The *Salmonella* virulence protein MgtC promotes phosphate uptake inside macrophages

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The MgtC virulence protein from the intracellular pathogen *Salmonella enterica* is required for its intramacrophage survival and virulence in mice and this requirement of MgtC is conserved in several intracellular pathogens including *Mycobacterium tuberculosis*. Despite its critical role in survival within macrophages, only a few molecular targets of the MgtC protein have been identified. Here, we report that MgtC targets PhoR histidine kinase and activates phosphate transport independently of the available phosphate concentration. A single amino acid substitution in PhoR prevents its binding to MgtC, thus abrogating MgtC-mediated phosphate transport. Surprisingly, the removal of MgtC’s effect on the ability to transport phosphate renders *Salmonella* hypervirulent and decreases a non-replicating population inside macrophages, indicating that MgtC-mediated phosphate transport is required for normal *Salmonella* pathogenesis. This provides an example of a virulence protein directly activating a pathogen’s phosphate transport inside host.
Phosphorus is one of the most abundant elements found in living organisms. As in the form of phosphate ions, it is found in lipids, nucleic acids, proteins, or carbohydrates; participates in many enzymatic reactions relying on the transfer of phosphoryl group; serves as a reservoir of chemical energy when incorporated into ATP or linked to other molecules. In bacteria, phosphate signaling is mediated by the PhoP/PhoR two-component regulatory system (the PhoP/PhoR for gram-positive bacteria), which has been most extensively studied in Escherichia coli [1]. PhoR is a histidine kinase that likely responds to the periplasmic phosphate concentration. PhoR is anchored to the inner membrane by an N-terminal transmembrane-spanning region (TM), which is followed by four cytoplasmic domains: a charged region (CR), a Per-ARNT-Sim (PAS) domain, a dimerization/histidine phosphotransfer (DHP) domain, and a catalytic/ATP-binding (CA) domain [2]. Like other histidine kinases, PhoR phosphorylates a histidine residue at the DHP domain and, at a single amino acid substitution in PhoR preventing MgtC binding renders Salmonella hypervirulent and decreases non-replicating Salmonella inside macrophages, suggesting that MgtC’s interaction with PhoR histidine kinase is required for the normal course of Salmonella infection. Our findings provide an example of a Salmonella virulence protein that targets a sensor kinase to activate phosphate transport even without sensing a cognate signal.

Results

**mgtC overexpression promotes mRNA levels of Pho genes.** To explore other targets of the MgtC protein, we used a Salmonella strain harboring a plasmid with the mgtC coding region expressed from an IPTG-inducible promoter. Then, we compared the RNA profiles of Salmonella expressing the mgtC ORF and those expressing the vector after IPTG induction for 1 h. Surprisingly, we identified that mRNA levels of 72 genes including the mgtC gene itself were significantly higher in Salmonella expressing the mgtC gene than those expressing the vector (≥4-fold; Fig. 1b and Supplementary Table 3). By contrast, mRNA levels of ten genes were downregulated in mgtC-expressing Salmonella (≥4-fold; Fig. 1b and Supplementary Table 3). Among the upregulated genes, we were particularly interested in 17 genes whose functions are involved in phosphate transport/homeostasis (Pho regulon), because little is known about the molecular connection between the MgtC virulence protein and Pho regulon. These genes include the phoE gene, pstSCABphoU operon, ugpBAECQ operon, phoBR operon, and phnSTUV operon. Elevated mRNA levels of each gene by expressing the mgtC gene were verified by quantitative real-time PCR (Fig. 1c–h). As control experiments, mRNA levels of the phoP and mgtB genes were unaffected by mgtC overexpression (Supplementary Fig. 1).

MgtC interacts with PhoR histidine kinase. As mgtC overexpression increases mRNA levels of the Pho regulon, we suspected that the mgtC gene might have an impact on the regulatory system that controls expression of Pho genes. In *E. coli*, the PhoB/PhoR two-component system controls transcription of genes involved in phosphate transport [1]. If this is true for Salmonella and MgtC affects expression of these identified genes via the PhoB/PhoR two-component system, we hypothesized that we
would not observe the increase in mRNA levels of these genes by mgtC overexpression in a phoB mutant. As expected, mgtC overexpression could not promote mRNA levels of the genes identified above in the phoB mutant (Fig. 1c–h), suggesting that MgtC increases mRNA levels of Pho regulon by impacting the signaling via the PhoB/PhoR two-component system.

Phosphate signaling might include additional components besides the PhoB/PhoR two-component system because the cytoplasmic PhoU protein links the PhoR histidine kinase and PstB protein in the PstSCAB high-affinity phosphate transporter, thus suggesting a possible engagement of the PhoU protein and PstSCAB transporter in phosphate signaling (Fig. 2a). To understand how MgtC promotes transcription of Pho regulon, we investigated whether MgtC physically interacts with one of the above proteins. We used a bacterial two-hybrid assay to assess the interaction between MgtC and phosphate signaling proteins by expressing a C-terminal fusion of the cyaA-T18 fragment to the mgtC gene and N-terminal fusions of the cyaA-T25 fragment to the phoR, phoU, pstB, and pstA genes in an E. coli strain lacking the CyaA adenylate cyclase. (Here, we focused on the interaction between MgtC and proteins either located in or associated with the inner membrane because MgtC is an inner membrane protein.) We then spotted cells on an LB plate containing X-gal and measured β-galactosidase production from

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**Diagram a**: Network representation of Pho regulon signaling pathways with MgtC interaction. Intracellular compartments are shown in gray.

**Diagram b**: Graph showing log2 (p_mgtC/Vector) for various genes under mgtC overexpression in a phoB mutant compared to Wild-type phoB.

