The lipopolysaccharide modification regulator PmrA limits *Salmonella* virulence by repressing the type three-secretion system Spi/Ssa

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The regulatory protein PmrA controls expression of lipopolysaccharide (LPS) modification genes in *Salmonella enterica* serovar Typhimurium, the etiologic agent of human gastroenteritis and murine typhoid fever. PmrA-dependent LPS modifications confer resistance to serum, Fe^{3+}, and several antimicrobial peptides, suggesting that the *pmrA* gene is required for *Salmonella* virulence. We now report that, surprisingly, a *pmrA* null mutant is actually hypervirulent when inoculated i.p. into C3H/HeN mice. We establish that the PmrA protein binds to the promoter and represses transcription of *ssrB*, a virulence regulatory gene required for expression of the Spi/Ssa type three-secretion system inside macrophages. The *pmrA* mutant displayed heightened expression of SsrB-dependent genes and faster Spi/Ssa-dependent macrophage killing than wild-type *Salmonella*. A mutation in the *ssrB* promoter that abolished repression by the PmrA protein rendered *Salmonella* as hypervirulent as the *pmrA* null mutant. The antivirulence function of the PmrA protein may limit the acute phase of *Salmonella* infection, thereby enhancing pathogen persistence in host tissues.

Salmonella pathogenicity island 2 | cytotoxicity

Pathogens possess virulence genes that enable them to invade nonphagocytic cells, to survive within host phagocytic cells, to resist antimicrobial agents and the immune response presented by the host, and/or to kill host cells (1, 2). Paradoxically, pathogens can also have genes that actually reduce their virulence. That is, inactivation of certain genes can decrease the median lethal dose (i.e., LD_{50}) and/or enhance pathogen growth in host tissues (3, 4). However, the function(s) of such antivirulence factors and the contribution(s) they make to a pathogen's lifestyle remain largely unknown. Here, we establish that an ancestral *Salmonella* regulator is an antivirulence protein that represses expression of a horizontally acquired locus necessary for survival inside phagocytic cells.

The ancestral two-component system PmrA/PmrB is the major regulator of lipopolysaccharide (LPS) modification genes in *Salmonella enterica* serovar Typhimurium, the etiologic agent of human gastroenteritis and murine typhoid fever. The regulator PmrA is activated when its cognate sensor PmrB detects mildly acidic pH (5) or the presence of Fe^{3+} or Al^{3+} (6), and also when *Salmonella* experiences low Mg^{2+}, which is detected by the sensor PhoQ (7). Together with the regulator PhoP, PhoQ forms a two-component system that governs virulence functions in *Salmonella* (8). The PhoP-activated PmrD protein (9) activates the PmrA protein at the posttranslational level (10). The PmrA/PmrB system is active when *Salmonella* is inside macrophages and during infection of mice (11). PmrA-dependent modifications of the LPS increase bacterial resistance to the antibiotic polymyxin B (6) and host-derived antimicrobial peptides (6, 12), to serum complement (13), and to high concentrations of Fe^{3+} (14); and they reduce the proinflammatory properties of the LPS (15). However, inactivation of the *pmrA* gene was reported to have minimal effects on *Salmonella*'s ability to cause a lethal infection in BALB/c mice when inoculated orally and no effect when inoculated i.p. (16).

Type-three secretion systems (T3SSs) are specialized molecular machines that certain Gram-negative bacteria use to deliver effector proteins into eukaryotic cells (17). These effectors typically manipulate host cell functions, thereby mediating bacterial entry to and/or survival within host tissues (17, 18). *Salmonella* encodes a T3SS—termed Spi/Ssa—on the *Salmonella* pathogenicity island (SPI)-2 that is unique in that it translocates effectors into and across the phagosomal membrane (19) and is necessary for bacterial survival within macrophages (20). Expression of *spi/ssa* genes depends on the SPI-2–encoded SsrB/SpiR two-component system (21), which consists of the sensor SpiR (also known as SsrA) and the DNA binding regulator SsrB (22). An *ssrB* null mutant is highly attenuated for virulence in mice inoculated by the i.p. route (23), consistent with the notion that the SsrB-dependent Spi/Ssa system is required for pathogen growth in deep tissues.

We now report that inactivation of the *pmrA* gene renders *Salmonella* hypervirulent in C3H/HeN mice. We establish that the PmrA protein down-regulates expression of *spi/ssa* genes by repressing transcription from the *ssrB* promoter. The hypervirulence of the *pmrA* null mutant can be recapitulated by rendering the *ssrB* promoter resistant to repression by PmrA. Our findings suggest that bacterial pathogens use antivirulence factors for optimal fitness in host tissues.

