Control of a *Salmonella* virulence operon by proline-charged tRNA^{Pro}

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The intracellular pathogen *Salmonella enterica* serovar Typhimurium requires the *mgtC* gene to cause disease. The *mgtC* transcript includes a long leader region that harbors a short proline codon-rich ORF—termed *mgtP*—the translation of which is predicted to favor formation of one of two alternative stem-loop structures. We now report that the *mgtP* proline codons are critical for expression of the *mgtC* coding region inside host cells, for *Salmonella* survival inside macrophages, and for virulence in mice. We determine that the *mgtP* proline codons mediate the response to proline-charged tRNA^{Pro}, the levels of which decrease under proline limitation and/or hyperosmotic stress. The host compartment harboring *Salmonella* appears to be limited in proline because proline auxotrophs were defective for intramacrophage survival and virulence in mice. *Salmonella* seems to experience hyperosmotic stress during infection because osmotically regulated genes were highly induced inside phagocytic cells. Replacing *mgtP* proline codons with codons specifying threonine converted the *mgtC* leader into a threonine-responding element. Our findings indicate that an attenuation-like mechanism governs transcription elongation into the *mgtCBR* coding region. Moreover, they highlight how pathogens construe host signals by the effect they have on bacterial constituents.

leader mRNA | transcription attenuation

MgtC is a virulence protein used by several unrelated intracellular pathogens to survive within acidic macrophage phagosomes and to cause a lethal infection in mice (1–5). In the bacterium *Salmonella enterica* serovar Typhimurium—the etiologic agent of human gastroenteritis and murine typhoid fever—MgtC inhibits *Salmonella*'s own F_1F_o ATP synthase, thereby preventing the decrease in cytosolic pH and the excessive ATP accumulation resulting from acidification of *Salmonella*'s surroundings (6). MgtC's virulence role is unique because, rather than inhibiting a host protein, it targets a bacterial complex (i.e., the F_1F_o ATP synthase) that itself is necessary for virulence (7).

Expression of the MgtC protein is regulated at multiple levels. Transcription from the *mgtC* promoter is dependent on PhoP/PhoQ (8), a major virulence regulatory system highly active inside phagocytic cells (Fig. 1) (9). Transcription elongation into the *mgtC* coding region is, in turn, controlled by the 296-nucleotide-long leader RNA that precedes the *mgtC* ORF (Fig. 1). The *mgtC* leader RNA responds to an increase in cytosolic ATP by stimulating transcription elongation into the *mgtC* coding region (10, 11) (Fig. 1), an ability critical for *Salmonella* pathogenicity (10, 11). However, an additional signal appears to act on the *mgtC* leader during infection of a mammalian host because a strain with an ATP-insensitive *mgtC* leader RNA exhibited residual *mgtC* induction inside macrophages and was not as attenuated in mice as a strain deleted for the coding regions of the *mgtC* and *mgtB* genes (10). *mgtB* specifies a Mg^{2+} transporter (12) and follows *mgtC* in the *mgtCBR* operon (1), whereas *mgtR* specifies a peptide that promotes the degradation of the MgtC protein (13).

The *mgtC* leader RNA also includes a short ORF—termed *mgtP*—with three consecutive proline codons, two of which are necessary to promote transcription elongation into the *mgtC* coding region when *Salmonella* experiences proline limitation or hyperosmotic stress (11) (Fig. 1). This portion of the *mgtC* leader appears to be part of a transcription attenuator because (*i*) translation of *mgtP* is predicted to favor formation of one of two alternative stem-loop structures, (*ii*) stop codon mutations early in *mgtP* abolish expression of the associated coding region, and (*iii*) expression could be restored to the *mgtP* stop codon mutants by additional mutations that lock the *mgtC* leader into one of the two alternative stem-loop structures (11). Interestingly, one of the stem-loop structures sequesters the ribosome binding sequence and the start codon of the *mgtC* gene, suggesting additional regulation at the level of translation (Fig. 1).

We now report that the ability of the *mgtC* leader RNA to respond to a decrease in the levels of proline-charged tRNA^{Pro} is critical for expression of the associated coding region inside host cells, for survival inside macrophages, and for virulence in mice. By replacing the *mgtP* proline codons with codons specifying threonine, we rendered expression of the *mgtC* coding region responsive to threonine. Taken together with the finding that proline limitation and hyperosmotic stress decrease the levels of proline-charged tRNA^{Pro}, these results indicate that *mgtP* controls gene expression by a transcription attenuation-like mechanism. Our data suggest that a compartment harboring *Salmonella* is limited in proline and that *Salmonella* experiences hyperosmotic stress inside macrophages.

Results

Replacing *mgtP*'s Proline Codons by Threonine Codons Converts the *mgtC* Leader into a Threonine-Responding Genetic Element. To investigate how the wild-type *mgtP* controls *mgtC* expression when *Salmonella* experiences proline limitation or hyperosmotic stress (11), we constructed a derivative of a proline auxotroph in which

Significance

Pathogens must express their virulence genes in the correct locales to cause disease. This task requires a pathogen's ability to sense host signals and to transduce this information to its expression machinery. Here we establish that the facultative intracellular pathogen *Salmonella enterica* responds to a decrease in the levels of proline-charged tRNA^{Pro} by promoting expression of the *mgtCBR* virulence operon. We determine that hyperosmotic stress and proline limitation reduce proline-charged tRNA^{Pro} levels and show that the compartment harboring *Salmonella* is limited in proline and that *Salmonella* experiences high osmolarity inside macrophages. Our findings indicate that *Salmonella* detects host cues by the changes they produce on bacterial constituents.