**Graph c-h**: Bar graphs depicting relative mRNA levels of phoE, phoB, pstS, upgB, and phoE genes for different conditions: Vector, pmgtC, phoB, and pmgtC in Wild-type and phoB mutant backgrounds.
Regulation of the Salmonella PhoB/PhoR two-component system by the MgtC virulence protein. **a** When external phosphate levels are low, PhoR histidine kinase phosphorylates the PhoB response regulator. Phosphorylated PhoB activates transcription of several genes involved in phosphate transport including the PhoB/PhoR two-component system itself, PhoE outer membrane phosphate channel, PstSCAB inner membrane phosphate transporter, PhoU-negative regulator, UgpBAECQ glycerol-3-phosphate ABC transporter, and PhnSTUV aminoethylphosphate transporter. Independently of available phosphate levels, the MgtC virulence protein binds to the Leu421 residue of PhoR histidine kinase, activates autophosphorylation of the PhoR protein, and subsequently induces transcription of the cognate PhoB-dependent genes. This results in an increase in phosphate transport even in the absence of low-phosphate signal. **b** Heterologous expression of the mgtC gene promotes mRNA levels of genes involved in phosphate transport. Scatter plot analysis of RNA sequencing in the mgtC+expressing versus the vector-expressing Salmonella. Genes involved in phosphate transport are indicated in red. The mgtC gene from the plasmid is indicated in green. **c–h** Relative mRNA levels of the phoE (c), phoB (d), pstS (e), phnS (f), ugpB (g), and mgtC (h) genes in either wild-type (14028s) or a mgtC deletion mutant Salmonella (KK10) harboring a plasmid with the mgtC gene (pUHE21-2lacIq). Bacteria were grown for 3 h in N-minimal media containing 10 mM Mg2+ and then for an additional hour in the same media containing 0.5 mM Mg2+ and 0.25 mM IPTG. Shown are the means and SD (n = 3, independent measurements). Relative mRNA levels represent (target RNA/rpsH RNA) ×10,000. See also Supplementary Fig. 1.
a cAMP-dependent promoter that could be produced when T18 and T25 fragments of the cyaA gene functionally complemented by a physical interaction between fused MgtC and the partner proteins. Among the strains we tested, the strain expressing MgtC-T18 and T25-PhoR showed a strong blue color on the LB X-gal plate, indicating that MgtC interacts with PhoR histidine kinase (Fig. 2b). By contrast, other proteins coexpressed with MgtC-T18 exhibited a weak or no interaction, similar to those coexpressing the empty T25 fragment (Fig. 2b). The MgtR peptide that interacts with MgtC17,28 was used as a positive control.

As an independent approach, we verified the interaction between MgtC and PhoR using an immunoprecipitation assay. A C-terminally GFP-fused PhoR protein immunoprecipitated MgtC in protein extracts prepared from a strain expressing PhoR-GFP and MgtC from a constitutive and an arabinose-inducible promoter, respectively (Fig. 2c, d). Likewise, C-terminally FLAG-tagged MgtC immunoprecipitated PhoR-GFP (Fig. 2e, f). These experiments demonstrated that MgtC interacts with PhoR histidine kinase.

Leu421 of the PhoR protein is required for MgtC interaction. Given that Asn92 in MgtC is required for interaction with both PhoR and F1Fo, ATP synthase14 (Supplementary Note and Supplementary Fig. 2), we started to search for a region or residue of PhoR that is required for MgtC interaction to assess the effect of MgtC binding to PhoR independently of its binding to F1Fo ATP synthase. To identify a region(s) of PhoR that interacts with MgtC, we constructed several T25-fused phoR fragments corresponding to the TM, PAS including the short CR, CA, or both the TM and PAS domains, and tested whether each domain could interact with coexpressed MgtC-T18 (Supplementary Fig. 3a). None of the domains exhibited β-galactosidase activity when coexpressed with MgtC-T18 (Supplementary Fig. 3b), suggesting that none of the domains in the PhoR protein are sufficient for MgtC interaction. We then created a series of T25-fused phoR derivatives deleted from their C-termini to determine the minimal requirement for MgtC interaction. Interestingly, phoR derivatives that harbor the coding region up to the amino acid 421 or more (421, 422, 423, 424, 425, 426, or full-length phoR) retained the ability to interact with MgtC, while phoR derivatives with coding regions up to amino acid 420 or less (420, 419, 418, or 417) lost this ability (Fig. 3a). These results indicate that the amino acid at position 421 in PhoR, which corresponds to leucine (Fig. 3b), is required for MgtC interaction.

Homology-based structure modeling suggested that Leu421 is located at the end of the fourth β-stand in the CA domain, which is followed by a disordered C-terminal region (422–431) (Fig. 3c). T25-PhoR variants in which the Leu421 residue is substituted by residues with smaller side chains, such as valine, alanine, and glycine lost the ability to interact with MgtC-T18, whereas a T25-PhoR variant with the Leu421 to Ile substitution retained a weak binding to coexpressed MgtC-T18 (Supplementary Fig. 3a). None of the domains exhibited binding and catalyzes the transfer of γ-phosphate from ATP to a key histidine residue in the DHF domain (His213 for Salmonella PhoR), a process called autophosphorylation29. As the position of Leu421 appears to be in parallel to that of His213 in the DHF domain (Fig. 3d), the location and configuration of the leucine residue led us to hypothesize that MgtC’s binding would promote autophosphorylation by making ATP accessible to His213 in the DHF domain. To test this, we prepared membrane fractions from wild-type cells expressing either PhoR alone or MgtC and PhoR together and measured the accumulation rate of phospho-PhoR after adding γ-radiolabeled ATP. Membrane fractions prepared from wild-type cells expressing both MgtC and PhoR accumulated PhoR-P faster than those prepared from cells expressing PhoR alone (Fig. 3e, f), supporting our hypothesis that MgtC promotes autophosphorylation of PhoR histidine kinase by binding to the Leu421 residue of PhoR.