Results

Inactivation of the *pmrA* Gene Renders *Salmonella* Hypervirulent. We reexamined the virulence behavior of a *pmrA* null mutant in C3H/HeN mice because the PmrA/PmrB system is activated in the presence of Fe^{3+} (6), and C3H/HeN mice harbor a functional allele of the *Nramp1* (also known as *Slc11a1*) gene, which encodes a protein that mediates iron transport across the phagosomal membrane (24). By contrast, BALB/c mice are $Nramp1^{-/-}$.

Unexpectedly, the *pmrA* mutant was more virulent than the isogenic wild-type strain following i.p. inoculation (Fig. 1*A*). The hypervirulence phenotype was manifested by an earlier time to death than animals infected with wild-type *Salmonella* (Fig. 1*A*). This result implied that the PmrA/PmrB system promotes expression of a gene(s) that antagonizes *Salmonella* virulence and/or represses expression of a virulence determinant(s).

PmrA Represses Transcription of the SPI-2 *ssaG* Gene. Given that the *pmrA* hypervirulence phenotype was observed in $Nramp1^{+/+}$ mice,

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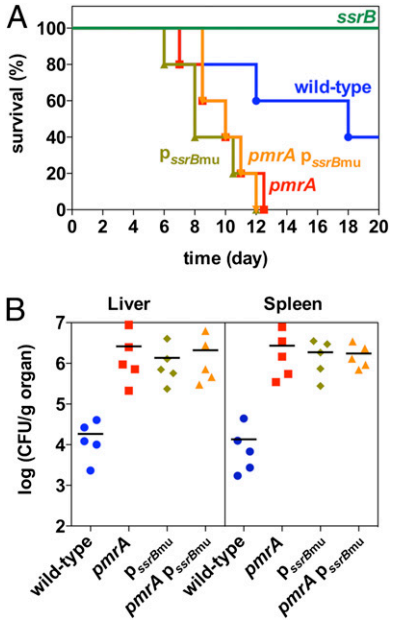

Fig. 1. Lack of PmrA or its binding site in the *ssrB* promoter increases *Salmonella* virulence in mice. C3H/HeN mice were inoculated i.p. with ~10^3 cfu of wild-type, *pmrA*, *ssrB*, *ssrB* promoter mutant (p_{ssrBmu}), and *pmrA ssrB* promoter double mutant (*pmrA* p_{ssrBmu}) *Salmonella*. Survival of mice was monitored daily (*A*). At 5 d after infection, the number of bacteria in the liver and spleen were determined (*B*).

we wondered whether the PmrA/PmrB system might regulate expression of SPI-2 genes. Genes located in the SPI-2 pathogenicity island are up-regulated in the spleens of *Nramp1*$^{+/+}$ mice relative to those of congenic *Nramp1*$^{-/-}$ animals (25). Thus, we investigated expression of the SsrB-activated *ssaG* gene, which is the first gene of a large operon (from *ssaG* to *ssaU*) and encodes a component of the Spi/Ssa T3SS (22). We used a set of isogenic strains harboring a plasmid containing the *ssaG* promoter region fused to a promoterless *gfp* variant that specifies an unstable GFP (i.e., GFPaav) (26).

There was more fluorescence in the *pmrA* mutant than in the wild-type strain when bacteria were grown in minimal media at pH 4.6 with high Mg^{2+} (Fig. 2*A*), a condition reported to activate SPI-2 genes (27). This behavior was also true when bacteria were grown in minimal media at pH 4.6 with low Mg^{2+}, although there was less of a difference between the two strains. By contrast, the expression was similar in wild-type and *pmrA Salmonella* in media containing neutral pH regardless of the Mg^{2+} concentration (Fig. 2*A*), which is a noninducing condition for SPI-2 genes (27, 28). Control experiments demonstrated that there was no detectable fluorescence in an *ssrB* mutant strain regardless of growth conditions (Fig. 2*A*), as anticipated by the established requirement for *ssrB* in *ssaG* transcription (21). Moreover, the elevated *ssaG* expression displayed by the *pmrA* mutant when grown in minimal media at pH 4.6 with high Mg^{2+} was restored to wild-type levels by a plasmid expressing the wild-type *pmrA* gene from a heterologous promoter but not by the plasmid vector (Fig. 2*B*).

