

Salmonella promotes virulence by repressing cellulose production

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Cellulose is the most abundant organic polymer on Earth. In bacteria, cellulose confers protection against environmental insults and is a constituent of biofilms typically formed on abiotic surfaces. We report that, surprisingly, *Salmonella enterica* serovar Typhimurium makes cellulose when inside macrophages. We determine that preventing cellulose synthesis increases virulence, whereas stimulation of cellulose synthesis inside macrophages decreases virulence. An attenuated mutant lacking the *mgtC* gene exhibited increased cellulose levels due to increased expression of the cellulose synthase gene *bcsA* and of cyclic diguanylate, the allosteric activator of the BcsA protein. Inactivation of *bcsA* restored wild-type virulence to the *Salmonella mgtC* mutant, but not to other attenuated mutants displaying a wild-type phenotype regarding cellulose. Our findings indicate that a virulence determinant can promote pathogenicity by repressing a pathogen's antivirulence trait. Moreover, they suggest that controlling antivirulence traits increases long-term pathogen fitness by mediating a trade-off between acute virulence and transmission.

biofilm | magnesium | ATP

Bacterial pathogens encode genes that promote virulence. Virulence genes increase the fitness of pathogens by fostering replication at the expense of their hosts (1). Typically, virulence genes function by providing protection from host antimicrobial products, enabling the synthesis of nutrients that are limiting in host tissues and by manipulating host pathways in ways that favor pathogen survival at preferred sites. Notably, pathogens also may encode antivirulence genes, that is, genes that hamper pathogens' virulence (2–5). Here we provide a singular example of a virulence protein that promotes pathogenicity by interfering with the production of an antivirulence factor.

Cellulose is a polysaccharide composed of $\beta(1\rightarrow4)$ -linked D-glucose units. As a major structural component of the cell walls of plants and many eukaryotic microorganisms, cellulose accounts for $\sim 1.5 \times 10^{12}$ tons of the annual biomass on Earth, making it the most abundant organic polymer on the planet (6). In bacteria, cellulose is an exopolysaccharide normally synthesized in the context of organized bacterial communities known as biofilms. Cellulose inhibits bacterial motility by hindering flagellar rotation (7), and provides cohesion and structural integrity to mature biofilms (8–10).

The facultative intracellular pathogen *Salmonella enterica* serovar Typhimurium causes gastroenteritis in humans and a systemic infection in mice that resembles typhoid fever (11). During systemic infection, *Salmonella* survives and replicates in specialized membrane-bound mildly acidic vacuoles within host phagocytic cells (12, 13). Growth within these specialized compartments requires the coordinated expression of an array of virulence determinants (14), including the MgtC protein (15). MgtC is a unique virulence factor because it interacts with and inhibits the activity of *Salmonella*'s F_1F_0 ATP synthase (16), a protein complex that is responsible for synthesis of the majority of the ATP in the bacterium (17) and is also required for virulence (18). MgtC's action prevents a nonphysiological increase in cytosolic ATP and decrease in cytosolic pH taking place during

growth in mildly acidic environments, such as that experienced by *Salmonella* inside a macrophage phagosome (16).

In addition to its role in promoting intramacrophage survival, MgtC enables *Salmonella* (15, 19) and a number of phylogenetically distant intracellular bacterial pathogens (20–24) to grow normally in low-Mg²⁺ laboratory media. In *Salmonella*, growth in low-Mg²⁺ media also promotes *mgtC* expression, even when *Salmonella* experiences a neutral pH (15, 19). Notably, the *mgtC* mutant harbors higher ATP levels than the wild-type (WT) strain when grown in low-Mg²⁺ media, similar to what it exhibits on mild acidification of its surroundings (16). These findings suggest that a rise in ATP levels leads to physiological alterations that hinder growth in low-Mg²⁺ media and attenuated virulence.

We now report that, surprisingly, *Salmonella* produces cellulose when inside macrophages. We establish that the MgtC protein promotes *Salmonella* virulence by limiting cellulose production during infection. We determine that MgtC controls both expression of the cellulose synthase complex and the intracellular levels of cyclic diguanylate (c-di-GMP), the cellulose synthase's allosteric activator. Virulence can be restored to the *mgtC* mutant simply by preventing cellulose biosynthesis, which does not affect ATP levels. Our findings illustrate how *Salmonella* uses a virulence protein to repress the expression of an antivirulence trait during infection of a mammalian host, and they define cellulose as an antivirulence determinant. Moreover, they suggest that pathogens use antivirulence traits to balance acute virulence and transmission.