**phoR<sup>L421A</sup> substitution prevents MgtC’s effect on PhoR.** Given that all experimental conditions tested above contain 10 mM phosphate, MgtC seems to promote PhoR autophosphorylation even when phosphate is not limiting. To get a further insight into the MgtC-mediated control of PhoR histidine kinase, we created a phoR chromosomal mutant where Leu421 was substituted by either the Ala or Gly (phoR<sup>L421A</sup> to phoR<sup>L421G</sup>)(Fig. 4a), lacking the ability to interact with MgtC (Fig. 2c–f, and Supplementary Fig. 4). The phoR<sup>L421A</sup> to phoR<sup>L421G</sup> substitutions were assumed to disrupt MgtC’s binding to PhoR independently of its binding to F1Fo ATP synthase. Therefore, we first checked whether these substitutions affect intracellular ATP levels. Intracellular ATP levels of the phoR substitution mutants were unaffected in all tested conditions (Supplementary Fig. 5), suggesting that these phoR substitutions have no effect on the interaction between MgtC and F1Fo, ATP synthase.

Next, because the mgtC gene is highly expressed in low Mg<sup>2+</sup> but repressed in high Mg<sup>2+</sup> by the PhoP/PhoQ two-component system19, we expected that we could observe MgtC’s regulatory action on PhoR only in low Mg<sup>2+</sup> but not high Mg<sup>2+</sup>. The following results demonstrate our hypothesis: First, the levels of PhoP in the membrane vesicles prepared from wild-type cells increased immediately after the addition of γ-labeled ATP in low Mg<sup>2+</sup> (Fig. 4b), but PhoP was not detected until 15 min in high Mg<sup>2+</sup> (Fig. 4c). This is due to MgtC’s binding to PhoR, because the phoR<sup>L421A</sup> to phoR<sup>L421G</sup> substitutions prevented the increase of PhoP levels in low Mg<sup>2+</sup> (Fig. 4c, d). Control experiments proved that PhoP levels were unaffected by the phoR<sup>L421A</sup> to phoR<sup>L421G</sup> substitutions in high Mg<sup>2+</sup> (Fig. 4f, g).

In agreement with elevated PhoP levels observed in low Mg<sup>2+</sup>, Salmonella grown in low Mg<sup>2+</sup> promoted expression of Pho regulon because wild-type Salmonella increased the amounts of the HA-tagged PhoB protein (Fig. 4h) and mRNA levels of the phoE gene in low Mg<sup>2+</sup> (Fig. 4k). The MgtC-mediated increases of the PhoB-HA protein and phoE mRNA were not observed in the phoR<sup>L421A</sup> to phoR<sup>L421G</sup> mutants (Fig. 4h, k). Control experiments were carried out as follows: the increases in the PhoB-HA protein levels and phoE mRNA levels were not detected in non-inducing media containing 10 mM Mg<sup>2+</sup> (Fig. 4h, k), the phoR<sup>L421A</sup> to phoR<sup>L421G</sup> substitutions did not affect the expression behaviors of mRNA or protein levels of the mgtC gene (Fig. 4i, l), and Fur protein levels were unaffected in all tested conditions (Fig. 4j). We pursued the phoR<sup>L421A</sup> substitution for further experiments because even though the phoR<sup>L421A</sup> substitution eliminates MgtC-mediated PhoR autophosphorylation, the phoR<sup>L421A</sup> variant appears to be fully functional in terms of phosphate signaling, given that it retained the ability to produce PhoR-P and promote phoE mRNA levels in low phosphate, a PhoB/PhoR-inducing condition (Fig. 4k). It is interesting to note that the phoR<sup>L421G</sup> substitution had no effect on PhoR autophosphorylation but showed a defect in the increase of phoE mRNA levels in low phosphate (Supplementary Fig. 6 and Fig. 4k), suggesting that it might compromise the ability of PhoR-P to transfer its phosphate to PhoB.

The fact that MgtC increases PhoR-P levels and thus induces mRNA levels of Pho regulon suggests that the phoR<sup>L421A</sup>
substitution might have an impact on Salmonella’s ability to transport phosphate. Salmonella expressing MgtC from the plasmid immediately increased uptake of $^{32}$P-labeled orthophosphate from the medium (Supplementary Fig. 7a). The increase in phosphate uptake is mediated by MgtC’s binding because the phoR$^{L421A}$ variant did not increase phosphate uptake in the presence of MgtC (Supplementary Fig. 7b). The vector-expressing Salmonella did not show uptake of $^{32}$P-labeled orthophosphate in either the wild-type or the phoR$^{L421A}$ substitution mutant (Supplementary Fig. 7). Similar to what we observed in Salmonella strains expressing MgtC from the plasmid, wild-type Salmonella started to accumulate inorganic phosphate immediately after the addition of $^{32}$P-labeled orthophosphate when grown in low Mg$^{2+}$ to express MgtC from its chromosomal location (Fig. 4m). However, the phoR$^{L421A}$ variant was defective in phosphate transport in the same media (Fig. 4m). Control experiments demonstrated that both cells did not increase phosphate transport when grown in non-inducing media containing 10 mM Mg$^{2+}$ and then for an additional hour in the same media containing 0.5 mM Mg$^{2+}$, 0.25 mM IPTG, and 1 mM l-arabinose and membrane vesicles were prepared as described in Methods

MgtC-PhoR binding is required for Pho expression inside host. Previous transcriptome analyses have reported that several genes controlled by the PhoB/PhoR two-component system are highly induced when Salmonella is inside macrophages.$^{18,30}$ We speculated that such an increase in the mRNA levels of the PhoB/PhoR-controlled genes during infection might be due to the presence of the MgtC protein because the mgtC gene is one of the most highly expressed genes inside macrophages,$^{18}$ and also because MgtC activates PhoR autophosphorylation to promote PhoB-dependent gene expression (Figs. 1 and 4). To explore this, we infected Salmonella strains into the macrophage-like cell line J774A.1 and measured mRNA levels of the phoE and phoB genes (Fig. 1c, d). In wild-type Salmonella, mRNA levels of both the phoE and phoB genes increased at 2 h, peaked at 6 h, slightly decreased at 9 h, and then further decreased at 21 h post infection (Fig. 5a, b). We ascribed these expression behaviors to MgtC’s action on PhoR because (i) the mRNA profiles of the phoE and phoB genes exhibited similar patterns to that of the mgtC gene (Fig. 5a–c) and (ii) either a disruption of the MgtC–PhoR interaction by introducing the phoR$^{L421A}$ substitution or a removal of the mgtC gene eliminated the increase in the mRNA levels of both the phoE and phoB genes inside macrophages (Fig. 5a–c). These data suggest that the interaction between MgtC and PhoR is required for a full induction of the mRNA levels of the phoE and phoB genes inside macrophages.