Because Fe^{3+} is a prime activator of the PmrA/PmrB system (6), we reasoned that it should be possible to modulate expression of SPI-2 genes in wild-type *Salmonella* by varying the concentrations of Fe^{3+} in the growth media. Indeed, *ssaG* expression was greatly reduced as the Fe^{3+} concentration increased (Fig. 3*A*). This effect was mediated by PmrA because *ssaG* expression was no longer responsive to the Fe^{3+} concentration in a *pmrA* mutant background (Fig. 3*B*).

Next, we examined whether the enhanced *ssaG* expression resulting from inactivation of the *pmrA* gene was also observed when *Salmonella* was inside macrophages. This test was important because expression of SPI-2 genes greatly increases upon *Salmonella* phagocytosis (29, 30), and the PmrA/PmrB system is active inside macrophages (11). We found *ssaG* expression to be ~30-fold higher at 4 h after internalization by J774A.1 macrophages relative to expression displayed by bacteria grown in LB (Fig. 2*C*), which is in agreement with previous results (22, 29). Inactivation of the *pmrA* gene further increased *ssaG* expression (Fig. 2*C*), as observed during growth in minimal media (Fig. 2*A*). We note that the increased *ssaG* expression does not depend on the presence of a functional Nramp1 protein because J774A.1 macrophages originated from an *Nramp1*$^{-/-}$ animal (31). Taken together, these results indicate that PmrA functions as a negative regulator of *ssaG* and potentially of other SPI-2 genes.

PmrA Represses *ssaG* Transcription Indirectly, by Hindering Expression of the *ssaG* Activator SsrB. In principle, PmrA could repress *ssaG* transcription directly, by binding to the *ssaG* promoter, or, indirectly, by hindering transcription of an *ssaG* activator(s) or by furthering expression of an *ssaG* repressor(s). Sequences resembling a PmrA box (i.e., a direct repeat of the sequence CTTAAT separated by 5 nt present in PmrA-activated promoters; refs. 32–34) were not found in the *ssaG* promoter region. By contrast, we identified a perfect match to the consensus PmrA box downstream of the mapped transcriptional start site of the *ssrB* gene (Fig. 4*A*), which encodes an activator necessary for *ssaG* transcription (22). This analysis suggested that PmrA might bind to the *ssrB* promoter hampering *ssrB* transcription.

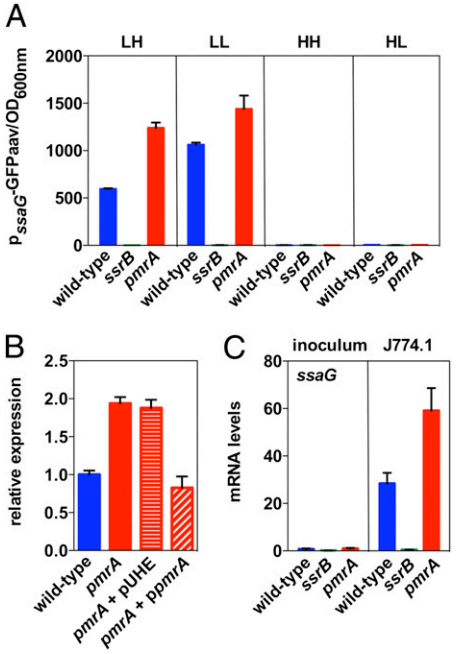

Fig. 2. PmrA decreases expression of the SPI-2 *ssaG* gene. (*A*) Fluorescence from a *ssaG-gfp* transcriptional fusion was determined in wild-type, *pmrA*, and *ssrB Salmonella* grown in *N*-minimal medium at pH 4.6 with 1 mM Mg^{2+} (LH) or 10 μM (LL), or pH 7.7 with 1 mM Mg^{2+} (HH) or 10 μM (HL) for 8 h. (*B*) Relative expression from a chromosomal *ssaG-lacZ* transcriptional fusion was determined in wild-type, *pmrA*, *pmrA* harboring the plasmid vector (pUHE21-2*lacI*^{q}), and *pmrA* harboring a plasmid expressing *pmrA* from a *lac* promoter derivative following growth in *N*-minimal medium at pH 4.6 with 1 mM Mg^{2+} (LH). Values are normalized relative to those displayed by the wild-type strain. (*C*) mRNA levels of *ssaG* produced by wild-type, *pmrA*, and *ssrB Salmonella* grown overnight in LB media (inoculum) and 4 h after infection of J774A.1 macrophages.