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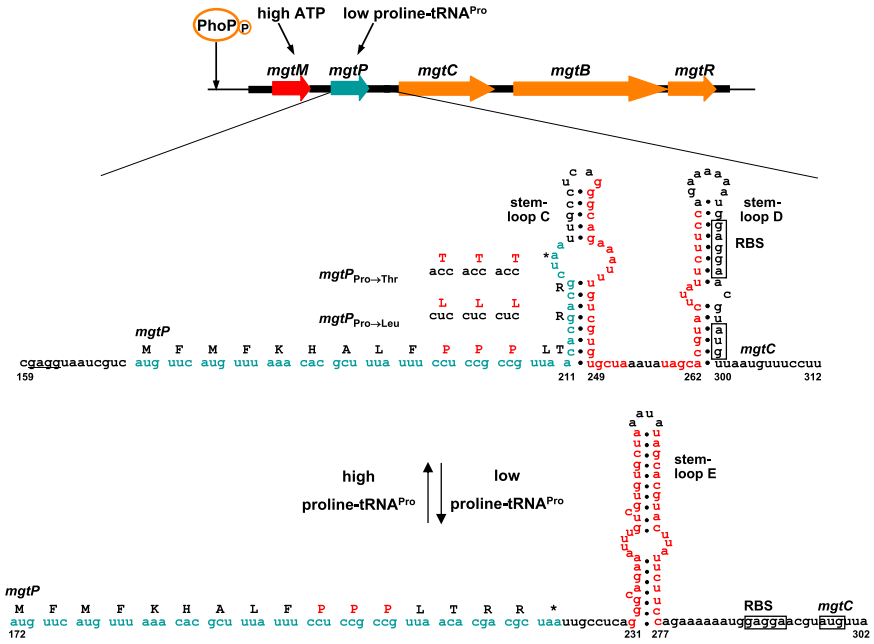

Fig. 1. Regulation of the *mgtCBR* virulence operon by ATP and proline tRNA^{Pro}. The phosphorylated PhoP protein binds to the promoter of the *mgtCBR* operon and stimulates transcription initiation. The 296-long *mgtCBR* leader mRNA harboring two short ORFs (termed *mgtM* and *mgtP*) controls transcription elongation into the coding region in response to ATP and proline tRNA^{Pro} levels. Proline tRNA^{Pro} levels determine the coupling/uncoupling between transcription of the *mgtCBR* leader and translation of *mgtP*, allowing formation of one of two alternative stem-loop structures (stem loop D versus E), hence controlling transcription elongation into the *mgtCBR* coding region. The sequences of *mgtP* variants used in this work are indicated above the Pro codons.

the three proline codons in the chromosomal copy of *mgtP* were substituted by threonine codons (Fig. 1). This derivative could no longer increase the mRNA levels of the *mgtC* coding region when experiencing proline limitation (Fig. 2 *A* and *B*, Fig. S1 *A* and *B*) or hyperosmotic stress (Fig. 2 *C* and *D*, Fig. S1 *C* and *D*). These results are in agreement with our previous report demonstrating that both proline limitation and hyperosmotic stress induced expression of the *mgtC* coding region in an organism harboring the wild-type *mgtP* sequence but not in one where the proline codons were substituted by codons specifying glycine (11). Control experiments revealed that substitution of the *mgtP* proline codons specifically affected the mRNA levels of the *mgtC* coding region because the levels of the *mgtA* coding region, which are also induced upon proline limitation (14), were similar in the *mgtP*$^{+}$ strain and in the variant with the *mgtP* proline codons replaced by threonine codons (Fig. 2 *A–D*, Fig. S1 *A–D*). Likewise, the mRNA levels of the *phoP* gene and of the *mgtC* and *mgtA* leader regions, which are not induced by proline limitation (11, 14), were not affected by substitutions in the *mgtP* codons (Fig. 2 *A–D*, Fig. S1 *A–D*).

Introduction of the *mgtP* allele with the proline codons replaced by threonine codons conferred the ability to promote *mgtC* expression in response to threonine limitation upon a threonine auxotroph (Fig. 2*F*, Fig. S1*F*). This ability was specific to the associated *mgtC* coding region as threonine limitation had no effect on the mRNA levels of the *mgtC* and *mgtA* leader regions or the *mgtA* and *phoP* coding regions (Fig. 2*F*, Fig. S1*F*). Moreover, it required the threonine codons in the *mgtP* variant because the mRNA levels of the *mgtC* coding region did not increase upon threonine limitation in the isogenic *mgtP*$^{+}$ strain (Fig. 2*E*, Fig. S1*E*). These results indicate that expression of the *mgtC* coding region responds to limitation for the particular amino acid having consecutive codons in *mgtP* rather than to nutrient limitation per se. Furthermore, they support the notion that the *mgtP* portion of the *mgtC* leader RNA controls transcription elongation into the *mgtC* coding region by a mechanism resembling transcription attenuation (15, 16).

