carried out with a DNA fragment carrying the *pbgP* promoter region and increasing amounts of purified phosphorylated PmrA (G211) or PmrA (E211) proteins. Excess unlabeled *pbgP* DNA (cold probe) released the labeled probe from the retarded complex. Addition of excess unlabeled *ompX* or *mgtA* DNA did not release the labeled probe. (B) Levels of PmrA protein determined by Western blotting analyses from *S. typhimurium* strains expressing C-terminally HA-tagged versions of the PmrA (G211) (EG18052) or PmrA (E211) (DC53) proteins. Bacteria were grown in N-minimal medium at pH 7.7 with low Mg\(^{2+}\) (i.e., 10 \(\mu\)M), or low Mg\(^{2+}\) (i.e., 10 \(\mu\)M) and high Fe\(^{3+}\) (i.e., 100 \(\mu\)M). The levels of RpoB were used as loading controls.

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Intragenic Suppression of pmrA (E211) Restores Transcription in Low Mg2+

An S. typhimurium strain harboring the pmrA505 allele can transcribe PmrA-activated genes in a pmrD mutant and under non-inducing conditions for the PhoP/PhoQ system [14] (Figure 5E). This is because the PmrA505-P protein, which harbors a histidine residue instead of arginine at position 81, is resistant to dephosphorylation by PmrB in vitro [17], presumably resulting in increased levels of PmrA-P in vivo. We hypothesized that this increase might be sufficient to overcome the DNA-binding defect of the PmrA (E211) protein, enabling it to activate expression of PmrA-dependent genes in response to the low Mg2+ signal. As predicted, the R81H substitution rescued the ability of PmrA E211 to promote phgP transcription. When bacteria experienced low Mg2+, the S. typhimurium strain with the pmrA (R81H E211) gene transcribed phgP to levels similar to those produced in response to Fe3+ (Figure 5E). The pmrA (R81H E211) strain expressed phgP when grown in high Mg2+, though not to the levels displayed by the isogenic strain harboring the pmrA (R81H G211) allele (Figure 5E). Furthermore, phgP transcription was ~5-fold higher in a pmrA (R81H E211) derivative deleted in pmrD than in the isogenic pmrA (E211) ΔpmrD strain when encountering low Mg2+ + high Fe3+ (Figure 5E). Yet, the levels were several fold lower than those produced by the pmrA (R81H G211) ΔpmrD strain (Figure 5E). These results indicate that the R81H substitution in PmrA can partially overcome the defect of the E211 allele.

The pmrA (E211) Allele Delays Expression of PmrA-Activated Genes

We previously reported that when an S. typhimurium (pmrA G211) strain experiences Fe3+, there is a surge in the mRNA levels of PmrA-activated genes, which increase, peak and then decrease to reach new steady-state levels in a manner reflecting the amount of PmrA-P protein [12]. Because the PmrA (E211) protein has a lower affinity for the phgP promoter in vitro than the PmrA (G211) protein (Figure 4A), we reasoned that a strain with the pmrA (E211) allele might differ in the kinetics with which PmrA-dependent transcripts are produced in vivo.

To test this idea, bacteria were grown under non-inducing conditions for the PmrA/PmrB system, shifted to media containing low Mg2+ + high Fe3+ and incubated for different extents of time. In the pmrA (G211) strain, the phgP and pmrC mRNAs peaked at 5 min and 10 min, respectively, before decreasing to steady-state levels (Figure 6A and 6B). By contrast, in the pmrA (E211) strain, the transcripts increased steadily over 60–90 min (Figure 6A and 6B). Deletion of the pmrD gene abolished phgP and pmrC expression in the pmrA (E211) strain (Figure 6C and 6D), but it decreased expression of these mRNAs only modestly in the pmrA (G211) strain (Figure 6C and 6D). Thus, the pmrA allele affects both the conditions in which PmrA-dependent genes are expressed and the kinetics with which genes are transcribed when bacteria experience inducing conditions.

The pmrA (E211) Allele Enhances Biofilm Formation by S. enterica

S. typhimurium B can cause chronic infections by persisting in the gallbladder for many years [37,38]. The ability of the related S. typhi to form biofilms on cholesterol-coated gallstones is believed to facilitate colonization of the gallbladder [39–41]. Thus, we investigated whether the pmrD allele altered S. enterica’s ability to form biofilms on cholesterol-coated surfaces using an assay developed by the Gunn laboratory [42].