phoR$^{L421A}$ substitution promotes Salmonella’s virulence. We then speculated whether MgtC-mediated expression of the Pho regulon has a physiological consequence(s) during Salmonella infection. To explore this, we measured the replication efficiency of Salmonella strains within macrophages. Unexpectedly, the phoR$^{L421A}$ substitution that prevents MgtC interaction increased Salmonella’s survival inside macrophages by up to ~300%, compared to that observed in wild-type Salmonella at 18 h post infection (Fig. 5d). Such an increase is likely due to the inability of Salmonella to express Pho genes because the phoB mutant responsible for transcription of the Pho genes showed a similar increase in intramacrophage survival (Fig. 5d). As previously reported,$^{14,31}$ the mgtC mutant showed a severe defect in macrophage survival (Fig. 5d). MgtC’s virulence function is mostly due to MgtC’s inhibitory action on the F$_1$F$_0$ ATP synthase because both the atpB mutant lacking the
a subunit of the F_{i}F_{o} ATP synthase and the atpB mgtC double mutant showed similar defects in intramacrophage survival\textsuperscript{14}. In contrast, a Salmonella strain with the phoR\textsuperscript{L421A} substitution in the mgtC mutant background showed a defect similarly to the mgtC deletion mutant (Fig. 5d) but differently to the mutant strains with the phoR\textsuperscript{L421A} substitution or phoB deletion. Therefore, MgtC’s effect on intramacrophage survival via its inhibitory interaction with the F_{i}F_{o} ATP synthase appears to be dominant over its effect via its stimulatory interaction with PhoR.
with macrophages. The percentage of cells expressing high levels of GFP (GFPHigh) was calculated from fluorescent fluorescence in Salmonella mises Pho genes via the PhoB/PhoR two-component system (Fig. 3), and subsequently activation of Pho genes by the (Fig. 5). Interestingly, the fact that the removal of MgtC-mediated regulation of mRNA levels of Pho genes inside macrophages mediated control of PhoR histidine kinase contributes to upregulation of MgtC virulence protein might impact on the formation of non-replicating Salmonella inside macrophages, because the increase in phosphate uptake apparently reduces replication inside macrophages (Fig. 5d), and also because the deletion of a gene involved in the phosphate signaling pathway has been reported to be involved in the formation of persisters, which are phenotypically similar to non-replicating cells.

To measure the formation of non-replicating Salmonella, we used a dual fluorescence-based assay by introducing a plasmid expressing GFP from an arabinose-inducible promoter and mCherry from a constitutive promoter (Supplementary Fig. 8). In principle, because GFP- and mCherry-expressing Salmonella infected macrophages without arabinose, replicating Salmonella would dilute the GFP fluorophore, leading to a decrease in GFP levels as they divide, whereas non-replicating Salmonella would retain high GFP levels (Supplementary Fig. 8). Wild-type Salmonella retained 33.9% and 15.0% of GFP-high cells inside macrophages at 6 and 9 h post infection, respectively (Fig. 5f).

If this is the case, then what would be the benefit(s) of such an increase in phosphate uptake during Salmonella infection? We reasoned that the increase in phosphate uptake mediated by the MgtC virulence protein might impact on the formation of non-growing/slow-growing Salmonella inside macrophages because the increase in phosphate uptake apparently reduces replication inside macrophages (Fig. 5d), and also because the deletion of a gene involved in the phosphate signaling pathway has been reported to be involved in the formation of persisters, which are phenotypically similar to non-replicating cells.

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The occurrence of GFP-high cells is partly due to the PhoR autophosphorylation (Fig. 3), and subsequently activation of Pho genes (Fig. 4). This MgtC-mediated control of PhoR histidine kinase contributes to upregulation of mRNA levels of Pho genes inside macrophages (Fig. 5). Interestingly, the fact that the removal of MgtC-mediated activation of Pho genes by the phoRL421A substitution promotes Salmonella virulence indicates that wild-type Salmonella activates phosphate transport despite the fact that this property compromises Salmonella’s ability to replicate within macrophages and to cause disease in mice.

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Similar to what we observed in intramacrophage survival, the phoRL421A substitution or phoB deletion rendered Salmonella hypervirulent when mice were injected with ~10^3 CFUs of Salmonella intraperitoneally (Fig. 5e). Collectively, this section of data demonstrates that MgtC’s regulatory action on expression of Pho genes via the PhoB/PhoR two-component system compromises Salmonella’s ability to survive within macrophages and virulence in mice.

**phoRL421A decreases non-replicating Salmonella inside host.** We identified that MgtC activates phosphate uptake by stimulating PhoR autophosphorylation (Fig. 3), and subsequently activating transcription of Pho genes (Fig. 4). This MgtC-mediated control of PhoR histidine kinase contributes to upregulation of mRNA levels of Pho genes inside macrophages (Fig. 5). Interestingly, the fact that the removal of MgtC-mediated activation of Pho genes by the phoRL421A substitution promotes Salmonella virulence indicates that wild-type Salmonella activates phosphate transport despite the fact that this property compromises Salmonella’s ability to replicate within macrophages and to cause disease in mice.