***mgtP* Proline Codons Are Necessary for Normal *mgtC* Expression Inside Macrophages.** To evaluate *mgtP*'s role during infection, we introduced the *mgtP* allele with the proline codons substituted by threonine codons into a wild-type genetic background (i.e., prototroph for both proline and threonine). The resulting variant failed to fully induce *mgtC* expression inside the macrophage-like cell line J774 A.1, achieving mRNA levels that were only one-third to one-half of those produced by the isogenic *mgtP*$^{+}$ strain (Fig. 3 *A* and *B*). This defect is due to the absence of codons specifying proline, as opposed to the presence of codons specifying threonine per se. This is because replacing the *mgtP* proline codons with codons specifying leucine prevented normal *mgtC* induction both in a proline auxotroph experiencing proline limitation (Fig. S2 *A* and *B*) and in an otherwise wild-type strain inside macrophages (Fig. S2 *C* and *D*). As expected, the strain harboring the *mgtP* allele with the proline codons substituted by leucine codons retained the ability to induce the *mgtA* coding region in a proline auxotroph experiencing proline limitation and had no effect on the expression of the *mgtC* and *mgtA* leader regions or the *phoP* coding region (Fig. S2 *A* and *B*). These data suggested that the levels of proline-charged tRNA^{Pro} decrease during *Salmonella* infection of a phagocytic cell.

***mgtP* Proline Codons Are Necessary for *Salmonella* Survival Inside Macrophages and Virulence in Mice.** The *mgtP* variant with the proline codons replaced by threonine codons was defective for survival inside J774 A.1 macrophages (Fig. 3*E*). In addition, the *mgtP* variants with proline codons replaced by either threonine or leucine codons were defective for virulence in C3H/HeN mice inoculated intraperitoneally (Fig. 3*F*, Fig. S2*E*). These *mgtP* mutants were attenuated to a similar extent as our previously reported mutant with an *mgtC* leader that is not able to respond to an increase in cytosolic ATP (Fig. 3 *E* and *F*) (10, 11), and thus not as attenuated as a strain deleted for both the *mgtC* and *mgtB* coding regions (Fig. 3*F*).

We determined that a *Salmonella* strain with an *mgtC* leader unable to respond to ATP and bearing the *mgtP* allele with the proline codons replaced by threonine codons failed to promote transcription of the *mgtC* coding region inside macrophages (Fig. 3*D*), was highly compromised for survival inside macrophages (Fig. 3*E*), and attenuated for virulence in mice (Fig. 3*F*). Actually, this mutant was as defective as the strain deleted for both the *mgtC* and *mgtB* coding regions (Fig. 3*F*). And this was true also for a mutant unable to respond to ATP and with the *mgtP* allele harboring leucine codons in place of the proline codons (Fig. S2*E*). These data indicate that the ability to respond to an increase in cytosolic ATP and to conditions predicted to decrease the levels of proline-charged tRNA^{Pro} is critical for *mgtCBR*-mediated virulence.

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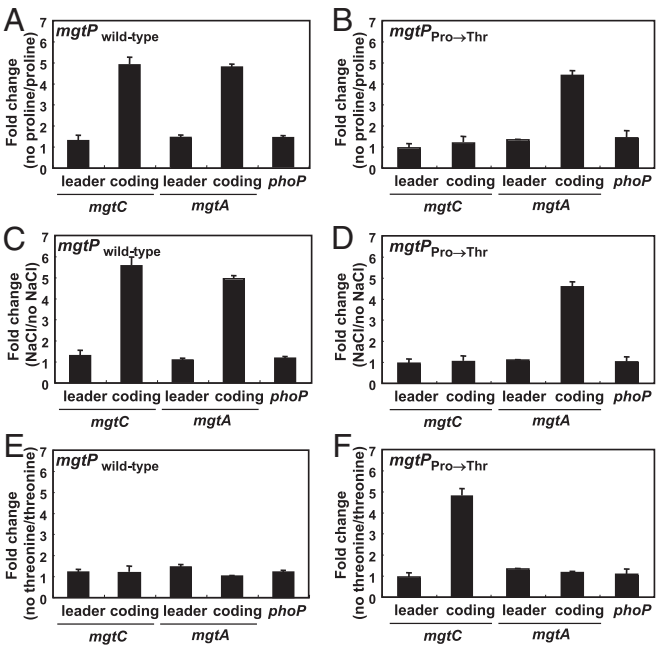