Biofilm formation was higher in S. paratyphi B (pmrA E211) compared to the isogenic pmrA (G211) strain (Figure 7A). Deletion of the pmrD gene further increased biofilm formation in the S. paratyphi B (pmrA E211) strain, which reached levels similar to those displayed by a pmrA null mutant (Figure 7A). This was expected because the ability of S. paratyphi B (pmrA E211) to express PmrA-dependent genes requires the PmrD protein (Figure 3A, Figure 6C and 6D). By contrast, the S. paratyphi B (pmrA G211) strain deleted for pmrD displayed low levels of attachment to cholesterol-coated surfaces, like the isogenic pmrD’ strain (Figure 7A). The growth rates of these strains are similar (Figure S4A) and therefore, are not responsible for the detected differences in biofilm formation. These phenotypes are mediated by the pmrA gene and do not appear to involve genes that are specific to S. paratyphi B because they can be recapitulated in an S. typhimurium strain background (Figure 7B and Figure S4B).

Discussion

Horizontally acquired genes typically endow a recipient organism with new capabilities. We have now determined that subtle variations in the amino acid sequence of an ancestral protein can have contrasting effects on the ability of a horizontally acquired gene product to confer a new trait, even within a species. A single residue difference between the ancestral PmrA protein from the serovars Paratyphi B and Typhimurium of S. enterica (Figure S1A) resulted in the former serovar becoming dependent on the horizontally acquired pmrD gene product to promote PmrA-dependent gene transcription (Figure 2 and Figure 3), but it expanded the environments where PmrA-activated genes are expressed in the latter serovar [14].

Allelic differences in orthologous ancestral proteins impact the functionality of horizontally acquired gene products not only within a species but also between species. For instance, the closely related bacterium E. coli behaves like S. paratyphi B in that it is unable to display PmrA-dependent gene expression when experiencing low Mg2+. Yet, the genetic basis for this behavior is different: the PmrB protein from E. coli exhibits ~9 times the PmrA-P phosphatase activity than that manifested by the S. typhimurium PmrB protein [15,18]. Because PmrA-P is the PmrA form that binds its target promoters, the hyperphosphatase activity of the E. coli PmrB hinders activation of the PmrA/PmrB system via PmrD [15,18].

Species-specific allelic differences between conserved ancestral proteins can impact whether horizontally acquired genes are retained in a new host. S. typhimurium and E. coli display distinct levels of DNA supercoiling due to the 3% amino acid difference between their ancestral GyrB proteins [43], likely contributing to the preferential retention of virulence-related prophages in S. typhimurium but not in E. coli. Hence, the capacity of a horizontally acquired gene product to bring about a new property can be affected by the allelic nature of an organism’s ancestral proteins.
Figure 5. PmrA-P levels are higher in *S. typhimurium* pmrA (E211) than in *S. typhimurium* pmrA (G211) experiencing PmrD- and PmrA-inducing conditions. (A–B) Levels of PmrA-P following incubation of PmrA (G211)-P or PmrA (E211)-P (10 μM) with PmrBc (5 μM) in the presence of 2.5 μM (A) or 1.25 μM (B) PmrD for the indicated times. The graph depicts the level of PmrA-P at the indicated times relative to levels at the start of the reaction. Data correspond to the mean values of at least three independent experiments and error bars show standard deviation. (C) Levels of phosphorylated versus unphosphorylated PmrA protein determined by Phos-tag gel analyses from *S. typhimurium* expressing C-terminally HA-tagged versions of the PmrA (G211) (EG18052) or PmrA (E211) (DC53) proteins. Bacteria were grown in N-minimal medium at pH 7.7 with low Mg²⁺ (i.e., 10 μM) and high Fe³⁺ (i.e., 100 μM). The total amounts of PmrA protein were determined on the same gels by boiling the samples to hydrolyze the phospho-Asp from PmrA-P. (D) Quantitation of the Western blot analyses shown in (C). The graph depicts the level of PmrA-P relative to total PmrA protein. Data correspond to the mean values of four independent experiments and error bars show standard deviation. These results are significantly different as determined by a two-tailed Student t-test (p<0.05). (E) β-galactosidase activity (Miller units) produced from a *pbgP-lac* transcriptional fusion in the following *S. typhimurium* 14028s strains: pmrA (G211) (EG9241), pmrA (R81H G211) (DC294), pmrA (E211) (EG11775), pmrA (R81H E211) (DC302), pmrA (G211) ΔpmrD (EG14331), pmrA (R81H G211) ΔpmrD (DC302), pmrA (E211) ΔpmrD (DC300), pmrA (R81H E211) ΔpmrD (DC304).
Allelic Differences in a Regulatory Protein Can Give Rise to Distinct Phenotypic Behaviors within a Species