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Discussion

We have established that the MgtC virulence protein targets the PhoR histidine kinase, leading to activation of genes involved in phosphate transport and an increase in the non-replicating Salmonella population during infection. MgtC binds to the CA domain of PhoR histidine kinase (Figs. 2 and 3), thereby activating PhoR autophosphorylation to promote transcription of Pho genes (Figs. 3 and 4). MgtC’s action toward PhoR allows Salmonella to ensure phosphate uptake independent of the presence of a cognate signal, which might not be guaranteed during infection. Phosphate acquisition seems to be critical for normal Salmonella virulence because the phoR substitution that prevented MgtC’s binding lost the ability to activate Pho regulon during infection and rendered Salmonella hypervirulent (Fig. 5). These findings reveal an unexpected molecular link between two previously observed phenomena: the increases in the mRNA levels of the mgtC gene and pstSCABphoU operon during Salmonella infection.

It was proposed that MgtC activates PhoB indirectly by decreasing cytoplasmic Pi levels via a decrease in translation that limits the release of free cytoplasmic Pi from ATP36. This conclusion was drawn from the data that the strain lacking MgtC, which was previously shown to destabilize ribosome assembly in low Mg2+ (ref. 38), accumulated steady-state levels of intracellular phosphate when cells were grown for 5 h in low phosphate and low Mg2+36. However, we could not detect the elevated phosphate levels of the mgtC mutant when we tested our strains in the same media (Supplementary Fig. 9). In addition, our findings unequivocally demonstrate that: (i) MgtC activates mRNA levels of the PhoB-dependent genes directly by interacting with PhoR histidine kinase and (ii) MgtC exerts its effects on the increase in PhoR autophosphorylation, mRNA levels of the PhoB-dependent genes, and phosphate uptake even when expressed from the IPTG-inducible promoter for 1 h without altering intracellular phosphate levels (Supplementary Fig. 10). Given that MgtC overexpression does increase radioactive phosphate uptake immediately (Supplementary Fig. 7) but does not alter the intracellular phosphate levels in high-phosphate medium (Supplementary Fig. 10), this result reflects that steady-state levels of intracellular phosphate are relatively high and less affected by the amount of Pi transported via MgtC in this condition. Cumulatively, these data argue that MgtC induces mRNA levels of the PhoB-dependent genes independent of cytoplasmic Pi levels.

PhoQ senses low Mg2+, acidic pH, or antimicrobial peptides20–22 that could be encountered within a macrophage phagosome and phosphorylates PhoP to promote transcription of the PhoP-dependent genes, including mgtC (Fig. 6)19,39. Likewise, PhoR senses low phosphate and activates PhoB via a phosphorelay to promote transcription of Pho genes, including phoE and pstSCAB1,2. MgtC allows Salmonella to promote transcription of Pho genes in response to the PhoP/PhoQ-activating signals, low Mg2+, in addition to the PhoB/PhoR-activating signal, low phosphate. The PhoP/PhoQ- and PhoB/PhoR-activating signals exert their effects on transcription of the Pho genes independently and additively because Salmonella grown in both low Mg2+ and low phosphate further increased phoE mRNA levels, compared to those grown in low Mg2+ or low phosphate alone (Fig. 4). However, PhoP-dependent transcription is not influenced by the PhoB/PhoR-activating signal because low phosphate does not affect mgtC transcription (Fig. 4). MgtC’s function that connects the PhoP/PhoQ and PhoB/PhoR two-component systems is reminiscent of that performed by the Salmonella PmrD protein. PmrD is produced by the PhoP/PhoQ two-component system in response to low Mg2+ and then binds to the PmrB histidine kinase, thereby promoting transcription of the PmrA-dependent genes involved in lipopolysaccharide (LPS) modification/resistance to antimicrobial peptides40,41. As PmrB normally responds to high Fe3+ (ref. 42), PmrD enables Salmonella to transcribe the PmrA-regulated genes in response to two different signals: low Mg2+ and high Fe3+. MgtC and PmrD are similar to each other in a sense that the post transcriptional regulation occurred by MgtC and PmrD allows Salmonella to produce phosphate uptake proteins and LPS-modifying
enzymes in low Mg²⁺, even in the absence of cognate signals for the second two-component systems. However, the ways in which these proteins achieve activation of the second two-component system clearly differ because MgtC binds to the PhoR histidine kinase and activates PhoB-dependent transcription by promoting PhoR autophosphorylation activity, whereas PmrD binds to the PmrB histidine kinase and activates PmrA-dependent transcription by inhibiting PmrB phosphatase activity towards phospho-PmrA. In addition, MgtC is an inner membrane protein, whereas PmrD is a small cytoplasmic protein, which belongs to a group called conductor/adaptor proteins.

One way to understand the signal(s) or environment during infection is to identify bacterial genes specifically expressed during infection. Previous large-scale transcriptome analyses or during infection are actually mediated by MgtC and low phosphate-induced non-replicating bacilli in this strain. Therefore, we identified the proteins that are most interesting in the context of systemic infection. Our findings highlight the critical role that phosphate metabolism might play in pathogens’ persistence.

Methods

Bacterial strains, plasmids, primers, and growth conditions. The bacterial strains and plasmids used in this study are listed in Supplementary Table 1. All Salmonella enterica serovar Typhimurium strains are derived from the wild-type strain 14028s and were constructed by phase P22-mediated transductions. All DNA oligonucleotides are listed in Supplementary Table 2. Bacteria were grown at 37 °C in Luria-Bertani broth (LB), and N-minimal media (pH 7.7) supplemented with 0.1% casamino acids, 38 mM glycerol, and the indicated concentrations of MgCl₂. In case of low-phosphate N-minimal media, 10 mM KH₂PO₄ in the N-minimal media was replaced by 0.01 mM KH₂PO₄. Escherichia coli DH5α was used as the host for the preparation of plasmid DNA, and BTH101 lacking the cya gene was used as the host for the bacteria for the hybrid two-stage system. For Supplementary Fig. 9, we used modified MOPS media lacking CaCl₂ and containing 0.5 mM Mg⁶⁺. Ampicillin was used at 50 μg ml⁻¹, chloramphenicol at 25 μg ml⁻¹, kanamycin at 50 μg ml⁻¹, or tetracycline at 10 μg ml⁻¹. IPTG (isopropyl β-D-thiogalactopyranoside) was used at 0.25 mM, i-arabinose at 1 mM, and X-Gal (5-bromo-4-chloro-3-indolyl β-D-galactopyranoside) at 80 μM.