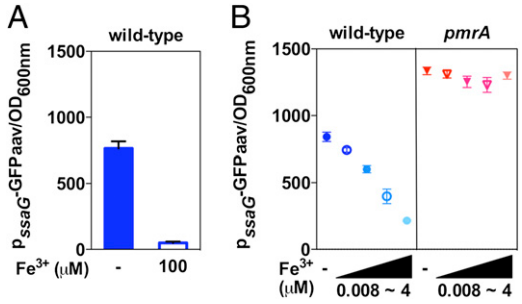

Fig. 3. Iron regulates expression of the SPI-2 *ssaG* gene in a PmrA-dependent manner. GFP activity from the *ssaG-gfp* transcriptional fusion was determined in wild-type *Salmonella* grown in *N*-minimal medium at pH 4.6 with 1 mM Mg^{2+} for 8 h with or without 100 μM Fe^{3+} (*A*); or in wild-type and *pmrA Salmonella* grown in *N*-minimal medium at pH 4.6 with 1 mM Mg^{2+} with various concentrations of iron (0.008, 0.04, 0.2, and 4 μM Fe^{3+}) or without iron (*B*).

The PmrA protein appears to repress *ssrB* directly because: First, the purified PmrA protein protected the −45 to −16 region from ATG of the *ssrB* gene in vitro (Fig. 4*B*), which contains the predicted PmrA binding site in the *ssrB* promoter (Fig. 4*A*). Second, chromatin immunoprecipitation experiments revealed that an HA-tagged PmrA expressed from its normal promoter and chromosomal location bound to the *ssrB* promoter in vivo but not to the *ssaG* promoter (Fig. 4*C*). Importantly, the enrichment in *ssrB* promoter DNA was comparable to that of other known PmrA-regulated genes, including *pmrC* (Fig. 4*C*). Third, substitution of four conserved nucleotides in the predicted PmrA box of the *ssrB* promoter (Fig. 4*A*) reduced PmrA binding to *ssrB* promoter DNA in vitro (Fig. 5*A*) and increased *ssaG* expression in vivo (Fig. 5*B*). Notably, the *ssaG* expression levels exhibited by the strain with the mutation of the PmrA-binding site in the *ssrB* promoter were similar to those displayed by the *pmrA* null mutant (Fig. 5*B*). Furthermore, nucleotide substitution of the PmrA-binding site in the *ssrB* promoter increased expression of both *ssrB* and *ssaG* within the J774A.1 macrophage-like cell line (Fig. 5*C*) and in bone marrow-derived macrophages prepared from C3H/HeN mice (Fig. S1). We note that the expression levels of the *ssrB* and *ssaG* genes in the *ssrB* promoter mutant were comparable to those in the *pmrA* mutant (Fig. 5*C* and Fig. S1) and that the *ssrB* promoter mutation had no effect on the expression of the PmrA-activated *pmrC* gene (Fig. 5C).

PmrA's action at the *ssrB* promoter is not simply to antagonize activation of *ssrB* transcription by the SsrB protein because the levels of β-galactosidase activity originating from a plasmid-linked *ssrB-lacZ* transcriptional fusion were higher in the *pmrA* mutant than in the wild-type strain and the *ssrB* mutant (Fig. 4*D*) even though this fusion was driven by the heterologous *lac* promoter derivative $p_{lac1–6}$ (35). The only *Salmonella* sequences present in the plasmid-linked *ssrB-lacZ* fusion were those located between the *ssrB* transcription start site and the *ssrB* start codon, which include the PmrA binding site. By contrast, inactivation of *pmrA* had no effect on the expression of a similar transcriptional fusion that included the leader region of the *spiR* gene instead of that of *ssrB* (Fig. 4*D*). In addition, similarly low fluorescence levels were displayed by isogenic *ssrB* and *ssrB pmrA* strains harboring a plasmid carrying a fusion between the *ssaG* promoter and a promoterless *gfp* (aav) gene (Fig. 4*E*). And inactivation of *pmrA* in the *ssrB* promoter mutant background did not alter the levels of *ssaG* expression (Fig. 5*B*). These results demonstrated that SsrB is necessary to activate the *ssrB* promoter even in the absence of PmrA repression. Finally, PmrA's role as a repressor of *ssrB* transcription is not simply to prevent the PhoP and OmpR proteins from activating *ssrB* transcription (21, 36) because there were similarly low levels of *ssaG* expression in *phoP*, *phoP pmrA*, *ompR*, and *ompR pmrA* mutants (Fig. S2). Taken together, the results presented in this section indicate that PmrA's effects on *ssaG* expression result from direct repression of *ssrB* transcription.