Why is the glutamate residue at position 211 of the PmrA protein evolutionarily conserved among *S. paratyphi* B (SPV) isolates that cause paratyphoid fever, which is in contrast to *S. typhimurium* and other natural isolates of *S. enterica* that contain a glycine residue at that position (Figure S1A)? It has been proposed that variation in polymyxin B resistance among enteric bacteria reflects an organism's lifestyle [44]. Therefore, the degree of antibiotic resistance conferred by the *pmrA* (E211) allele might be sufficient for *S. paratyphi* B to proliferate in its particular ecological niche. Furthermore, bacteria harboring the *pmrA* (E211) allele exhibited enhanced biofilm formation on cholesterol-coated surfaces (Figure 7), which constitutes an *in vitro* model that mimics bacterial attachment to the surfaces of human gallstones [45]. This property may promote *S. paratyphi* B's survival in the gallbladder lumen, where it establishes chronic infection [37,38,40,46]. Such fitness benefits might contribute to the maintenance of the *pmrA* (E211) allele in *S. paratyphi* B natural populations (Figure S1A). Yet, *S. typhi* and *S. paratyphi* A also persist within the gallbladder [37,47] in spite of the fact that they encode PmrA proteins identical to that of *S. typhimurium* (Figure S1A). This suggests that serovars Typhi and Paratyphi A utilize different regulatory strategies for colonization of and/or survival within the gallbladder than serovar Paratyphi B (SPV).

Bacterial biofilms are major contributors to persistent infections [48]. We determined that the heightened activity of the PmrA/PmrB system inhibits biofilm development (Figure 7), even though PmrA does not appear to affect the expression of genes encoding major components of *S. enterica* biofilms [49–51] (Figure S4C). This adds to the diversity of regulatory mechanisms that control *S. enterica* biofilm formation on cholesterol-coated surfaces [40,42,52]. Our results, together with the finding that PmrA-dependent genes are downregulated in *S. typhimurium* biofilms compared to planktonic cells [53], suggest that PmrA-regulated gene products interfere with *S. enterica* biofilms. Yet, others have detected expression of PmrA-dependent transcripts encoding LPS modification enzymes in biofilms formed by *Pseudomonas aeruginosa* [54].
genes requires the accumulation of sufficiently high levels of PmrA-P, the active form of the protein that promotes gene transcription [34]. We determined that the PmrA (E211) protein binds with lower affinity in vitro (Figure 4A). Yet, the levels of PmrA (E211)-P are enhanced by PmrD to a larger extent that those of PmrA (G211)-P (Figure 5A and 5B). This builds up PmrA (E211)-P to high enough levels in vivo (Figure 5C and 5D) thereby advancing its binding to target promoters and gene expression during growth in low Mg\(^{2+}\) and high Fe\(^{3+}\) (Figure 3A). By contrast, the amount of PmrA (E211) protein, and thus active PmrA (E211)-P, is insufficient to promote transcription when bacteria are incubated in low Mg\(^{2+}\) alone (Figure 3A and Figure 4B). Consistent with this notion, an amino acid substitution in the PmrA (E211) protein that was previously shown to render PmrA (G211)-P resistant to PmrB-promoted dephosphorylation [17] restored plegB transcription in the presence of low Mg\(^{2+}\) or under repressing conditions for the PmrA/PmrB system (Figure 5E).

We suggest that the dissimilar affinities displayed by the PmrA orthologs for target promoters distinguish the ability of bacteria to survive in their particular niches upon experiencing the presence of Fe\(^{3+}\). The decreased affinity of PmrA (E211) for target DNA (Figure 4A) might result in it promoting transcription of genes with high affinity binding sites but not those with low affinity sites. Hence, an organism harboring the PmrA (E211) protein will not necessarily promote expression of all the genes activated by an organism harboring the PmrA (G211) protein. The lower affinity of the PmrA (E211) protein for target promoters also impedes positive autoregulation of the pmrAB operon (Figure 4B), a property that governs the transient increase in PmrA activity when bacteria initially experience Fe\(^{3+}\) [12]. Consequently, the PmrA (E211) protein confers slower PmrA-dependent gene expression kinetics than the PmrA (G211) protein when bacteria first encounter Fe\(^{3+}\), even though the levels of PmrA-activated mRNAs eventually reach similar steady state levels (Figure 6A and 6B).