Fig. 2. Proline limitation promotes transcription of the *mgtCBR* coding region in a manner dependent on the *mgtP* proline codons. (*A* and *B*) Fold change in the mRNA levels of the leader regions of the *mgtC* and *mgtA* transcripts and the coding regions of the *mgtC*, *mgtA*, and *phoP* genes produced by a proline auxotroph harboring either the wild-type *mgtCBR* leader (EG19886) (*A*) or a derivative in which the three *mgtP* Pro codons were substituted by Thr codons ($mgtP_{Pro\rightarrow Thr}$; EL417) (*B*). Bacteria were grown in *N*-minimal media with 500 μM Mg^{2+} in the presence of 1 mM proline for 1 h and then grown for 45 min in media containing or lacking proline. The expression levels of target genes were normalized to that of 16S ribosomal RNA *rrs* gene. Fold change was calculated by dividing the mRNA levels of cells grown in the absence of proline by that of cells grown in the presence of proline. Shown are the means and SDs from two independent experiments. (*C* and *D*) Fold change in the mRNA levels of the leader regions of the *mgtC* and *mgtA* transcripts and the coding regions of the *mgtC*, *mgtA*, and *phoP* genes in strains with the wild-type *mgtCBR* leader (14028s) (*C*) or a derivative with the three *mgtP* Pro codons substituted by Thr codons (EL611) (*D*) that were subjected to hyperosmotic stress. The RNA values were normalized relative to those corresponding to the *rrs* gene. Bacteria were grown for 1 h in modified *N*-minimal medium without casamino acids and containing 500 μM Mg^{2+} or in media that also had 0.3 M NaCl. Shown are the means and SDs from two independent experiments. (*E and F*) Fold change in the mRNA levels of the mRNA regions listed in *A* and *B* produced by a threonine auxotroph harboring either the wild-type *mgtCBR* leader (EL601) (*E*) or a derivative in which the three *mgtP* Pro codons were substituted by Thr codons ($mgtP_{Pro\rightarrow Thr}$; EL621) (*F*) under threonine limitation conditions analogous to those described above for proline limitation. Shown are the means and SDs from two independent experiments. mRNA levels relative to those of the 16S rRNA *rrs* gene are presented in Fig. S1.

***Salmonella* Experiences Hyperosmotic Stress Inside Phagocytic Cells in a Compartment That Is Low in Proline.** The data presented above raised the question: What condition(s) promotes a decrease in the levels of proline-charged $tRNA^{Pro}$ during infection? *Salmonella* may reside in a compartment that is low in proline and experience a diminished ability to synthesize proline. Alternatively or in addition, *Salmonella* might be subjected to hyperosmotic stress, which would decrease the availability of proline to charge $tRNA^{Pro}$ because proline can serve as an osmoprotectant (17).

To explore the possibility of the compartment harboring *Salmonella* during infection being low in proline, we investigated the behavior of proline auxotrophs harboring mutations in the *proB* and *proC* genes, which specify proteins that carry out the first and third steps in proline biosynthesis, respectively (17). Both the *proB* and *proC* single mutants were defective for survival inside J774 A.1 macrophages (Fig. 4*A*) and for virulence in mice inoculated intraperitoneally (Fig. 4*B*). The virulence defect of the *proC* mutant is due to its inability to synthesize proline (as opposed to a potential effect of the *proC* mutation affecting a neighboring gene) because its macrophage survival defect could be partially corrected by a plasmid harboring the wild-type *proC* gene but not by the plasmid vector (Fig. 4*C*). Furthermore, proline addition to the tissue culture media rescued growth of the *proC* mutant inside macrophages (Fig. 4*C*).

We were surprised to find that the *proC* mutant was more attenuated than the *proB* mutant inside macrophages (Fig. 4*A*). This virulence difference could be due to the accumulation of gamma-glutamyl semialdehyde (i.e., the ProC substrate) in the *proC* mutant because a *proB proC* double mutant displayed the same attenuation as the *proB* single mutant (Fig. 4*A*). The *proC* single mutant grew like the *proB* single mutant and the *proB proC* double mutant in defined media containing casamino acids and 1 mM proline (Fig. 4*D*). The different phenotypes displayed by the *proC* mutant when *Salmonella* is inside host cells versus grown in defined media could reflect a lower accumulation of gamma-glutamyl semialdehyde in the latter growth condition due to feedback inhibition of proline on the enzyme encoded by the *proB* gene (18, 19).

Next, we examined the mRNA levels of the hyperosmolarity-induced *proV* and *ompC* genes, which specify a component of a glycine betaine/proline uptake system and an outer membrane porin, respectively. In agreement with previous reports (20), the mRNA levels of the *proV* and *ompC* genes rose in response to increasing osmolarity (Fig. 5*A*). Both *proV* and *ompC* were highly induced when *Salmonella* was inside macrophages (Fig. 5*B*). Differences in induction kinetics between *proV* and *ompC* could be due to an additional signal(s) acting on *proV* and/or *ompC*. Cumulatively, these data indicate that *Salmonella* faces high osmolarity inside phagocytic cells, residing in a compartment of low proline.

Proline Limitation and Hyperosmotic Stress Decrease the Levels of Proline-Charged $tRNA^{Pro}$. Taken together with our published data (11), the results presented above suggest that *mgtP*-mediated transcription elongation into the *mgtC* coding region is controlled by an attenuation mechanism that entails formation of one of two alternative stem-loop structures. Which structure forms is determined by the coupling/uncoupling between transcription of the *mgtCBR* leader and translation of the *mgtP* ORF. Uncoupling of transcription and translation, and the resulting transcription elongation into the *mgtC* coding region, may occur if ribosomes stall at the *mgtP* proline codons due to low levels of proline-charged $tRNA^{Pro}$.

To test this model, we examined the levels of proline-charged $tRNA^{Pro}$ in *Salmonella* subjected to proline limitation and/or hyperosmotic stress. The levels of proline-charged tRNA were lower in a *proB* mutant in media lacking proline compared with media with 1 mM proline (Fig. 6*A*), and the latter were reduced upon addition of 0.3 M of sodium chloride (Fig. 6*A*). The levels of proline-charged $tRNA^{Pro}$ decreased also in wild-type *Salmonella* when incubated in 0.3 M of sodium chloride (Fig. 6*C*), and they were partially restored upon addition of 1 mM proline (Fig. 6*C*). Hyperosmotic stress and proline limitation appear to affect the levels of proline-charged $tRNA^{Pro}$ specifically because the levels of threonine-charged $tRNA^{Thr}$ were not altered under these conditions (Fig. 6 *B* and *D*).