Results

Growth in Low Mg²⁺ Requires MgtC to Reduce Intracellular ATP Levels. ATP exists as a Mg²⁺ salt in living cells (25). Therefore, when Mg²⁺ is limiting, bacteria may need to reduce their ATP levels. Given

Significance

Salmonella enterica and a number of phylogenetically distant intracellular bacterial pathogens require the MgtC virulence protein for both intraphagosomal replication and normal growth in magnesium-limited conditions. MgtC operates by interacting with and inhibiting the F_1F_0 ATP synthase, reducing ATP levels within the bacterium. We show that by lowering ATP levels, MgtC prevents a rise in cyclic diguanylate, a second messenger that promotes biofilm formation in bacteria. We demonstrate that MgtC represses the biosynthesis of cellulose, a major structural component of *Salmonella* biofilms, and that cellulose interferes with replication inside macrophages and virulence in mice. Our results indicate that virulence genes can function to repress the expression of traits that interfere with virulence.

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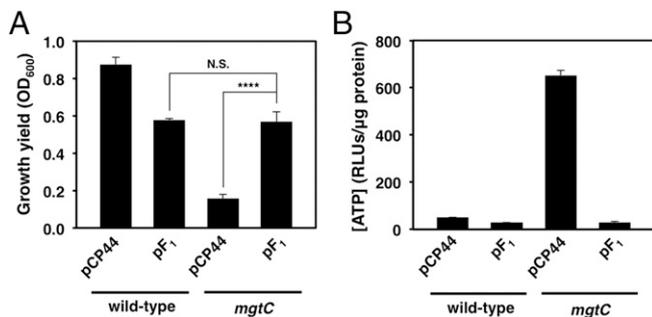


Fig. 1. High ATP levels inhibit bacterial growth in low-Mg²⁺ media. (A) Growth yield of WT (14028s) and *mgtC* (EL4) *Salmonella* strains harboring a plasmid expressing the α , β , and γ components of the ATP synthase (pF₁) or the plasmid vector (pCP44) after 24 h in low-Mg²⁺ liquid medium. Error bars represent SDs. *****P* < 0.0001, not significant (N.S.), two-tailed *t* test. Results are representative of at least six independent experiments. (B) ATP levels of WT (14028s) and *mgtC* (EL4) *Salmonella* strains harboring pF₁ or pCP44. Results are representative of at least six independent experiments.

that the function of MgtC is to lower ATP levels (25), we reasoned that the growth defect of the *mgtC* mutant in low Mg²⁺ (15, 19) might be overcome by decreasing ATP levels in an MgtC-independent fashion. To test this notion, we examined the effect of a plasmid coexpressing the genes encoding the α , β , and γ components of the F₁ subunit of the F₁F_o ATP synthase, which is known to result in elevated ATPase activity (26). This plasmid

restored growth of the *mgtC* mutant to the levels of the WT strain carrying the same plasmid (Fig. 1A). By contrast, the plasmid vector had no effect on the growth yield of the *mgtC* mutant (Fig. 1A), which retained elevated ATP levels (Fig. 1B). WT *Salmonella* carrying the plasmid with the F₁ subunit genes displayed a lower growth yield than that carrying the plasmid vector (Fig. 1A), presumably because of increased ATP hydrolysis (26) (Fig. 1B). Taken together, these results indicate that *Salmonella* growth in low Mg²⁺ requires the MgtC protein to reduce the ATP concentration.

MgtC Represses Cellulose Biosynthesis During Growth in Low Mg²⁺.

WT and *mgtC* *Salmonella* grew similarly in low-Mg²⁺ media during the first 4 h (Fig. S1A). However, starting at 6 h, the optical density of the WT culture increased for another 18 h, whereas that of the *mgtC* mutant decreased dramatically (15, 19) (Fig. S1A). The decrease in optical density of the *mgtC* mutant was accompanied by the formation of cellular aggregates at the liquid–air interface and at the bottom of the tube (24) (Fig. 2A). The formation of these aggregates is due to absence of MgtC protein because a plasmid expressing the *mgtC* gene, but not the vector control, reverted this phenotype (Fig. 2A). Given that the aggregates resembled structures deployed in *Salmonella* biofilms (8, 10), we wondered whether the MgtC protein curbs production of an extracellular structure(s) required for biofilm formation.