Our findings raise the possibility that such disparate expression dynamics in bacteria harboring the pmrA (G211) versus the pmrA (E211) allele lead to distinct cellular behaviors, as previously demonstrated in other signal transduction systems [12,56–58].

Our work provides a singular example of how different alleles of a conserved transcription factor can display disparate signal prerequisites for activating gene expression. Importantly, these differences are independent of both the signal-sensing domain of the upstream sensor protein that controls the activity of the transcription factor [59,60] and of the network architecture of these signaling systems [61,62]. Similarly, amino acid substitutions in the transcription factor CEBPB from placental mammals reorganized the location of key phosphorylation sites, changing the way the protein responds to signaling pathways compared to the non-mammalian ortholog [63]. Allelic variation in transcription factors can also affect the ability of orthologous regulatory pathways to control gene expression in response to signal availability. For instance, a single amino acid difference between the E. coli strain B and the E. coli K12 arginine repressor results in transcription of arginine biosynthesis genes in E. coli strain B even when arginine is present, whereas repression of these very genes is selected for in E. coli K12 [64]. Therefore, in addition to modifying protein-protein interactions and altering the recognition of particular DNA-binding motifs [65–68], allelic variation among transcription factors results in different interpretations of signals, leading to phenotypic diversity.

Figure 7. S. enterica strains harboring the S. paratyphi B pmrA allele display enhanced biofilm formation. (A) Ability of isogenic S. paratyphi B SARA46 strains to attach to cholesterol-coated surfaces in a tube biofilm assay. The ability of bacteria to form biofilms was determined in the SARA46 strain harboring either the pmrA (E211) (DC282) or pmrA (G211) gene (DC280), in a ΔpmrD mutant expressing the pmrA (E211) (DC287) or pmrA (G211) gene (DC285), and in a ΔpmrA mutant (DC167). Data correspond to the mean values of three independent experiments. Asterisks indicate statistically significant differences based on a two-tailed Student t-test (p<0.05). (B) Ability of isogenic S. typhimurium strains to attach to cholesterol-coated surfaces in a tube biofilm assay. The ability of bacteria to form biofilms was determined in the wild-type (14028s), in a strain harboring a 3′ FLAG-tagged S. typhimurium pmrD gene and either the pmrA (E211) (EG16279) or pmrA (G211) gene (EG13404), in a ΔpmrD mutant expressing the pmrA (E211) (DC46) or the pmrA (G211) gene (EG14088), and in a pmrA mutant (EG7139). Data correspond to the mean values of three independent experiments and error bars show standard deviation. Asterisks indicate statistically significant differences based on a two-tailed Student t-test (p<0.05).

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and commensal Escherichia coli [55]. Thus, it would appear that complex and distinct gene expression programs underlie biofilm formation in various bacterial species, which differ depending on the environmental signal(s) and the type of surface to which bacteria attach [51].

Why does the S. paratyphi B PmrA require the PmrD protein to promote expression of PmrA-activated genes even when bacteria encounter the Fe\(^{3+}\) signal that is directly detected by the PmrB sensor [10]? The ability of bacteria to express PmrA-dependent
Interplay of Response Regulator Domains Impacts Phosphorylation, Dimerization, and DNA Binding

We established that a single amino acid difference in the response regulator PmrA impacts several of its biochemical properties. First, substitution of the neutral glycine residue by the negatively charged glutamate at position 211 of the C-terminal DNA-binding domain decreased PmrA’s association with its target promoters (Figure 4A). This could be ascribed to electrostatic repulsion with DNA. Second, we determined that an amino acid substitution in the DNA-binding domain of PmrA allosterically affects biochemical activities ascribed to the N-terminal receiver domain in other response regulators [69]. Specifically, the substitution at position 211 reduced the PmrA (E211)-P protein’s propensity to dimerize (Table 1), likely contributing to its decreased DNA binding affinity since response regulator dimerization promotes binding to target DNA [29,30].

Third, PmrA (E211)-P was more resistant to PmrB-mediated dephosphorylation than PmrA (G211)-P when PmrD was present (Figure 5A and 5B). Therefore, an amino acid substitution in the C-terminal domain of PmrA renders this protein dependent on PmrD, which was previously shown to interact with the N-terminal domain of PmrA-P [17]. Finally, we demonstrated that the PmrA (E211)-P protein exhibits lower affinity for a target promoter than PmrA (G211)-P (Figure 4A) even though the levels of PmrA (E211)-P are higher than those of PmrA (G211)-P protein in vivo (Figure 5C and 5D). These results argue against the proposal that DNA binding stimulates response regulator phosphorylation [70].