Discussion

We have identified a singular example in which proline-charged $tRNA^{Pro}$ acts as a regulatory signal controlling virulence gene expression. Specifically, we established that *Salmonella*'s ability to sense a decrease in proline-charged $tRNA^{Pro}$ levels via a proline codon-rich ORF in the leader region of the *mgtCBR* transcript is required for bacterial survival inside macrophages (Fig. 3) and for causing disease in mice (Fig. 3, Fig. S2). Moreover, an *mgtC* leader RNA that was unable to sense both proline-charged

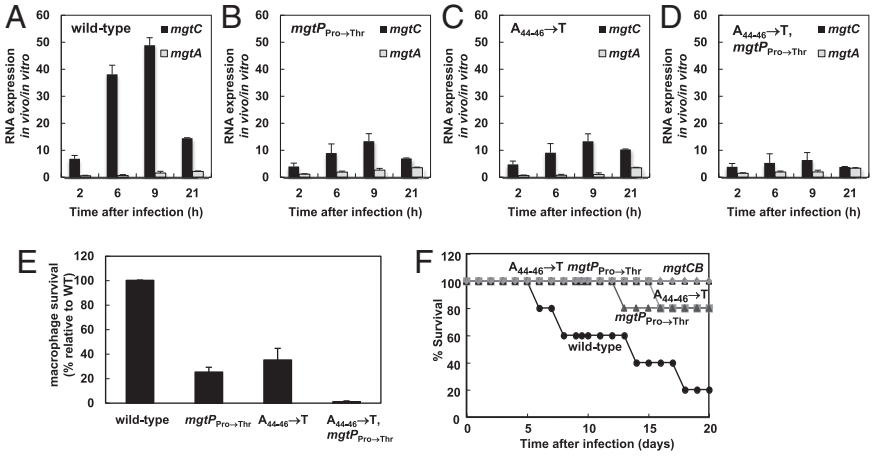

Fig. 3. The *mgtP* proline codons are necessary for *mgtC* expression and survival inside macrophages as well as virulence in mice. (*A–D*) Relative mRNA levels of the *mgtC* and *mgtA* coding regions produced by wild-type *Salmonella* (14028s) (*A*), an *mgtP* mutant with the Pro codons replaced by Thr codons (EL611) (*B*), an ATP-sensing defective leader mutant (EL341) (*C*), and a mutant defective for sensing both signals (EL602) (*D*) inside J774 A.1 macrophages at the indicated times after infection. (*E*) Survival inside J774 A.1 macrophages of the *Salmonella* strains listed in *A–D* at 18 h after infection. (*F*) Survival of C3H/HeN mice inoculated intraperitoneally with ~10^3 colony-forming units of the *Salmonella* strains listed in *A–D* and of a mutant *Salmonella* deleted for both the *mgtC* and *mgtB* genes (EL6).

tRNA^{Pro} levels and ATP, which is detected by a different portion of the *mgtCBR* leader (10), failed to induce *mgtC* expression inside macrophages (Fig. 3*D*) and was as attenuated as a strain deleted for the *mgtC* and *mgtB* coding regions (Fig. 3*F*, Fig. S2*E*). These data demonstrate the critical role that both proline-charged tRNA^{Pro} and ATP play in expression of a critical virulence determinant during infection.

***mgtP* Is Part of a Transcriptional Attenuator.** We previously proposed that the *mgtP*-including portion of the *mgtC* leader RNA controls transcription elongation into the associated coding region by an attenuation-like mechanism (11). This proposal was based on the ability of the *mgtP* portion of the *mgtC* leader to adopt two alternative stem-loop structures (Fig. 1), on *mgtP* translation predicted to favor formation of one of the two structures (Fig. 1), and on the results of genetic experiments in which full *mgtP* translation was compromised and/or the leader RNA locked into one of the two structures (11).