To identify the nature of the cellular aggregates produced by the *mgtC* mutant, we carried out a suppression analysis by inactivating genes involved in the biosynthesis of extracellular polymers that are implicated in biofilm formation or that have

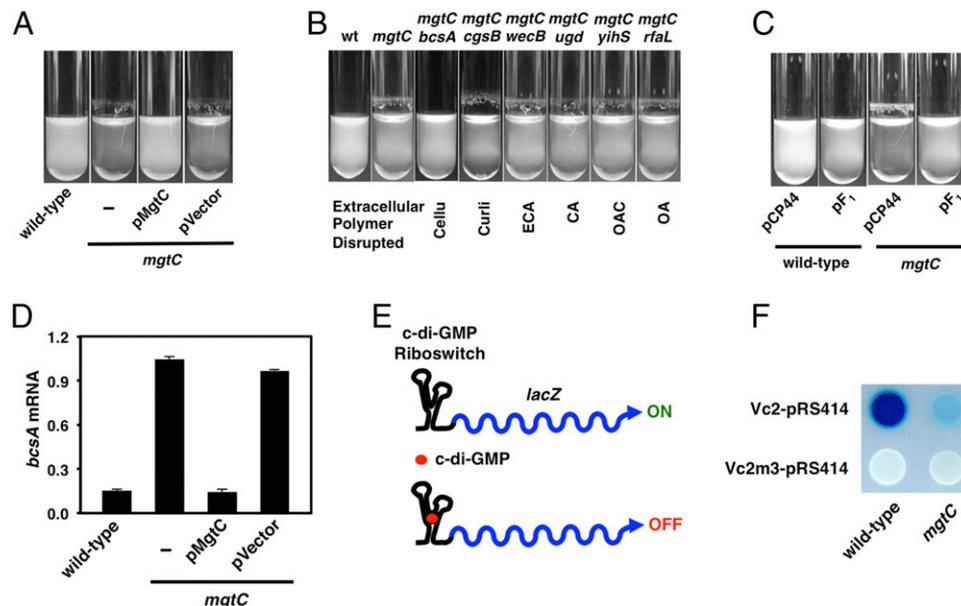


Fig. 2. The MgtC protein represses cellulose production during growth in low-Mg²⁺ media. (A) Formation of cellular aggregates at the liquid–air interface in WT (14028s), *mgtC* (EL4), and *mgtC* *Salmonella* strains harboring the *mgtC*-expressing plasmid (pMgtC) or the plasmid vector (pUHE21-2-*lacI*^q) after 24 h of growth in low-Mg²⁺ liquid medium. (B) Suppression analysis of the *mgtC* mutant (EL4) with mutations that disrupt the biosynthesis of cellulose (Cellu; MP142), curli (MP363), enterobacterial common antigen (ECA; MP118), colanic acid (CA; MP125), O-antigen capsule (OAC; MP120) or the O-antigen (OA; MP72). (C) A plasmid that expresses the F₁ subunit genes (pF₁), but not the plasmid vector (pCP44), suppresses the formation of cellular aggregates in the *mgtC* mutant (EL4), resulting in growth similar to that of the isogenic WT strain (14028s) in low-Mg²⁺ medium. (D) mRNA levels of the *bcsA* gene produced by WT (14028s), *mgtC* (EL4), and *mgtC* *Salmonella* strains harboring the *mgtC*-expressing plasmid or the plasmid vector after 8 h in low-Mg²⁺ liquid medium. Error bars represent SDs. Values were normalized to those of the *dnaK* gene. (E) Schematic of reporter for c-di-GMP levels present in plasmid Vc2-pRS414 where the *V. cholerae*'s Vc2 riboswitch (which turns off expression of a downstream gene on c-di-GMP binding) to a promoterless *lacZ* gene from *E. coli*. (F) WT (14028s) and *mgtC* (EL4) *Salmonella* colonies harboring plasmid Vc2-pRS414 with the c-di-GMP–responding riboswitch (Upper) or a derivative with a mutant riboswitch that does not respond to c-di-GMP (Vc2m3-pRS414) (Lower) after a 48-h incubation at 37 °C on 2.5 μ M Mg²⁺ N-minimal medium plates containing 5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside (X-Gal; 80 μ g/mL). The *mgtC* mutant harboring plasmid Vc2-pRS414 is of a lighter blue color (i.e., lower β -galactosidase) than the isogenic WT strain, reflecting the presence of higher c-di-GMP levels. By contrast, both WT and *mgtC* *Salmonella* form white colonies when harboring plasmid Vc2m3-pRS414 with a mutant riboswitch that is unresponsive to c-di-GMP. Results are representative of at least three independent experiments.

the potential to contribute to biofilm development (8, 10). Inactivation of the *bcsA* gene, which encodes a catalytic subunit of the cellulose synthase complex (27), suppressed aggregation (Fig. 2B) and partially rescued the growth yield of the *mgtC* mutant (Fig. S1B). By contrast, inactivation of the genes required for the biosynthesis of curli, enterobacterial common antigen, colanic acid, O-antigen capsule, or the O-antigen had no effect on the aggregation behavior (Fig. 2B) or growth yield (Fig. S1B) of the *mgtC* mutant grown in low-Mg²⁺ liquid medium.