Concluding Remarks

The continuous increase in genomic information has resulted in organismal behavior being deduced from the presence/absence of genes whose biochemical activity was experimentally determined in orthologs, usually in a model organism. However, our work illustrates the potential danger in adopting this approach, even for closely related organisms belonging to the same species. We established that a single amino acid difference in a natural allele of a 222 amino acid long transcription factor affected its dependence on a horizontally acquired gene product. Consequently, this changes the environments in which the regulon controlled by the transcription factor is expressed, giving rise to phenotypic differences between closely related bacteria. Our findings, and those of others [65,71], underscore that subtle amino acid differences among orthologous proteins, which cannot be readily predicted from sequence conservation and computational comparisons of related genomes, contribute to the existing phenotypic diversity within and across species.

Materials and Methods

Bacterial Strains, Plasmids, and Growth Conditions

Bacterial strains and plasmids used in this study are listed in Table S2. *S. enterica* serovar Typhimurium strains were derived from the wild-type strain 14028s. *S. enterica* serovar Paratyphi B strains were derived from SARA46 [21], unless otherwise indicated. Bacteria were grown at 37°C with aeration in Luria-Bertani (LB) broth or in N-minimal media (pH 7.7) and supplemented with 0.1% casamino acids, 38 mM glycerol, 10 μM or 10 mM MgCl₂ and 100 μM FeSO₄ [72]. When necessary, antibiotics were added at the following final concentrations: ampicillin, 50 μg/ml; chloramphenicol, 20 μg/ml; kanamycin, 50 μg/ml; and tetracycline, 10 μg/ml. Phage P22-mediated transduction of *S. enterica* strains was performed as described [73]. *E. coli* DH5α was used as a host for the preparation of plasmid DNA.

Construction of *S. typhimurium* Chromosomal Mutants

Strain EG14331, which has a MudJ transposon insertion in *phaP* and expresses the *pmrA* (E211) gene from the normal chromosomal location, was constructed by combination of the one-step inactivation method [74] and Lac⁺ selection. A PCR fragment encompassing the coding region of the *pmrA* (E211) gene was amplified using primers 2426 and 2428 (Table S3) and *S. paratyphi* B genomic DNA as template and recombined into the *pmrA* region in the EG14326 chromosome. Lac⁺ colonies were selected on N-minimal media plates (pH 5.8) with 0.1% casamino acids, 10 μM MgCl₂, and 100 μM FeSO₄ and supplemented with 1.3% lactose as the sole carbon source.

Strain EG18502, which harbors a C-terminal HA-tagged version of the *S. typhimurium* *pmrA* gene, was constructed using a modification of the one-step inactivation protocol [74]. A CmR cassette was amplified from plasmid pKD3 using primers 7994 and 7995 (Table S3). The PCR product was gel purified and electroporated into *S. typhimurium* containing plasmid pKD46 [74] selecting for chloramphenicol-resistant transformers at 37°C. The resultant strain (EG18501) harbored an HA sequence immediately upstream of the stop codon of the *pmrA* coding region followed by a CmR cassette. The CmR cassette was removed using plasmid pCP20 as described [74].

Strain DG53, which harbors a C-terminal HA-tagged version of the *S. paratyphi* B *pmrA* gene, was constructed using a modification of the one-step inactivation protocol [74]. DNA fragments that encompassed the *S. paratyphi* B *pmrA* ORF and a CmR cassette downstream of the *pmrA* gene were generated by performing two sequential PCR reactions. The *S. paratyphi* B *pmrA* ORF was amplified with primers 2426 and 11363 using 14028s genomic DNA as a template; the CmR cassette was amplified from plasmid pKD3 using primers 7995 and 11269 (Table S3). A second PCR product was gel purified and electroporated into *S. typhimurium* containing plasmid pKD46 [74] selecting for chloramphenicol-resistant transformers at 37°C. The resultant strain (DC51) harbored a HA sequence immediately upstream of the stop codon of the *pmrA* coding region followed by a CmR cassette. The CmR cassette was removed using plasmid pCP20 as described [74].