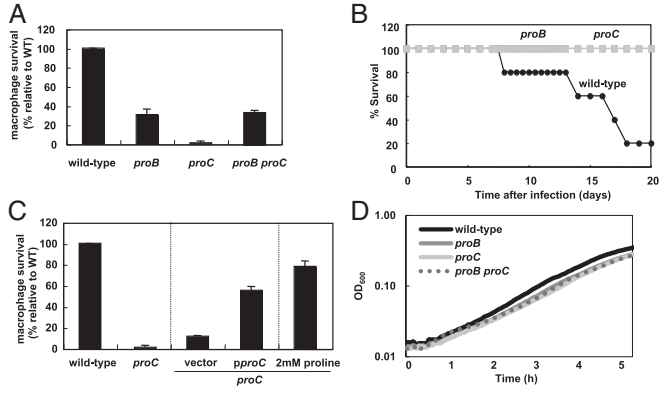

Fig. 4. Proline auxotrophs are defective for survival inside macrophages and virulence in mice. (*A*) Survival inside J774 A.1 macrophages of wild-type (14028s), *proB* (EG19886), *proC* (EL605), and *proB proC* (EL625) *Salmonella* at 18 h after infection. (*B*) Survival of C3H/HeN mice inoculated intraperitoneally with ~10^3 colony-forming units of wild-type (14028s), *proB* (EG19886), or *proC* (EL605) *Salmonella*. The data are representative of two independent experiments, which gave similar results. (*C*) Survival inside J774 A.1 macrophages of wild-type (14028s), *proC* (EL605), *Salmonella*, and a *proC* mutant *Salmonella* harboring either the plasmid vector or the wild-type *proC* gene, or in tissue culture media with 2 mM proline at 18 h after infection. (*D*) Growth curves of wild-type (14028s), *proB* (EG19886), *proC* (EL605), and *proB proC* (EL625) *Salmonella* in *N*-minimal medium containing 1 mM proline. Bacteria were grown at 37 °C for 5 h in a 96-well plate with orbital shaking and absorbance measured at OD_{600} every 2.5 min.

Two unique sets of independent experiments now provide further support for the notion that transcription attenuation controls expression of the *mgtC* coding region. First, we established that an *mgtP* allele in which the three proline codons were substituted with threonine codons gained the ability to respond to threonine limitation (Fig. 2*F*). And second, we determined that proline limitation and hyperosmotic stress decrease the levels of proline-charged tRNA^{Pro} (Fig. 6).

Differential Intramacrophage Expression Behavior of *Salmonella* Genes Regulated by Leader Sequences Harboring Short Proline Codon-Rich ORFs. The leader regions of the *mgtA* and *mgtCBR* transcripts harbor two different short proline codon-rich ORFs, designated *mgtL* and *mgtP*, respectively, which enable proline limitation and hyperosmotic stress to induce expression of their associated coding regions (11, 14). Given that *Salmonella* experiences hyperosmotic stress (Fig. 5) during infection in a compartment with proline levels so low that it cannot support growth of proline auxotrophs (Fig. 4), why is the *mgtA* coding region not induced inside macrophages whereas *mgtC*'s is?

The *mgtA* coding region might not be induced inside macrophages because of several nonmutually exclusive possibilities. First, *mgtL*'s proline codons are not consecutive, being located at the third, fifth, seventh, and ninth positions (14), whereas *mgtP* harbors three consecutive proline codons (Fig. 1). The presence of consecutive proline codons might enhance ribosome stalling at *mgtP*. In this context, it is interesting that elongation factor P is required to alleviate ribosome stalling in ORFs harboring consecutive proline codons (21–24) and that a functional elongation factor P is necessary for *Salmonella* virulence (25). Second, *mgtP* is preceded by a large RNA segment, whereas that is not the case for *mgtL*. And third, the levels of the *mgtA* transcript are affected by RNase E, which targets the *mgtA* leader RNA (26). The different expression behavior of the *mgtC* and *mgtA* genes makes sense because *mgtC* is required for *Salmonella* virulence, whereas *mgtA* is not (1).

Nutritional Environment of the *Salmonella*-Containing Vacuole. *Salmonella* resides within a membrane-bound compartment inside macrophages (27). This compartment appears to be a nutrient-poor locale because *Salmonella* auxotrophs for purine, pyrimidine, histidine, and aromatic amino acid are avirulent (28, 29). We have now demonstrated that proline auxotrophs are attenuated for survival inside macrophages and virulence in mice (Fig. 4), indicating that this compartment is also limited for proline. It is interesting that *Legionella pneumophila*, which also resides within a phagosome, induces proline biosynthetic genes when inside macrophages (30). Thus, other bacterial pathogens that remain within phagosomes may also experience low proline. By contrast, high proline stimulates virulence gene expression in

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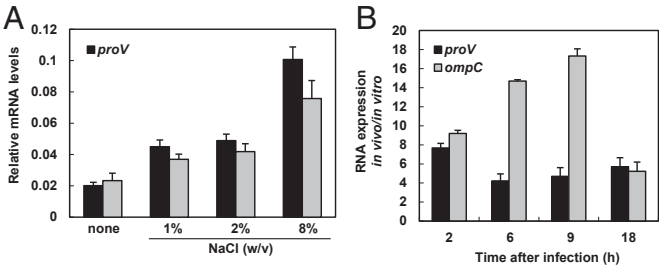

Fig. 5. Hyperosmotic stress-inducible *proV* and *ompC* genes are highly expressed inside macrophages. (*A*) Relative mRNA levels of the coding regions of the *proV* and *ompC* genes in wild-type *Salmonella* (14028s) under different osmotic conditions. Bacteria were grown for 1 h in modified *N*-minimal medium without casamino acids and containing 500 μM Mg^{2+} or in media that also had 1%, 2%, or 8% NaCl (wt/vol). Shown are the means and SDs from two independent experiments. (*B*) Relative mRNA levels of the *proV* and *ompC* coding regions of wild-type *Salmonella* (14028s) inside J774 A.1 macrophages at the indicated times after infection. mRNA levels are relative to those of the 16S rRNA *rrs* gene.

entomopathogenic *Photorhabdus* (31), indicating that different pathogens may respond to a given signal in opposite ways.