That the aggregates formed by the *mgtC* mutant contained cellulose was further supported by three independent approaches. First, the cellulose-hydrolyzing enzyme cellulase dissolved the aggregates (Fig. S2A); second, the cellulose-binding dye calcofluor (28) stained the aggregates (Fig. S2B); and third, when grown on low-Mg²⁺ solid medium, the seemingly asymptomatic *mgtC* mutant (19) (Fig. S2C) accumulated calcofluor and the cellulose-binding dye Congo red (28) in a *bcsA*-dependent manner (Fig. S2D). These data indicate that the MgtC protein prevents cellulose biosynthesis and/or its surface deployment.

MgtC Represses Cellulose Biosynthesis by Lowering ATP levels. The increased cellulose levels displayed by the *mgtC* mutant are a downstream effect of ATP accumulation because expression of the α , β , and γ components of the F₁ subunit of the ATP synthase prevented cellulose production in the *mgtC* mutant (Fig. 2C). These results suggest that ATP alters the levels and/or activity of *Salmonella*'s cellulose synthase complex. As proposed, inactivation of the *mgtC* gene resulted in a sevenfold increase in the amount of *bcsA* mRNA (Fig. 2D), a phenotype that was corrected to WT by the plasmid expressing the *mgtC* gene, but not by the plasmid vector (Fig. 2D). The increase in *bcsA* mRNA is due to heightened cytosolic ATP because *bcsA* mRNA levels were lowered on introduction of the plasmid expressing the F₁ subunit genes, but not the vector control, into the *mgtC* mutant (Fig. S3).

The bacterial cellulose synthase complex requires allosteric activation by the second messenger c-di-GMP (27, 29). To explore whether inactivation of the *mgtC* gene alters c-di-GMP levels, we used as a reporter a plasmid harboring the c-di-GMP-responsive Vc2 riboswitch from *Vibrio cholerae* (30) fused to a promoterless *lacZ* gene from *Escherichia coli*. Because c-di-GMP binding to this riboswitch turns off *lacZ* expression, the higher the intracellular c-di-GMP concentration, the lower the β -galactosidase activity (30) (Fig. 2E). We determined that β -galactosidase activity was lower in the *mgtC* mutant harboring the reporter plasmid than in the isogenic WT strain (Fig. 2F), indicative of higher c-di-GMP levels in the *mgtC* mutant than in WT *Salmonella*. Control experiments demonstrated similar β -galactosidase activity in WT and *mgtC* *Salmonella* harboring a plasmid with a mutant riboswitch derivative unable to respond to c-di-GMP (30) (Fig. 2F). Taken together, these data indicate that the higher ATP concentration present in the *mgtC* mutant promotes cellulose production by enhancing the levels of active cellulose synthase complex.

MgtC Represses Cellulose Biosynthesis Within Phagocytic Cells. The *mgtC* gene is one of the most highly induced genes when *Salmonella* is inside a macrophage (14). We thus wondered whether *Salmonella* produces cellulose when inside a host cell and whether MgtC exerts a regulatory effect on cellulose production in this environment, similar to what was observed during growth in low-Mg²⁺ laboratory media (Fig. 2 and Fig. S2). To look specifically for cellulose inside host cells, we carried out fluorescent microscopy of macrophages infected with *Salmonella* following permeabilization and incubation with a recombinant peptide consisting of a cellulose-binding module fused to a 6-His tag (31) and an anti-6-His antibody conjugated to a fluorescent probe. We detected cellulose in macrophages containing WT and *mgtC* *Salmonella* but not in a mutant that cannot make cellulose due to inactivation of the