Strain DC274, which has a CmR cassette immediately downstream of the *pmrA* ORF, was constructed by a modification of the one-step inactivation protocol [74]. A PCR product encompassing the CmR cassette was generated using primers 12255 and 12437 (Table S3) and pKD3 as template. The PCR product was gel purified and electroporated into *S. typhimurium* containing plasmid pKD46 [74] selecting for chloramphenicol-resistant transformers at 37°C.

Construction of *S. paratyphi* B Chromosomal Mutants

Strain EG16275, which has a MudJ transposon insertion in *phaP* and expresses the *pmrA* (G211) gene from the normal chromosomal location, was constructed by combination of the one-step inactivation method [74] and Lac⁺ selection. A PCR fragment encompassing the coding region of the *pmrA* (G211) gene was amplified using primers 2426 and 2428 (Table S3) and *S. typhimurium* 14028s genomic DNA as template and recombined into the *pmrA* region in the DC306 chromosome. Lac⁺ colonies were selected on N-minimal media plates (pH 5.8) with 0.1% casamino acids, 10 μM MgCl₂, and 100 μM FeSO₄ and supplemented with 1.3% lactose as the sole carbon source.

To construct an *S. paratyphi* B derivative of strain SARA46 harboring the *pmrA* (G211) ORF from the normal chromosomal location (DC280), we used a modification of the one-step...
Ancestral Gene Requires Horizontally Acquired Gene

The DNA fragments were gel-purified with QIAquick columns (Qiagen), and 150 ng of DNA was labeled using T4 polynucleotide kinase (NEB) and (γ–32P) ATP at 37°C. Unincorporated (γ–32P) ATP was removed using G-50 microcolumns (Amersham). 10 μM His-tagged PmrA-H6 was incubated for 60 min at room temperature with 5 μM H6-PmrB, T156R (a PmrB mutant that was shown to possess autokinase and phosphotransferase activity but lacks phosphatase activity [17]) in the presence or absence of 1 mM ATP to generate phosphorylated or unphosphorylated PmrA, respectively. 106 cpm of labeled probe, 200 ng poly (dl-dC) (Amersham) and 0, 100, 200 or 300 pmol of phosphorylated or unphosphorylated His-tagged PmrA (E211) or PmrA (G211) proteins were mixed with binding buffer (20 mM Hapes (pH 8.0), 10 mM KCl, 2 mM MgCl2, 0.1 mM EDTA, 0.1 mM DTT, 50 μg/ml BSA, and 10% glycerol) in a final volume of 30 μl and incubated at room temperature for 20 min. Samples were run on 4–20% TBE gels (Invitrogen), dried and then autoradiographed using a BAS-5000 imaging system and phosphor imaging plate (Fuji Film).

Analytical Gel Filtration

180 μl PmrA (50 μM) was phosphorylated using pGEX-PmrB, T156R beads as described [17]. Fast performance liquid chromatography (FPLC) experiments were conducted with an AKTA FPLC system (GE Healthcare) at 4°C. 100 μl of phosphorylated PmrA (G211) or PmrA (E211) was individually applied to a Superdex 200 10/300 GL column (GE Healthcare) that had been pre-equilibrated with 1× TBS/1 mM MgCl2/1 mM DTT/10% glycerol. Proteins were then eluted in the same buffer at a flow rate of 0.5 ml min−1. Absorbance was monitored at 280 nm and fractions were analyzed by SDS-PAGE. The column was calibrated with a mixture of protein molecular mass standards (GE Healthcare), containing aprotinin (6.5 kDa), ribonuclease A (13.7 kDa), carbonic anhydrase (29 kDa), ovalbumin (44 kDa) and conalbumin (75 kDa), which was applied to the column under similar conditions.

Western Blotting Analyses to Determine Total PmrA Levels

Bacteria from overnight cultures grown in N-minimal medium at pH 7.7 with 10 mM MgCl2 were washed twice with N-minimal medium containing no Mg2+ and added into fresh N-minimal medium at pH 7.7 with 10 μM MgCl2 and 100 μM FeSO4 with 1:50 dilution. The bacterial cultures were grown to OD600 0.4 in a shaking water bath at 37°C before harvesting the cells at 4°C and resuspending the cell pellet with 1 ml ice-cold 20 mM Tris, pH 7.0 (Ambion). The samples were then added to a 2 ml Lysing Matrix Tube (MP Biomedicals) and lysed three times for 40 s at 6 m/s using the FastPrep-24 instrument (MP Biomedicals). The tubes were spun down to remove the Lysing Matrix beads and 100 μl cell lysate was added to 100 μl 2× Laemeli buffer (Biorad) and boiled for 3 min. Equivalent amounts of each sample (normalized to OD600) were run on a 4–12% Bis-Tris gel (Invitrogen) in 1× MES buffer (Invitrogen), transferred to a PVDF membrane, and analyzed by Western blotting with an anti-HA monoclonal antibody (Sigma) or an anti-RpoB antibody (Neoclone). Western blots were developed using anti-mouse IgG horseradish peroxidase-linked antibodies (GE Healthcare) and Supersignal west femto (Pierce).
Phos-Tag Analyses of Phosphorylated Response Regulator In Vivo