RNA sequencing to identify genes affected by mgtC expression. Bacteria were grown overnight in N-minimal medium containing 10 mM Mg²⁺. A 1/100 dilution of the overnight culture was used to inoculate 10 ml of the same medium, and grown for 3 h. Cells were then washed and transferred to 10 ml of N-minimal medium containing 0.5 mM Mg²⁺ and 0.25 mM IPTG to induce mgtC expression, and then grown for an additional hour. Bacteria were harvested and RNA was isolated for further analysis. Total RNA was prepared by using RNeasy mini kit (Qiagen) and the integrity of the RNA samples was measured by using BioAnalyzer 2100 (Agilent Technologies). Samples with an RNA integrity number of over 8.0 were used for the next step. For ribosomal RNA depletion, 5 μg of the total RNA was processed by Ribo-Zero rRNA Removal Kit (Bacteria) (Illumina, Inc.) and the rRNASeq assay (Illumina, Inc.). Sequencing library for RNA-Seq was constructed using Truseq Stranded Total RNA Library Prep Kit (Illumina, RS-122-2201), following the manufacturer’s instructions. Final sequencing libraries were quantified by PicoGreen assay (Life Technologies) and visualized using BioAnalyzer 2100. Sequencing was performed at ChunLab Inc. (Korea) using a HiSeq 2500 and NextSeq 500 instrument (illumina Inc.,). The following manufacturer’s protocol, which generated 100 bp single-end reads (HiSeq 2500) and 75 bp paired-end reads (NextSeq 500) for each sample. The sequencing adapter removal and quality-based trimming for the raw data were performed using Trimmomatic v. 0.36 with TruSeq adapter sequences. Cleaned reads were mapped to the reference genome using bowtie2 with a default parameter. For counting the reads mapped to each CDS (coding sequences), featureCounts was used. Finally, the count from each CDS was normalized to FPKM (fragments per kilobase million) and TPM (transcripts per million) value. Processed data were deposited in the Gene Expression Omnibus (GEO) database with accession number GSE103153.

Plasmid construction. pTGF- pho and pTGF- pho-Leu 421 Ala plasmids expressing the C-terminally GFP-fused PhoR and PhoR Leu 421 Ala proteins from the constitutive pR _p promoter promoters were constructed as follows. The pho and pho-Leu 421 Ala genes were amplified by PCR using primers KHU450 and KHU451 and 14028s or EN499 (pho-Leu 421 Ala) genomic DNA as templates. The PCR products were purified and introduced into EcoRI and BamHI restriction sites of PMBL2.

For the bacterial two-hybrid assays, the plasmids pUT18-mgc, pUT18-mgc, pUT18-mgc, pUT18-mgc, and pUT18-mgc were amplified by PCR using primers KHU450 and KHU451 and 14028s or EN499 (pho-Leu 421 Ala) genomic DNA as templates. The PCR products were purified and introduced into EcoRI and BamHI restriction sites of PMBL2.
Construction of the pKT25-verified strains were constructed by removing the CmR cassettes from Leu421Ala, Leu421Val), and KHU363/KHU545 (for phoR 1-420, Leu421Gly). Cells were grown overnight at 30 °C in LB supplemented with ampicillin (50 μg mL⁻¹) and kanamycin (50 μg mL⁻¹). Then, 2 μl of cells was spotted on solid LB medium containing 100 μM IPTG, 100 μM ampicillin, 100 μM kanamycin, and 80 μM X-Gal, followed by incubation at 30 °C for 40 h. For more detailed quantitative analysis, β-galactosidase assays were performed.

**Protein structure modeling and protein docking modeling.** We used Protein Homology/analogY Recognition Engine V 2.0 (Phyre2) to model the structure of the cytoplasmic portion of the PhoR protein (corresponding to amino acids 52–431 from Salmonella enterica serovar Typhimurium 14028s. The structure of the Salmonella PhoR protein was modeled based on the structure of VicK from Streptococcus mutans (PDB: 4R55)60. Then, we used the ChouPro webserver67,68, to dock a dimer structure of the Salmonella PhoR protein.

**Western blot analysis.** Cells were grown for 5 h in 15 ml of N-minimal medium containing 10 mM or 0.01 mM Mg2⁸⁺. Cells were normalized by measuring the optical density of the culture medium at a wavelength of 600 nm (OD600). Crude extracts were prepared in TBS (Tris-buffered saline) buffer by sonication. Whole-cell lysates were resolved on 12% sodium dodecyl sulfate (SDS)-polyacrylamide gels, and the separated proteins were transferred onto nitrocellulose membranes and incubated with monoclonal anti-HA antibodies (Santa Cruz, 1:10,000 dilution, sc-8051). The Western blots were developed by incubation with anti-rabbit IgG horseradish peroxidase-linked antibodies (ThermoFisher, 401-2150) for 1 h, and were visualized using the ECL detection system (SuperSignal® West Femto Maximum Sensitivity Substrate, Thermo). The unprocessed scans of blots with the locations of molecular weight markers are provided in the Source Data file.