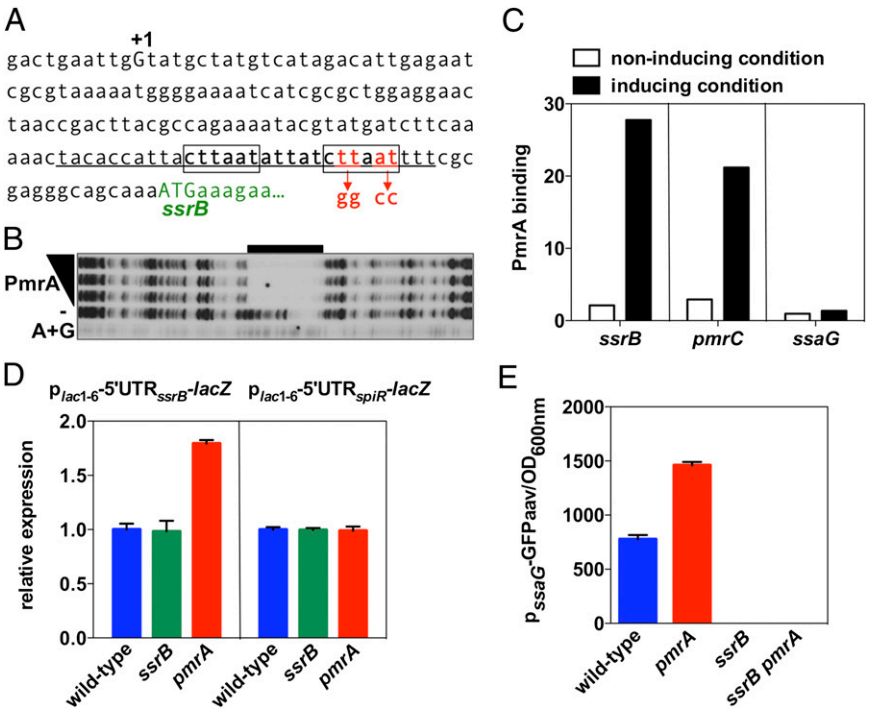

Fig. 4. PmrA binds to the promoter of the *ssrB* gene. (*A*) DNA sequence of the promoter region of the *ssrB* gene. A +1 corresponds to the reported transcriptional start site, the boxed sequences indicate those matching the PmrA-binding consensus sequence, and the underlined sequence indicates the region protected by the PmrA protein in the *ssrB* promoter shown in *B*. Mutated nucleotides at the PmrA binding site in the *ssrB* promoter mutant strain are indicated beneath the sequence. (*B*) DNase I footprinting analysis of the *ssrB* promoter using purified PmrA-His6 protein (0, 100, 200, and 300 pmoles) was performed as described in *Materials and Methods*. Lane A+G corresponds to dideoxy chain-termination sequences for the *ssrB* promoter DNA. The black bar indicates the region protected by the PmrA-His6 protein. (*C*) In vivo occupancy of the *ssrB*, *pmrC*, and *ssaG* promoter regions by the PmrA-HA protein when *Salmonella* strains expressing PmrA-HA from its original chromosomal location and promoter were switched from noninducing conditions (*N*-minimal medium at pH 7.7 with 1 mM Mg^{2+}) into noninducing or inducing conditions (*N*-minimal medium at pH 7.7 with 10 μM Mg^{2+} and 100 μM Fe^{3+}). (*D*) β-galactosidase activity expressed by wild-type, *ssrB*, and *pmrA Salmonella* harboring a plasmid with a $p_{lac1–6}$-driven transcriptional fusion of the 5′ untranslated region (5′UTR) of *ssrB* or *spiR* to a promoterless *lacZ* gene. Bacteria were grown in *N*-minimal medium at pH 7.7 with 10 μM Mg^{2+}. Values are normalized by that of the wild-type strain. (*E*) Fluorescence originating from a plasmid-linked *ssaG-gfp* transcriptional fusion was determined in wild-type, *pmrA*, *ssrB*, and *ssrB pmrA Salmonella* grown in *N*-minimal medium at pH 4.6 with 1 mM Mg^{2+} (LH) for 8 h.