To determine the levels of PmrA-P in vivo, samples were prepared as described in the previous section. 100 μl of cell lysate was added to 100 μl chilled 2× Laemeli buffer (Biorad) (to detect phosphorylated PmrA) or to 100 μl 2× Laemeli buffer (Biorad) and boiled (to detect total PmrA). Samples were analyzed on a Phos-Tag gel as described [36]. Briefly, Phos-Tag acrylamide gels containing 10% (w/v) 19:1 acrylamide/Bis solution, 330 mM Tris-HCl, pH 8.8, 75 μM Phos-tag and 150 μM MnCl2 were prepared. Stacking gels contained 4% (w/v) 19:1 acrylamide/Bis solution and 130 mM Tris, pH 6.8. Equivalent amounts of each boiled or unboiled sample (normalized to OD600) were loaded onto the gel and run for 2 h at 4°C under constant voltage (150 V) using chilled running buffer containing 1% (v/v) SDS, 25 mM Trizma base and 192 mM glycine and 25 mM Trizma base, with 10 min with chilled transfer buffer containing 20% (v/v) methanol, 192 mM glycine and 25 mM Trizma base, with 1 mM EDTA to remove Mn2⁺ from the gel. Gels were incubated for an additional 10 min in transfer buffer without EDTA. Transfer to nitrocellulose membranes was performed using the Bio-Rad wet transfer apparatus under constant voltage (100 V) for 60 min. Western blotting was carried out as described in the previous section.

RNA Isolation and Real-Time PCR to Determine Transcript Levels

Bacteria from overnight cultures grown in N-minimal medium at pH 7.7 with 10 mM MgCl₂ were washed twice with N-minimal medium containing no Mg²⁺ and added into fresh N-minimal medium at pH 7.7 with 10 mM MgCl₂ with 1:50 dilution. The bacterial cultures were grown to OD₆₀₀ 0.4 in a shaking water bath at 37°C, spun down and resuspended in 100 μl N-minimal medium at pH 7.7 with 1 mM MgCl₂ and then added into N-minimal medium at pH 7.7 containing no Mg²⁺ and 100 μM FeSO₄. 0.5 μl aliquots of cells were removed at the indicated time points, mixed with RNAProtect Bacteria Reagent (Qiagen) for stabilization of RNA, and total RNA was isolated using RNeasy Kit (Qiagen) with on-column DNase treatment. cDNA was synthesized using TaqMan (Applied Biosystems) and random hexamers. Quantification of transcripts was carried out by real-time PCR using SYBR Green PCR Master Mix (Applied Biosystems) in an ABI 7500 Sequence Detection System (Applied Biosystems). The following primers (Table S3) were used to analyze transcript levels: rs (3023, 3024), phgP (6522, 6523), pmrC (3007, 3008) and pmrD (4491, 4492). The relative amount of cDNA was determined using a standard curve obtained from PCR with serially diluted genomic DNA, and results were normalized to the levels of 16S ribosomal RNA. Data correspond to the mean values of at least three independent experiments.

Growth on Agarose Plates Containing Polymyxin B

The ability of bacteria to grow in the presence of polymyxin B was determined as follows. Bacteria were streaked onto N-minimal media plates, pH 5.8, containing 1% agarose, 38 mM glycerol, 10 μM MgCl₂ and 2.5 kg/ml polymyxin B with or without 100 μM FeSO₄ and incubated at 37°C overnight before examination of the plates for bacterial growth.