**Immunoprecipitation assay.** The interaction between the MgtC and PhoR proteins was investigated in wild-type Salmonella expressing the mgtC gene from an arabinose-inducible plasmid (pBAD33-mgtC) or pBAD33-mgtC-FLAG) and C-terminally gfp-tagged phoR gene, its derivatives (pTGFP-phoR, pTGFP-phoR Leu241Ala, or pTGFP-phoR Leu241Gly), or Salmonella expressing the empty vector (pGTGF) from the constitutive promoter62. Cells were grown overnight in N-minimal media containing 10 mM Mg2⁺. A 1/100 dilution of the bacterial culture was inoculated in 15 ml of N-minimal medium containing 10 mM Mg2⁺, and grown for 3 h. Then, washed and transferred to 15 ml of N-minimal media containing 0.5 mM Mg2⁺ and 1 mM l-arabinose, and grown for 1 h. Cells were normalized by measuring OD600. Crude extracts were prepared in TBS (Tris-buffered saline) buffer by sonication. For a pull-down assay with anti-GFP antibody, 50 μl of the crude extract was kept for input and 500 μl of the protein extracts were mixed with 25 μl of anti-GFP-Trap® A beads (Chromotek) for 1 h at 4 °C on nutor (BenchMark), according to the manufacturer’s instructions. After washing the beads, the bound proteins were eluted in SDS sample buffer, separated on 12% SDS-polyacrylamide gel, and analyzed by western blotting using anti-Myc (9E10) or anti-HA antibodies raised against purified recombinant proteins (anti-GFP, anti-1:1000 dilution, Rockland, 600-401-215) antibodies for 2 h. For a pull-down assay with anti-FLAG antibodies, 50 μl of the crude extract was kept for input and 500 μl of the protein extracts were mixed with 25 μl of EZview® Red anti-FLAG™ M2 affinity gel (Sigma-Aldrich, F2426) for 1 h at 4 °C on nutor (BenchMark), according to the manufacturer’s instructions.

**Membrane vesicle preparation.** Cells were grown for 5 h in 15 ml of N-minimal medium containing 10 mM or 0.01 mM Mg2⁺ and 10 μM or 0.01 μM Pi. Cells were normalized by measuring OD600. Crude extracts were prepared in TBS (Tris-buffered saline) buffer by sonication. After removing cell debris, the membranes were isolated by centrifugation for 2 h at 40,000 x g at 4 °C (Optima™ TLX Ultracentrifuge, Type 90Ti Rotor, BeckmanCoulter). The pellets were resuspended in 500 μl of TBS buffer. The protein concentration was determined using a Nanodrop machine (ThermoFisher).

**Autophosphorylation assay.** Fifty microliters of membrane vesicles expressing PhoR, PhoR Leu241Ala, or PhoR Leu241Gly were incubated in 100 μl of TBS (Tris-buffered saline) buffer containing 1 mM MgCl₂ at room temperature. The reaction was started with the addition of [γ-32P] ATP (10 μCi, PerkinElmer) to the mixture69. A 10 μl aliquot was mixed with 10 μl of 5x SDS loading buffer (Biosegang) at different time points to stop the reaction. The samples were kept on ice until they were loaded onto a 12% SDS-polyacrylamide gel. After electrophoresis, the gel was dried on the membrane using Model 583 gel dryer (Bio-Rad) and then autoradiographed. Phosphorylated PhoR proteins were identified using samples prepared from wild-
type and the phoR mutant Salmonella grown for 5 h in N-minimal medium containing 0.01 mM Pi, a PhoB/PhoR-inducing condition.

Phosphate transport assay. Salmonella strains with the wild-type PhoR and phoR gene isolated using RNeasy Kit (QIAGEN), according to the manufacturer using Synergy H1 plate reader (BioTek). Then, 0.5 mM Mg²⁺ and soaked in 4 ml of scintillation fluid (HIDEX) using the 32P-window and by counting each vial for 10 s. The amount of Pi taken up by the cells was determined with a scintillation counter (Triathler multilabel tester, HIDEX) using the 32P-window and by counting each vial for 10 s. The amount of Pi taken up by the cells was calculated from the counts on the filters at each time-point relative to a control without cells, and data were plotted as specific activity [nmol Pi per mg protein].

Measuring intracellular phosphate levels. Salmonella strains were grown for 5 h in N-minimal medium with 10 mM and 0.01 mM Mg²⁺. Salmonella strain harboring a plasmid with the mgc gene were grown for 3 h in N-minimal medium with 10 mM Mg²⁺, and then for an additional 1 h in the same medium containing 0.5 mM Mg²⁺ and 0.25 mM IPTG. Cells were normalized by measuring OD₅₀₀ washed twice with 2 ml of 0.1 M LiCl, and then air-dried in 20 ml scintillation vials and soaked in 4 ml of scintillation fluid (HIDEX). The amount of radioactivity taken up by the cells was determined with a scintillation counter (Triathler multilabel tester, HIDEX) using the 32P-window and by counting each vial for 10 s. The amount of Pi taken up by the cells was calculated from the counts on the filters at each time-point relative to a control without cells, and data were plotted as specific activity [nmol Pi per mg protein].

Measuring intracellular ATP levels. For measuring the intracellular ATP levels, we used the BacTiter-Glo™ Microbial Cell Viability Assay (Promega, G8230), according to the manufacturer’s instruction with slight modifications. Briefly, bacteria were grown overnight in N-minimal medium containing 10 mM Mg²⁺. Then, 50 µl of the overnight culture was washed in N-minimal media without Mg²⁺ and grown for 5 h in 5 ml of N-minimal media containing 0.01 mM or 10 mM Mg²⁺. Cells were normalized by measuring OD₅₀₀ and resuspended in 1 ml PBS (phosphate-buffered saline). Then, 80 µl of this cell suspension was dispensed into an opaque 96-well plate (PerkinElmer), followed by the addition of 80 µl of BacTiter-Glo™ Reagent. The contents were then mixed briefly by pipetting and incubated for 5 min. The luminescence of the samples was measured by using Synergy H1 plate reader (BioTek).