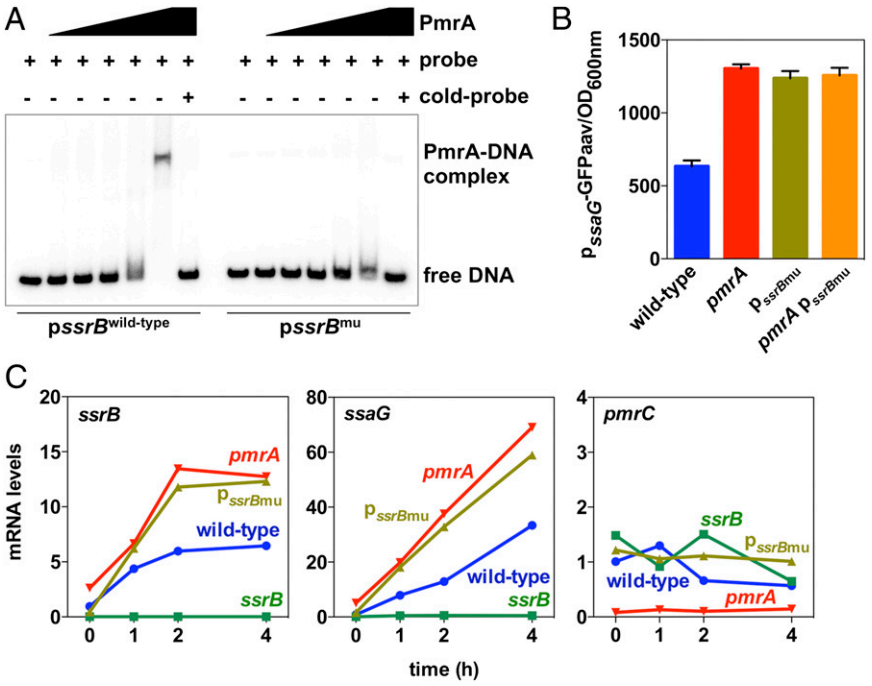

Fig. 5. Binding of PmrA to the *ssrB* promoter is required for PmrA-mediated repression of the SPI-2 *ssaG* gene. (*A*) Electrophoretic mobility shift assays of wild-type or mutated *ssrB* promoter region DNA fragments using purified PmrA-His6 protein (0, 75, 150, 300, 600, and 1,200 pmoles) were carried out as described in *Materials and Methods*. The shifted bands can be competed out by the corresponding cold probe (unlabeled DNA) in the presence of the maximum amount of PmrA-His6 protein used. (*B*) Fluorescence from a plasmid-linked *ssaG-gfp* transcriptional fusion was determined in wild-type, *pmrA* mutant, the *ssrB* promoter mutant (p_{ssrBmu}), and *pmrA ssrB* promoter double mutant (*pmrA* p_{ssrBmu}) *Salmonella* grown in *N*-minimal medium at pH 4.6 with 1 mM Mg^{2+}. (*C*) J774A.1 macrophages were infected with wild-type, *pmrA* mutant, *ssrB* mutant, and the *ssrB* promoter mutant (p_{ssrBmu}) strains. mRNA levels of the *ssrB*, *ssaG*, and *pmrC* genes were determined at the indicated times after infection.

PmrA Delays *Salmonella*-Induced Macrophage Death. To examine the physiological consequences of PmrA repression of *ssrB* transcription, we examined the kinetics of SPI-2–promoted macrophage death, which is critical for *Salmonella* virulence (37). We infected macrophages with various *Salmonella* strains and evaluated cell death by measuring lactate dehydrogenase released from dead or damaged cells. Cell death took place earlier in macrophages infected with the *pmrA* mutant than with the wild-type strain (Fig. 6). The faster cell death kinetics was also displayed by macrophages infected with the *ssrB* promoter mutant (Fig. 6). Control experiments showed reduced cell death in macrophages infected with the *ssrB* mutant (Fig. 6), in agreement with previous reports (38). These data indicate that the PmrA protein delays macrophage death induced by wild-type *Salmonella*.

PmrA Reduces *Salmonella* Virulence by Repressing *ssrB* Transcription. The results presented above suggested that the hypervirulence phenotype of the *pmrA* null mutant might be due to PmrA repression of *ssrB* transcription. If this notion were the case, one would expect: First, that the *ssrB* promoter mutant that is refractory to repression by the PmrA protein would display the same hypervirulence phenotype as the *pmrA* null mutant. And second, that a *pmrA ssrB* promoter double mutant would behave just like *pmrA* or *ssrB* promoter single mutants.

We determined that C3H/HeN mice infected with the *pmrA* mutant or the *ssrB* promoter mutant died earlier than those infected with the wild-type strain (Fig. 1*A*). This behavior was also true for the *pmrA ssrB* promoter double mutant (Fig. 1*A*). As an independent assay of virulence in vivo, we determined that 5 d after infection the number of bacteria in the liver and spleen of animals infected with the *pmrA* mutant, the *ssrB* promoter mutant, and the *pmrA ssrB* promoter double mutant were similar and were ~100-fold higher than those infected with the wild-type strain (Fig. 1*B*). These results support the notion that PmrA dampens virulence primarily by repressing transcription of the major regulator of SPI-2 genes.