Concluding Remarks. Our findings illustrate how a pathogen integrates signals that operate in different compartments into the decision to express a virulence determinant. For example, transcription initiation from the *mgtC* promoter is under direct control of the virulence regulatory protein PhoP, which is activated by its cognate sensor PhoQ when *Salmonella* experiences low Mg^{2+} (32, 33), mildly acidic pH (34), and/or antimicrobial peptides (35) in its surroundings. Because PhoP-activated promoters are highly induced inside macrophages (36), the phagosome containing *Salmonella* is likely to display one or more of these (and potentially other) signals that activate PhoQ in the periplasm.

The leader sequences of certain PhoP-activated transcripts control transcription elongation into the associated coding regions in response to cytosolic signals. For instance, the leader sequence of the *mgtCBR* transcript responds not only to ATP (10) and conditions predicted to alter the levels of proline-charged $tRNA^{Pro}$ (11) but also to cytoplasmic Mg^{2+} (26, 37). This raised the possibility of the defects in *mgtC* expression during infection (Fig. 3 *A*–*D* and Fig. S2*D*), survival inside macrophages (Fig. 3*E*), and virulence in mice (Fig. 3*F* and Fig. S2*E*) exhibited by the *mgtP* mutants with substitutions in the proline codons resulting from a compromised ability of the mutant *mgtC* leader to respond to cytoplasmic Mg^{2+}. However, we can rule out this possibility because the *mgtP* variant with the leucine codons replacing the proline codons responded to changes in Mg^{2+} like the isogenic $mgtP^{+}$ strain (Fig. S3). Moreover, a *Salmonella* strain with an *mgtC* leader unable to respond to an increase in cytosolic ATP retained a wild-type ability to respond to a decrease in cytosolic Mg^{2+} (10). Furthermore, the *mgtA* coding region is not induced inside macrophages (10) despite harboring a long leader RNA acting as a Mg^{2+}-sensing riboswitch (37). Cumulatively, these data argue that cytoplasmic Mg^{2+} is not a key signal promoting the preferential expression of the *mgtC* coding region inside macrophages.

In sum, that conditions decreasing the levels of proline-charged $tRNA^{Pro}$ induce expression of a virulence operon in *Salmonella* reinforces the notion that bacteria that associate with a eukaryotic host may respond to host cues indirectly, by the impact such cues have on bacterial constituents. In other words, a proline-limited environment coupled with a reduced proline biosynthetic ability and/or hyperosmotic stress result in a decrease in the levels of proline-charged $tRNA^{Pro}$ (Fig. 6), which denotes the environment experienced by *Salmonella* inside a macrophage. Finally, that proline-charged $tRNA^{Pro}$ controls expression of a virulence operon by a transcription attenuation-like mechanism demonstrates that this regulatory strategy has been appropriated by *Salmonella* to regulate functions unrelated to nutrient biosynthesis or transport (15, 16).

Materials and Methods

Bacterial Strains, Plasmids, Oligodeoxynucleotides, and Growth Conditions. Bacterial strains and plasmids used in this study are listed in Table S1. All *Salmonella enterica* serovar Typhimurium strains are derived from the wild-type strain 14028s (28) and were constructed by phage P22-mediated transductions as described (38). All DNA oligonucleotides are listed in Table S2. Bacteria were grown at 37 °C in Luria–Bertani broth (LB), *N*-minimal media (pH 7.7) (12) supplemented with 0.1% casamino acids, 38 mM glycerol, and the indicated concentrations of $MgCl_2$. When indicated, we used a modified *N*-minimal medium containing 0.2% glucose instead of 38 mM glycerol. *Escherichia coli* DH5α was used as the host for preparation of plasmid DNA. Ampicillin was used at 50 $\mu g \cdot ml^{-1}$, chloramphenicol at 20 $\mu g \cdot ml^{-1}$, and tetracycline at 10 $\mu g \cdot ml^{-1}$.

Effect of Proline Limitation on Gene Expression. The proline limitation experiment was performed as described (11).

Effect of Threonine Limitation on Gene Expression. Threonine limitation was performed as described above except that we used a threonine auxotroph and a 19-amino-acid mixture (all essential amino acids except threonine).

Effect of Hyperosmotic Stress on Gene Expression. Experiment was performed as described (14).

Quantitative Real Time PCR. Total RNA was isolated using RNeasy Kit (Qiagen) according to the manufacturer's instructions. The purified RNA was quantified