Quantitative real-time polymerase chain reaction (qRT-PCR). Total RNA was isolated using RNeasy Kit (QIAGEN), according to the manufacturer’s instructions with slight modifications. Briefly, bacteria were grown overnight in N-minimal media containing 10 mM Mg²⁺. Then, 50 µl of the overnight culture was washed in N-minimal media without Mg²⁺ and grown for 5 h in 5 ml of N-minimal media containing 0.01 mM or 10 mM Mg²⁺. Cells were normalized by measuring OD₅₀₀ and resuspended in 1 ml PBS (phosphate-buffered saline). Then, 80 µl of this cell suspension was dispensed into an opaque 96-well microplate (PerkinElmer), followed by the addition of 80 µl of BacTiter-Glo™ Reagent. The contents were then mixed briefly by pipetting and incubated for 5 min. The luminescence of the samples was measured by using Synergy H1 plate reader (BioTek).

Macrophage survival assay. Intramacrophage survival assays were conducted using the macrophage-like cell line J774.A1.51. Briefly, 5 x 10⁴ macrophages in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum (FBS) were seeded in 24-well plates and cultured at 37 °C. Overnight grown bacteria were added to the macrophages at a multiplicity of infection (MOI) of 10:1. The infected macrophages were centrifuged at 1000 rpm for 5 min at room temperature and incubated for an additional 20 min. Then, the extracellular bacteria were washed three times with PBS (phosphate-buffered saline) and killed by incubation with DMEM supplemented with 10% FBS and 120 µg ml⁻¹ gentamicin for 1 h. For measuring the number of bacteria at 1 h, cells were lysed with PBS containing 0.1% Triton X-100 and plated on Luria-Bertani broth plates with appropriate antibiotic for 18 h. For measuring the number of bacteria at 18 h, the DMEM was replaced after 1 h with fresh DMEM containing 12 µg ml⁻¹ gentamicin, and the incubation was continued at 37 °C. After 18 h, cells were lysed with PBS containing 0.1% Triton X-100 and plated on Luria-Bertani broth plates and Luria-Bertani broth plates with appropriate antibiotic for 18 h. The percentage survival was obtained by dividing the number of bacteria recovered after 18 h by the number of bacteria present at 1 h. All experiments were performed in duplicate and the results are representative of at least three independent experiments.

Measuring gene expression inside macrophages. Gene expression inside macrophages was measured with the following modifications. Macrophage infection was performed as described in the previous section, except that RPMI tissue culture medium was used for RNA extraction. At each time-point, the infected macrophages were lysed and stabilized with Tri reagent (Applied Biosystems) and RNA was extracted according to the manufacturer’s instructions. Control RNA was obtained from Salmonella grown to exponential growth phase (OD₅₀₀ = 0.5) in N-minimal media. RNA concentration in vivo was determined by measuring (mRNA levels of each gene inside macrophages/mRNA levels of rrsH inside macrophages)/(mRNA levels of each gene in the RPMI media/mRNA levels of rrsH grown in the RPMI media).

Mouse virulence assay. Six-to-eight-week-old female C3H/HeN mice were inoculated intraperitoneally with ~10⁹ colony-forming units of Salmonella strains. Mouse survival was followed for 21 days. Virulence assays were conducted three times with similar outcomes and the data correspond to groups of five mice. All animals were housed in a temperature- and humidity-controlled room, in which a 12 h light/12 h dark cycle was maintained. All procedures were performed according to the protocols (KW-181010-1) approved by the Institutional Animal Care and Use Committee of the Kangwon National University.

Measuring non-replicating Salmonella inside macrophages. Macrophage infection and assessment of non-replicating Salmonella were performed with the following modifications. J774 A1.1 macrophages were grown in Dulbecco modified Eagle medium (DMEM; PAA Laboratories) supplemented with 10% (vol/vol) fetal bovine serum (FBS) and 1% (vol/vol) antibiotic-antimycotic solution at 37 °C and 5% (vol/vol) CO₂ conditions in a 775 flask. Prior to infection, 7 x 10⁵ macrophages were seeded in 24-well plates and incubated with 10% (vol/vol) fetal bovine serum (FBS) and 1% (vol/vol) antibiotic-antimycotic solution at 37 °C and 5% (vol/vol) CO₂ conditions for 20 h. Salmonella carrying pFCgG, an mCherry-constitutive and GFP-inducible plasmid, were grown overnight in Luria-Bertani medium with 10 mM L-arabinose to induce GFP expression, and used to infect macrophages at a multiplicity of infection of 10:1. At the indicated time points, infected macrophages were washed and lysed with PBS solution containing 0.1% Triton X-100 (Sigma) to release the intracellular bacteria. The remaining bacterial cells were pelleted and resuspended in PBS solution. The fluorescence of the bacterial population was subsequently assessed by FACS analysis. Samples were analyzed on a NovoCyte™ Flow Cytometer (ACEA) using NovoExpress® software (ACEA). On the NovoCyte™ Flow Cytometer, fluorophores were excited at a wavelength of 488 nm, and green and red fluorescence were detected at 530 and 615 nm, respectively. Data were analyzed with NovoExpress® software. To analyze the fluorescence dilution, bacteria were identified after gating on the constitutive mCherry-positive signal. A grid for chasing the remaining non-replicating cells at the indicated times was drawn based on the GFP high populations of wild-type Salmonella at 1 h post infection. The percentage of cells expressing high levels of GFP (GFP10⁵) inside J774 A1.1 macrophages at the indicated times was calculated based on the following formula: (GFP10⁵/mCherrypositive Salmonella inside the grid)/(total number of Salmonella expressing mCherry and GFP fluorescence in Q-2 area, see Supplementary Fig. 8f).

Reporting summary. Further information on research design is available in the Nature Research Reporting Summary linked to this article.

Data availability
RNA sequencing data have been deposited in the Gene Expression Omnibus (GEO) database with accession number GSE10153. All other relevant data are provided as Source Data Files or available from the corresponding author upon reasonable requests.

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**Author contributions**

E.-J.L. designed the research; S.C. and E.C. performed most experiments; Y.-J.C. performed the RNA sequencing and data deposition; D.N. and J.L. performed the initial experiments; Y.-J.C. and E.-J.L. analyzed the data; and E.-J.L and S.C. wrote the paper.

**Additional information**

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