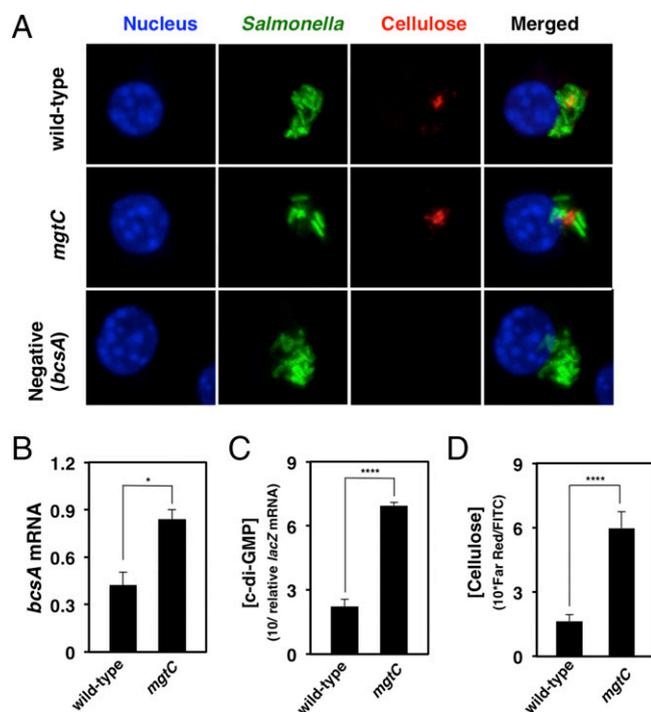


Fig. 3. *Salmonella* produces cellulose inside macrophages, and the MgtC protein reduces the levels of active cellulose synthase inside macrophages. (A) Fluorescent microscopy images of J774A.1 macrophages at 9 h post-infection with WT (14028s), *mgtC* (EL4), or *bcsA* (MP140) *Salmonella* strains harboring a plasmid (pFPV25.1) that expresses GFP constitutively. Representative images depict signals derived from the nucleic acid stain DAPI (blue), GFP-expressing *Salmonella* strains (green), and cellulose-CBM3a peptide-anti-His Alexa Fluor 647 complex (red). (B) *bcsA* mRNA levels in WT (14028s) and *mgtC* (EL4) *Salmonella* strains harvested from macrophages at 9 h postinfection. (C) Relative c-di-GMP concentrations in WT (14028s) and *mgtC* (EL4) *Salmonella* strains harboring the plasmid reporter (Vc2-prS414) harvested from macrophages at 9 h postinfection. The concentration of c-di-GMP is expressed as 10⁴/*lacZ* mRNA level. (D) Cellulose concentration in WT (14028s) and *mgtC* (EL4) *Salmonella* strains in macrophages at 9 h post-infection. The concentration of cellulose is expressed as 10⁴ · [Far Red signal]/[FITC signal]; *n* = 9. Error bars represent SDs. **P* < 0.05, *****P* < 0.0001, two-tailed *t* test. Results are representative of three independent experiments.

bcsA gene (Fig. 3A). The *mgtC* mutant had higher levels of *bcsA* mRNA (Fig. 3B), c-di-GMP (Fig. 3C), and cellulose (Fig. 3D) compared with WT *Salmonella* during growth within macrophages. Taken together, these data indicate that *Salmonella* makes cellulose inside host cells, and that MgtC controls this process by dictating the amount of active cellulose synthase.

Cellulose Hinders *Salmonella* Replication Inside Macrophages. If the virulence attenuation of the *mgtC* mutant results from enhanced cellulose levels, then inactivation of the cellulose synthase *bcsA* gene should rescue the *mgtC* mutant. As predicted, deletion of the *bcsA* gene increased replication of the *mgtC* mutant inside the macrophage-like cell line J774A.1 (Fig. 4A). Complementation experiments demonstrated that a plasmid expressing the *bcsA* gene from a heterologous promoter recovered cellulose production (Fig. S2E) and suppressed intramacrophage survival in the *mgtC bcsA* double mutant (Fig. 4A), whereas the plasmid vector had no effect (Fig. 4A and Fig. S2E). The *bcsA* mutation appears to suppress the replication defect of the *mgtC* mutant specifically because mutations in *proC* and *atpB*, which do not promote the cellulose-mediated aggregation displayed by the *mgtC* mutant (Fig. S4) but hinder intramacrophage replication due to an inability to synthesize proline (32) and to carry out