Discussion

The genes present in a bacterial pathogen can be divided into three distinct groups based on their virulence roles. The best understood group is constituted by the virulence genes, which are genes that upon inactivation reduce the pathogenicity of a pathogen. A second group includes those genes that do not appear to affect virulence because they do not alter pathogenicity when mutated. The third group encompasses the antivirulence genes. These genes are of particular interest because, puzzlingly, their inactivation renders a pathogen hypervirulent.

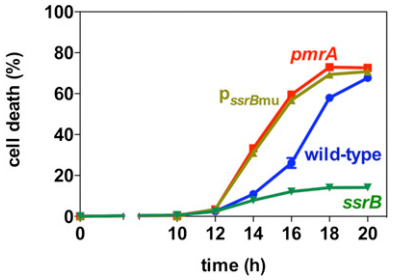

Fig. 6. PmrA delays SPI-2–promoted macrophage death. J774A.1 macrophages were infected with wild-type, *pmrA*, *ssrB*, and the *ssrB* promoter mutant (p_{ssrBmu}) *Salmonella*. The percentage of macrophage death was determined by detecting the amount of lactate dehydrogenase released into the supernatants at the indicated times after infection.

We have now determined that the regulatory protein PmrA is an antivirulence factor because inactivation of the *pmrA* gene exacerbated *Salmonella* virulence in C3H/HeN mice (Fig. 1*A*). This result was unexpected because *pmrA* null mutants were known to be more susceptible to serum (13) and to polymyxin B (6), an antibiotic believed to mimic the action of mammalian cationic antimicrobial peptides (12), and also because a *pmrA* mutant was mildly attenuated when inoculated into BALB/c mice (16), which are exquisitely susceptible to *Salmonella* infection ($LD_{50} < 10$). Unlike BALB/c mice, C3H/HeN mice carry functional Nramp1, which localizes to the phagosomal membrane (39) and hinders growth of several intracellular pathogens that remain within a phagosome (40).

How PmrA Decreases *Salmonella* Virulence. The antivirulence function of PmrA can be ascribed to its role as transcriptional repressor of the regulatory gene *ssrB*, which is essential for expression of genes required for proliferation inside macrophages and systemic infection in mice (23, 41). This antivirulence function is supported by the following: First, the hypervirulence phenotype of the *pmrA* null mutant could be phenocopied by mutating the PmrA binding site in the *ssrB* promoter (Fig. 1*A*). Second, a *pmrA ssrB* promoter double mutant exhibited the same hypervirulence phenotype of either single mutant (Fig. 1*A*). And third, SPI-2–dependent macrophage killing was accelerated in both the *pmrA* mutant and the *ssrB* promoter mutant (Fig. 6).

The SsrB protein is necessary for transcription of the genes specifying the T3SS encoded in the SPI-2 pathogenicity island as well as its secreted effectors (19, 20). Inactivation of the SPI-2 T3SS enhances *Salmonella*'s growth inside phagocytes during infection of an animal (42). These results suggest that PmrA repression of *ssrB* might enable *Salmonella* to control bacterial proliferation within a cell and to favor dissemination to neighboring cells. Alternatively or in addition, PmrA may enable proper repression of SPI-2 in the intestine, which is a PmrA-inducing environment (43, 44).

What is the identity of the SsrB-regulated gene(s) that must be tightly controlled for a normal course of *Salmonella* infection? We considered the possibility of the SsrB-repressed *sciS* being such a gene because its inactivation was reported to increase *Salmonella* virulence in BALB/c mice infected orally (4). However, we established that an *sciS* mutant is attenuated for virulence in C3H/HeN mice inoculated i.p. (Fig. S3), which is in contrast to the hypervirulence phenotype exhibited by the *pmrA* null mutant in the same mouse strain and route of inoculation (Fig. 1*A*).

Repression of *ssrB*, the master regulator of SPI-2 genes, constitutes a unique and unexpected function for the PmrA protein, whose best-described role was as the major activator of LPS modification genes (6, 16, 33). Whereas PmrA-dependent LPS modifications can reduce the risk of *Salmonella* detection by the Toll-like receptor-4 in a mammalian host (15, 45), its role in *ssrB* repression may reduce expression of Spi/Ssa-secreted effectors that dampen the host immune response (46, 47).