Tube Biofilm Assay

The ability of S. enterica strains to form biofilms on cholesterol-coated microcentrifuge tubes was determined as described [42]. Bacteria from overnight cultures grown in LB were added into fresh LB medium with 1:50 dilution and grown at 37°C to OD₆₀₀ 0.5. 100 μl of cells were added to cholesterol-coated Eppendorf tubes (Fisher Scientific) and incubated on a Nutator shaker at room temperature for 6 days. Each day, spent medium was removed, and the tubes were washed twice with LB medium before fresh LB medium was added. On day 6, after incubating tubes at 60°C for 1 hr to fix the attached bacteria, 200 μl of 0.1% crystal violet was added to stain cells for 5 min at room temperature. The tubes were then washed with 1 ml 1× PBS until the solution ran clear, and 200 μl of 33% acetic acid was added to extract the crystal violet dye, which was quantified at OD₅₇₀ using a Victor 3 1420 Multilabel counter (Perkin Elmer). Data correspond to the mean values of three independent experiments performed in duplicate.

Sequencing of the pmrA Gene from S. enterica Natural Isolates

The pmrA gene was amplified with high fidelity AccuPrime Tag DNA polymerase (Invitrogen) by using primers 2876 and 2877 (Table S3), which are upstream and downstream the pmrA ORF respectively. PCR products were purified with the QIAquick PCR purification kit (Qiagen). Sequencing reactions were initiated by using primers 2878, 2879 or 2880, performed using Big Dye 3.1 (Applied Biosystems) and analyzed on a 310 Genetic Analyzer (Perkin-Elmer). DNA sequences were translated by using Editseq 3.92 (DNASTAR). The sequences of these pmrA genes as well as those previously determined in [18] were aligned using ClustalX 77. The pmrA gene sequences have been deposited at GenBank under the accession numbers listed in Table S4.

Supporting Information

Figure S1 The S. paratyphi B PmrA differs from that of other S. enterica strains at position 211. (A) Alignment of the deduced amino acid sequences of the pmrA gene from 33 S. enterica isolates. A red arrow indicates the amino acid residue at position 211. (B) Homology model of the PmrA DNA-binding domain in complex with DNA, which was predicted using a homology-modeling program (Phyre) [78] and based on the crystal structure of the E. coli PhoB (purple) complexed to DNA [29]. The amino acid at position 211 is located in a flexible loop predicted to be in close contact with negatively charged DNA. (TIF)

Figure S2 The PmrA (E211) protein has a lower propensity to form dimers in solution than the PmrA (G211) protein. Gel filtration chromatogram of phosphorylated PmrA (G211) or PmrA (E211) proteins that were individually applied to a Superdex 200 10/300 GL column. Absorbance was monitored at 280 nm. (TIF)

Figure S3 The PmrA (G211) and PmrA (E211) proteins are similarly phosphorylated by PmrB, and their phosphorylated forms dephosphorylated by PmrBc. (A) Levels of PmrBc-P and PmrA-P following incubation of PmrBc (5 μM) in the presence of 2.5 μM PmrBc-P and their phosphorylated forms dephosphorylated by PmrBc. (B) Levels of PmrBc-P and PmrA-P following incubation of PmrA (G211)-P or PmrA (E211)-P (2.5 μM) with PmrBc (5 μM) in the presence of 2.5 μM (C) or 1.25 μM (D) PmrD for the indicated times. (TIF)
**Figure S4** PmrA does not regulate the expression of hcd or csgA in *Salmonella typhimurium*. (A–B) Growth of *S. paratyphi B* (A) or *S. typhimurium* (B) strains used for biofilm analyses in Figure 7. Bacteria were grown in 18 µL LB in a 96-well microtiter plate and OD600 was determined using a Victor + 1420 Multilabel counter (Perkin Elmer). (C) mRNA levels of the hcd and csgA genes from wild-type (14028s) or *pmrA* (EG7139) *S. typhimurium* strains determined by reverse-transcription-qPCR analysis. Bacteria were grown in N-minimal medium containing 10 µM Mg2+ and 100 µM Fe3+ and harvested to prepare RNA. Expression levels were normalized to those of the 16S ribosomal RNA gene. Data correspond to at least two independent experiments and error bars show standard deviation. (TIF)

**Table S1** Susceptibility of *S. typhimurium* strains to polymyxin B. (DOC)

**Table S2** Bacterial strains and plasmids used in this study. (DOC)

**Table S3** Primers used in this study. (DOC)

**Table S4** GenBank accession numbers for the *pmrA* genes from *S. enterica* natural isolates. (DOC)

**Author Contributions**
Conceived and designed the experiments: HDC MWJ EAG. Performed the experiments: HDC MWJ. Analyzed the data: HDC MWJ EAG. Contributed reagents/materials/analysis tools: HDC MWJ EAG. Wrote the paper: HDC EAG.
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