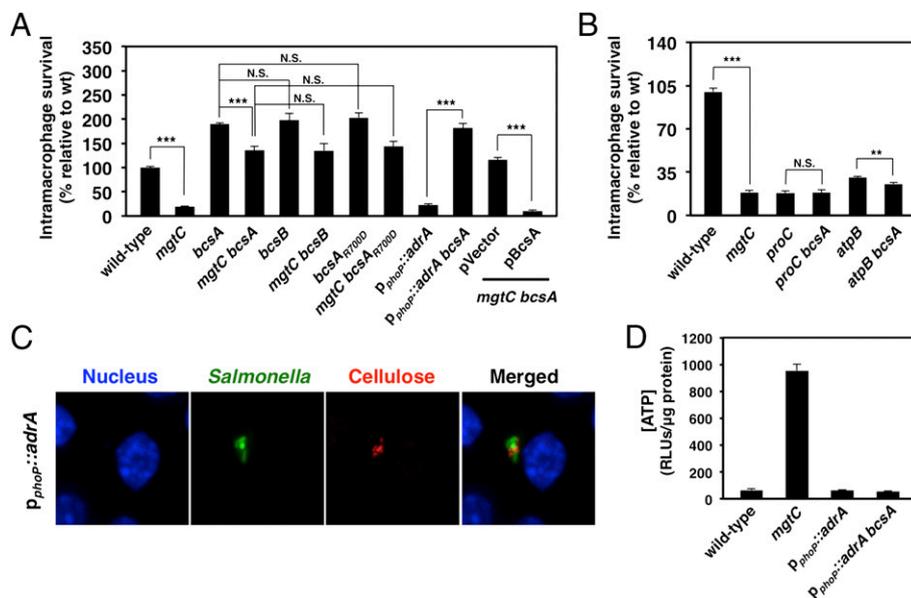


Fig. 4. Prevention of cellulose production restores intramacrophage replication to an *mgtC* null mutant. (A) Replication within J774A.1 macrophages of WT (14028s), *mgtC* (EL4), *bcsA* (MP140), *bcsA mgtC* (MP142), *bcsB* (MP654), *bcsB mgtC* (MP655), *bcsA_{R700D}* (MP518), *bcsA_{R700D} mgtC* (MP520), *P_{phoP}::adrA attTn7* (MP269), and *P_{phoP}::adrA attTn7 bcsA* (MP291) *Salmonella* strains and of the *bcsA mgtC* (MP142) mutant harboring a plasmid expressing the *bcsA* gene (*pBcsA*) or the vector control (*pUHE21-2lac^R*) at 18 h postinfection. Error bars represent SDs. ****P* < 0.001, *****P* < 0.0001, two-tailed *t* test. Results are representative of at least four independent experiments. (B) Replication within J774A.1 macrophages of WT (14028s), *mgtC* (EL4), *proC* (EL605), *proC bcsA* (MP358), *atpB* (EL515), and *atpB bcsA* (MP715) at 18 h postinfection. Error bars represent SDs. ****P* < 0.001, *****P* < 0.0001, two-tailed *t* test. Results are representative of two independent experiments. (C) Cellulose biosynthesis by strain *P_{phoP}::adrA attTn7* (MP269) inside macrophages. (D) ATP levels of WT (14028s), *mgtC* (EL4), *P_{phoP}::adrA attTn7* (MP269), and *bcsA P_{phoP}::adrA attTn7* (MP291) *Salmonella* strains after 8 h of growth in low-Mg²⁺ medium. Results are representative of two independent experiments.

oxidative phosphorylation (18), respectively, were not corrected by the *bcsA* mutation (Fig. 4B).

The enhanced virulence resulting from deletion of the *bcsA* gene is related to the inability to synthesize cellulose, as demonstrated by several findings. First, replacement of the WT copy of the *bcsA* gene by a variant specifying a BcsA protein with a single amino acid substitution in the c-di-GMP-binding site, which renders BcsA unable to synthesize cellulose (7), was sufficient to increase replication of the *mgtC* mutant inside macrophages (Fig. 4A). Second, deletion of the *bcsB* gene, which encodes the second catalytic subunit of the cellulose synthase complex (27), abolished cellulose production (Fig. S2F) and enhanced intramacrophage replication of the *mgtC* mutant (Fig. 4A). And third, the *bcsA* mutation did not restore the ATP concentration of the *mgtC* mutant to WT levels (Fig. S1C), and the same was true for mutations that prevented the biosynthesis of other extracellular polymers (Fig. S1C). These results demonstrate that excess cellulose, as opposed to the accumulation of ATP per se, is responsible for the attenuated virulence of the *mgtC* mutant.

To further examine the hypothesis that excess cellulose results in virulence attenuation, we investigated the behavior of an engineered strain with the macrophage-activated *phoP* promoter (33) fused to a promoterless *adrA* gene, which encodes a c-di-GMP synthase (34) (Fig. S5A and B). The goal was to create a *Salmonella* strain displaying increased c-di-GMP levels and resulting elevated cellulose content, but normal ATP levels, inside macrophages. The engineered strain produced cellulose in response to the low Mg²⁺ signal activating the *phoP* promoter (Fig. S5C) in a *bcsA*-dependent manner (Fig. S5D) and inside macrophages (Fig. 4C). The strain was defective for replication inside macrophages (Fig. 4A), and this defect was overcome on deletion of the *bcsA* gene (Fig. 4A). Notably, the engineered strain displayed similar ATP levels (Fig. 4D) and was phagocytosed at similar rates (Fig. S5E) as its isogenic *bcsA* counterpart,

indicating that its intramacrophage growth defect is related to excessive cellulose production as opposed to aberrant cytosolic ATP levels and/or the efficiency with which *Salmonella* is phagocytized by macrophages. Taken together, these results demonstrate that MgtC promotes intramacrophage replication by limiting cellulose biosynthesis.