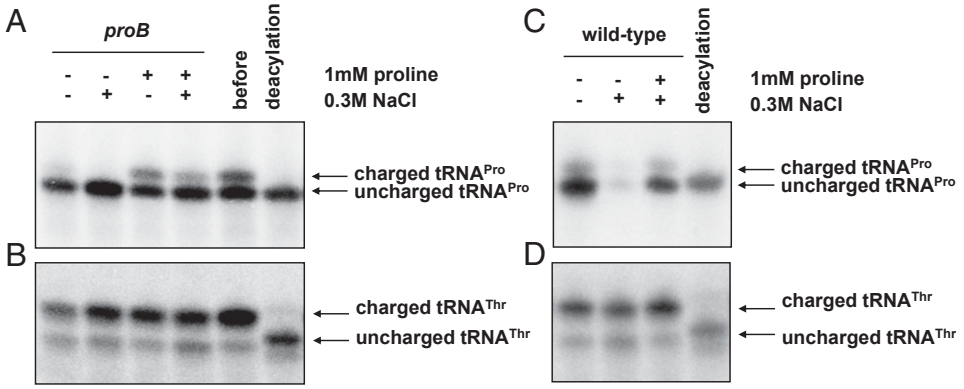

Fig. 6. Proline limitation and hyperosmotic stress decrease the levels of proline $tRNA^{Pro}$. (*A* and *B*) Northern blot analysis using a probe detecting $tRNA^{Pro}$ (*A*) and $tRNA^{Thr}$ (*B*) in *proB Salmonella* (EG19886) before or after switching bacteria to media containing or lacking 1 mM proline or 0.3 M NaCl. Bacteria were grown for 30 min in *N*-minimal media containing 500 μM Mg^{2+} in the presence or absence of 1 mM proline or 0.3 M NaCl. (*C* and *D*) Northern blot analysis using a probe detecting $tRNA^{Pro}$ (*C*) and $tRNA^{Thr}$ (*D*) in wild-type *Salmonella* (14028s) treated with 0.3 M NaCl or 0.3 M NaCl together with 1 mM proline. The levels of $tRNA^{Thr}$ in *B* and *D* were used as internal controls for the levels of $tRNA^{Pro}$ in *A* and *C*, respectively. Bacteria were grown for 1 h in *N*-minimal modified media containing 500 μM Mg^{2+} in the presence or absence of 1 mM proline or 0.3 M NaCl.

using a Nanodrop machine (NanoDrop Technologies). cDNA was synthesized using High Capacity RNA-to cDNA Master Mix (Applied Biosystems). The mRNA levels of the *mgtC*, *mgtA*, *phoP*, *proV*, *ompC*, and *rrs* genes were measured by quantification of cDNA using SYBR Green PCR Master Mix (Applied Biosystems) and appropriate primers (*mgtC* leader, 6962/6963; *mgtC* coding, 7530/7531; *mgtA* leader, 7225/7226; *mgtA* coding, 4308/4309; *phoP* coding, 4489/4490; *proV*, W688/W689; and *ompC*, W696/W697) and monitored using a Fast ABI7500 machine (Applied Biosystems). Data were normalized to the levels of 16S ribosomal RNA *rrs* gene amplified with primers 6970 and 6971.

Examining Survival Inside Macrophages. Intramacrophage survival assays were conducted with the macrophage-like cell line J774 A.1 as described (1).

Mouse Virulence Assays. Six- to eight-week-old female C3H/HeN mice were inoculated intraperitoneally with $\sim 10^3$ colony-forming units. Mouse survival was followed for 21 d. Virulence assays were conducted twice with similar outcomes, and data correspond to groups of five mice. All procedures were performed according to approved protocols by the Institutional Animal Care and Use Committee from Yale University.

Measurement of the Levels of Proline-Charged tRNA^{Pro}. We measured levels of proline-charged tRNA^{Pro} or threonine-charged tRNA^{Thr} as described (39) with slight modifications. Wild-type and proline auxotrophic (EG19886) *Salmonella* were grown as described (11, 14). We mixed 5 mL of cultures with 5 mL of 10% (wt/vol) trichloroacetic acid and kept it on ice. All subsequent steps were carried out at 0–4 °C. Cells were pelleted and resuspended in 0.3 mL of buffer A (0.3 M sodium acetate pH 4.5, 10 mM EDTA). tRNAs were extracted with 0.3 mL of phenol equilibrated with buffer A by vortexing vigorously (150 s with intervals) and centrifuged for 15 min at 20,817 × *g*. The aqueous phase was transferred to new tube containing 0.3 mL of phenol and repeated as above. The nucleic acids were precipitated with ethanol at –18 °C for 15 min followed by centrifugation for 30 min at 20,817 × *g*. The pellet was dissolved in 60 μL of buffer A, and the nucleic acids were precipitated as above. The pellet was resuspended in 20 μL of buffer B (10 mM sodium acetate pH 4.5, 1 mM EDTA).

Before gel electrophoresis, 4 μL of tRNA was deacylated by treating with 0.1 M Tris·HCl (pH 9.0) in a total volume of 50 μL for 1 h at 37 °C. Then, the tRNA was neutralized by adding 50 μL of buffer A, and precipitated as above and dissolved in 4 μL of buffer B. We mixed 4 μL of either untreated or deacylated sample with 6 μL of loading buffer [0.1 M sodium acetate pH 5.0, 8 M urea, 0.05% (wt/vol) bromophenol blue, and 0.05% (wt/vol) xylene cyanol], and electrophoresed it through a 6.5% polyacrylamide (acrylamide to bisacrylamide, 19:1) gel containing 0.1 M sodium acetate (pH 5.0) and 8 M urea. The electrophoresis was carried out 500 V at 4 °C until the bromophenol blue reached the bottom of the gel. The area of the gel between the two dyes was electroblotted onto a positively charged membrane. The tRNA was crosslinked to the membrane by 0.12 J of UV light. The membranes were prehybridized at 42 °C for 1 h in 12 mL of ULTRAhyb-oligo buffer (Invitrogen). We used radiolabeled W718 and W722 primers to detect tRNA^{Pro} and tRNA^{Thr}, respectively. Hybridization was at 42 °C overnight and the membranes were washed in wash buffer (0.3 M NaCl, 30 mM sodium citrate, 0.5% SDS) for 30 min at 42 °C twice. The membranes were analyzed by a phosphor imager scanner.

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