Control of the SPI-2 Virulence Locus. Several negative regulatory factors have been implicated in transcriptional control of SPI-2 genes. These factors include H-NS (22), YdgT (48), and Hha (49), all of which are nucleoid-associated proteins that recognize and selectively silence the expression of foreign DNA with higher adenine and thymine content relative to the resident genome (50). The PmrA protein directly binds to a specific sequence in the promoter of *ssrB*, repressing its expression (Figs. 4 and 5). Deletion of the genes specifying the nucleoid-associated protein attenuates *Salmonella*'s virulence in mice (48, 49), whereas preventing PmrA-mediated negative regulation of SsrB increases virulence (Fig. 1). This behavior could perhaps reflect that the regulatory effect of the nucleoid-associated proteins is not limited to SPI-2 genes (50, 51).

That iron limitation increases expression of SPI-2 genes (25, 52) could reflect that under such conditions the PmrA protein is not active (6). Indeed, we could decrease SPI-2 expression by increasing the iron concentration in the growth media (Fig. 3*A*); this effect was PmrA-dependent because it was not observed in the *pmrA* null mutant (Fig. 3*B*). By contrast, the ferric uptake regulator (Fur) does not appear to be involved in this process because an increase in iron would promote Fur dimerization and repression of its target promoters. Because one of Fur's target promoters is that corresponding to the *hns* gene (53), and H-NS is known to silence expression of SPI-2 genes (22), the increase in iron should increase expression of SPI-2 genes; however, we observed decreased expression (Fig. 3*A*).

It is remarkable that the PhoP protein, which is a major regulator of virulence functions in *Salmonella* (8), exerts two distinct and opposite effects on *ssrB* expression. It directly activates transcription of the *ssrB* gene (21). However, it promotes expression of PmrD, a posttranslational activator of PmrA (34), which represses *ssrB* transcription (Fig. 2). However, expression of PhoP-activated genes is maximal at 5–6 h after internalization, whereas repression of SPI-2 genes by PmrA was detected already 1 h after internalization (Fig. 5*C*). This result indicates that PmrA is probably activated in response to the signals detected by its cognate sensor PmrB (11). Moreover, it suggests that one of the roles of PhoP in promoting *ssrB* transcription might be to overcome PmrA-dependent repression of *ssrB*.

That a given regulator (e.g., PhoP) would promote expression of transcripts that favor virulence and reduce virulence is rare but not unprecedented. First, PhoP is required for transcription of the *pcgL* gene (7), the inactivation of which increases *Salmonella* virulence (3), as well as of several genes necessary for virulence (8). And second, PhoP is an activator of both the *mgtCBR* operon (7), which specifies the virulence protein MgtC (54), as well as of AmgR (55), an antisense RNA that down-regulates the levels of the *mgtC* transcript (55).

Cumulatively, our data demonstrate that the iron-inducible PmrA/PmrB two-component regulatory system properly tunes the level of SPI-2 induction in phagocytes by repressing *ssrB* transcription. This regulation is critical for *Salmonella*'s pathogenicity because lack of PmrA or its binding site on the *ssrB* promoter results in hypervirulence. Thus, bacterial pathogenicity is determined not only by virulence proteins, but also by antivirulence factors, which may contribute to the overall fitness of a pathogen.

Materials and Methods

Bacterial Strains, Plasmids, Primers, and Growth Conditions. *S. enterica* serovar Typhimurium strains were derived from wild-type strain 14028s. Unless otherwise stated, bacteria were grown at 37 °C in LB broth or in *N*-minimal medium (pH 7.4 or 4.6) (56) supplemented with 0.1% casamino acids, 38 mM glycerol, 1 mM or 10 μM of $MgCl_2$, and the indicated concentration of $FeSO_4$. 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 P22 transduction of *Salmonella* strains was performed as described (57). *Escherichia coli* DH5α was used as a host for the preparation of plasmid DNA. Bacterial strains and plasmids used in this study are listed in Table S1. Primers used in this study are listed in Table S2.

GFP Assay *Salmonella*. Cells expressing a *gfp* variant from the *ssaG* promoter were grown in minimal media. The expression of the GFP was measured by using multidetector (VICTOR3; PerkinElmer) with OD_{600} values. The measured values for GFP expression were divided by 1,000 times the OD_{600} values.

Identification of PmrA's Binding to the *ssrB* Promoter. To identify the binding of PmrA to the promoter region of the *ssrB* gene, chromatin immunoprecipitation (55), electrophoretic mobility shift assay (55), and DNase I footprinting assay (34) were carried out as described.

Detailed methods are described in *SI Materials and Methods*.

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