Inactivation of Cellulose Biosynthesis Renders *Salmonella* Hypervirulent in Mice. Deletion of the *bcsA* gene renders WT *Salmonella* hypervirulent. This is because (i) there were twice as many *bcsA* mutant bacteria as WT *Salmonella* inside macrophages at 18 h postinfection (Fig. 4A), and (ii) C3H/HeN mice inoculated intraperitoneally with 10⁴ or 10⁵ colony-forming units (CFU) of the *bcsA* mutant died within 5 d, whereas those that received WT *Salmonella* died within 20 d and 7 d, respectively (Fig. 5). The *bcsA mgtC* double mutant behaved like the *bcsA* single mutant: inoculation with 10⁴ or 10⁵ CFU of either strain resulted in death of the animals within 5–7 d postinoculation (Fig. 5). This behavior is in contrast to that exhibited by the *mgtC* mutant, which was highly attenuated at the same doses (Fig. 5). We conclude that WT *Salmonella* curtails its virulence potential by producing some cellulose during infection.

Discussion

Bacterial cellulose has been traditionally associated with the formation of biofilms (8, 10, 29, 35, 36). Even though biofilm formation is antithetical to acute virulence (37), we have now established that *Salmonella* produces cellulose inside macrophages and that one of *Salmonella*'s virulence proteins prevents the synthesis of excessive levels of cellulose during infection (Fig. 3). By reducing the intracellular concentrations of ATP, the MgtC virulence protein prevents the onset of a c-di-GMP signaling network that activates cellulose biosynthesis. In spite of the pleiotropic effects resulting from increased ATP levels displayed by the attenuated *mgtC* mutant (16), inactivation of the cellulose synthase gene *bcsA* was sufficient to restore virulence to the

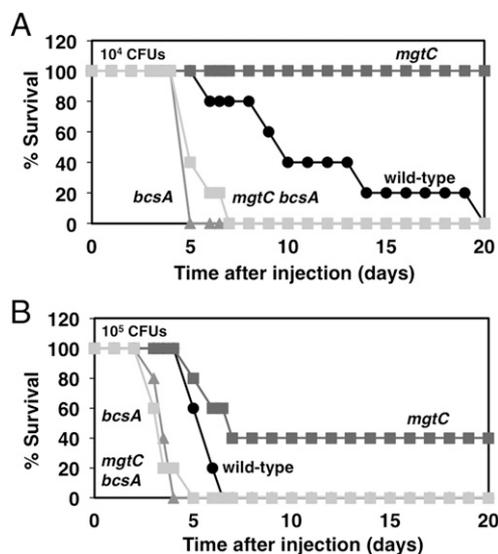


Fig. 5. Inactivation of the cellulose synthase gene *bcsA* restores virulence to an *mgtC* null mutant and increases virulence of WT *Salmonella*. Shown is survival of C3H/HeN mice injected intraperitoneally with WT (14028s), *mgtC* (EL4), *bcsA* (MP140), or *bcsA mgtC* (MP142) *Salmonella* strains at doses of 1×10^4 CFU (A) and 1×10^5 CFU (B). WT vs. *mgtC*, $P < 0.01$; WT vs. *bcsA*, $P < 0.01$; *bcsA* vs. *mgtC bcsA*, not significant (N.S.), Mantel–Cox test. Results are representative of two independent experiments.

mgtC mutant (Figs. 4 and 5) without correcting ATP levels (Fig. 5C). The idea that cellulose levels must be tightly controlled during infection is further supported by the attenuation displayed by a strain designed to overproduce cellulose inside macrophages without altering ATP levels (Fig. 4A, C, and D).

The second messenger c-di-GMP is used by a large number of bacterial species to regulate a wide range of physiological processes, including the transition from a motile planktonic state to a sessile existence in biofilms (37). The repression of c-di-GMP signaling pathways is critical during the establishment of acute infections, when bacteria must often silence traits associated with biofilm formation and environmental persistence and display those that promote virulence (37). In agreement with this notion, artificial induction of c-di-GMP signaling via the biofilm regulator CsgD impairs *Salmonella*'s ability to invade epithelial cells during the initial stages of colonization of the mammalian host (38, 39). Our findings now challenge this paradigm by establishing that *Salmonella* engages in c-di-GMP signaling that feeds into cellulose biosynthesis when the bacterium is within host phagocytic cells, and that the MgtC protein regulates one of the outputs of this signaling system (i.e., cellulose production) by reducing ATP levels within the bacterium (Fig. 1B and Fig. 2C).

The findings that the MgtC protein decreases transcription of the cellulose synthase gene *bcsA* (Fig. 2D) and lowers c-di-GMP levels (Fig. 2F) help explain why inactivation of the *phoP* gene results in heightened biofilm formation through a pathway that requires the ability of cells to produce cellulose (40). This is because the PhoP protein, a major regulator of *Salmonella* virulence (41), activates *mgtC* transcription directly by binding to the *mgtC* promoter and recruiting RNA polymerase (42).

The *bcsA mgtC* double mutant replicated better than WT and *mgtC Salmonella* inside macrophages but slightly less than the *bcsA* mutant (Fig. 4A). Moreover, during growth in low-Mg²⁺ liquid medium, the *bcsA mgtC* double mutant grew better than the *mgtC* mutant but not as well as the WT and *bcsA Salmonella* (Fig. S6). Taken together, these results suggest that MgtC controls an additional cellular function(s) that contributes to intramacrophage replication and growth in low-Mg²⁺ media.

An increase in virulence is expected to maximize the short-term rate of pathogen reproduction within the host. However, our results demonstrate that *Salmonella* reduces its virulence by expressing cellulose within host tissues. Because pathogens increase their overall reproductive rate by increasing their transmission (1), expression of cellulose, and potentially of other antivirulence traits (2–5), might allow *Salmonella* to exploit host resources slowly, which could prolong infection and increase the chance of transmission to new hosts. This hypothesis is in agreement with evolutionary models predicting that pathogens evolve levels of virulence that maximize a trade-off between exploitation of host resources (short-term fitness) and transmission (long-term fitness) (1).

Our results identify two ways in which pathogens can achieve a successful infection. First, they provide a singular example of a virulence protein (i.e., MgtC) that, rather than targeting the host, operates within the bacterium to repress production of a factor (i.e., cellulose) that antagonizes acute virulence. Second, they demonstrate that the dichotomy between virulence and biofilm formation can be implemented by a virulence protein as part of the virulence program of a pathogen.

Materials and Methods

Bacterial Strains, Plasmid Constructs, Primers, and Growth Conditions. The bacterial strains and plasmids used in this study are listed in Table S1, and oligonucleotide sequences are presented in Table S2. Single gene knockouts and deletions were carried out as described (43). Mutations generated via this method were subsequently moved into clean genetic backgrounds via phage P22-mediated transduction as described (44). Details of strain constructions are presented in SI Materials and Methods. Bacterial strains used in recombination and transduction experiments were grown in LB medium at 30 °C or 37 °C (43, 44). When required, the LB medium was supplemented with ampicillin (100 µg/mL), chloramphenicol (20 µg/mL), kanamycin (50 µg/mL), erythromycin (200 µg/mL), and/or L-arabinose (0.2% wt/vol). Growth under conditions of defined Mg²⁺ concentration was carried out in N-minimal medium (16). For strains containing mutations in F₁F₀ ATP synthase, 30 mM of D-glucose was used as the carbon source in N-minimal medium. When required, N-minimal medium was supplemented with ampicillin (25 µg/mL), erythromycin (200 µg/mL), isopropyl β-D-1-thiogalactopyranoside (IPTG) (250 µM), and either 10 mM (high) or 10 µM (low) MgCl₂.

For physiological experiments, after overnight growth in high-MgCl₂ liquid medium, cells were washed three times in Mg²⁺-free medium and inoculated (1:50) into low-MgCl₂ liquid medium.

Estimation of ATP Levels from Bacterial Samples. ATP measurements were carried out with heat-inactivated cells (70 °C for 20 min) after 8 h of growth in low-Mg²⁺ medium. Measurements were performed in a Synergy H1 Reader (BioTek) with a BacTiter-Glo Microbial Cell Viability Assay Kit (Promega) according to the manufacturers' instructions. Protein concentration in cell samples were estimated from heat-lysed cells (98 °C for 10 min) using a BCA Protein Assay Kit (Pierce). ATP measurements were normalized by the protein content of the samples.

Macrophage Survival Assay. Macrophage survival assays were conducted with the macrophage-like cell line J774A.1 as described previously (15), except that the macrophage medium was supplemented with IPTG (1 mM) when these cells were infected with *Salmonella* strains harboring plasmid pUHE-21-2*lacI*^q or its derivatives.

Mouse Virulence Assays. Mice virulence assays were carried out with 5- to 6-wk-old C3H/HeN female mice as described previously (5). Experiments were performed according to protocols approved by the Yale University Institutional Animal Care & Use Committee.

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