Supporting Information

Pontes et al. 10.1073/pnas.1500989112

SI Materials and Methods

Digestion and Calcofluor Staining of Cellulose Fibers. After overnight growth in high-Mg^{2+} (10 mM) liquid medium, bacteria were washed three times in Mg^{2+}-free medium, inoculated (1:50) into low-Mg^{2+} (10 μM) liquid medium, and grown for 24 h. Culture supernatants were then removed, and the tubes containing cellular aggregates were rinsed three times with PBS. For cellulase treatment, the tubes were filled with 4 mL of either citrate buffer (9 mM citric acid and 41 mM sodium citrate, pH 5.0), serving as a negative control, or citrate buffer supplemented with 0.1% cellulase (Sigma-Aldrich). Tubes were incubated overnight at 37 °C. The next morning, the contents of the tubes were mixed briefly to remove any loosely attached cellulose fibers from the glasses, and then the tubes were photographed.

For calcofluor staining, tubes were filled with 5 mL of either PBS, serving as a negative control, or PBS supplemented with 200 μg/mL calcofluor. After a 30-min incubation at 37 °C in the dark, the liquid was removed and the flasks were washed three times with PBS. Tubes were then visualized under white or UV (365 nm) light. Visualization of cellulose on solid medium was carried out as follows. Single colonies grown on LB plates were resuspended in 100 μL of dH_2O. Then 3 μL of the suspension was spotted on N-minimal solid medium (1) supplemented with either 10 μg/mL calcofluor or 20 μg/mL Congo red. Plates were then incubated at 37 °C for 1 d (high Mg^{2+}) or 2 d (low Mg^{2+}) and photographed.

mRNA Extraction and Quantification. Samples from macrophages infected with *Salmonella* were harvested at 9 h postinfection using TRIzol solution. Total RNA from TRIzol and *Salmonella* cell samples collected during growth in N-minimal medium were purified using the RNeasy Kit (Qiagen) according to the manufacturer's instructions. cDNA was synthesized from RNA samples using the SuperScript VILO cDNA Synthesis Kit (Life Technologies) according to the manufacturer's instructions. Quantification of transcripts was performed by quantitative PCR (qPCR) using SYBR Green PCR Master Mix (Applied Biosystems) in an ABI 7500 Sequence Detection System (Applied Biosystems). Relative amounts of cDNA were determined using a standard curve obtained from qPCR with serially diluted genomic DNA. Transcript levels of *dnaK* served as a normalizing, internal control.

Assessment of Intracellular c-di-GMP Levels in Low-Mg^{2+} Medium. Overnight cultures grown in high-Mg^{2+} (10 mM) liquid medium were washed three times in Mg^{2+}-free medium and inoculated (1:50) into low-Mg^{2+} (10 μM) liquid medium. After 8 h of growth, the optical densities of the cultures were normalized to an OD_{600} of 0.5, and 3 μL was spotted on 2.5 μM Mg^{2+} N-minimal solid medium supplemented with 25 μg/mL ampicillin and 80 μg/mL X-Gal. Plates were then incubated at 37 °C for 2 d and photographed.

Construction of Strains Containing the $bcsA_{R700D}$ Allele. A single amino acid substitution in the BcsA c-di-GMP binding site (2) was generated in the chromosomal copy of the *bcsA* gene in WT *Salmonella* (strain 14028s) as described (3) using primers listed in Table S2. An *mgtC* deletion was then introduced into this strain by transducing the Δ*mgtC*::Km^R allele from strain EL1 using phage P22 and subsequently removing the Km^R marker with the pCP20 helper plasmid (4).

Construction of the p_{phoP}::*adrA attTn7* Strain. Primers W1110 and W1111 were used to generate a gene fusion between the *phoP* promoter and a promoterless diguanylate cyclase *adrA* gene from *Salmonella* (p_{phoP}::*adrA*) by qPCR using *Salmonella* genomic DNA as a template. The PCR product was ligated into pCR-XL-TOPO (Life Technologies) and transformed into DH5α cells. The plasmid insert was then digested with NotI and XhoI and subcloned into pGRG36 (5) digested with the same restriction enzymes. The resulting construct was transformed into DH5α cells, which were maintained at 30 °C in the presence of 10 mM glucose. The pGRG36- p_{phoP}::*adrA* plasmid was then extracted from the *Escherichia coli* host (strain DH5α), and the insert was integrated into *Salmonella*'s Tn*7* attachment site as described (5). Integration of the insert into the chromosomal Tn*7* attachment site was verified by qPCR and DNA sequencing using primers 9454 and 9455. A clone containing a single base pair substitution between the putative *phoP* ribosome-biding site and the *adrA* translation start site (generated during the cloning process) exhibited reduced cellulose production at high Mg^{2+} concentrations (data not shown) and was used in our experiments.

Construction of pUHE-BcsA Plasmid. Primers W897 and W898 were used in qPCR with *Salmonella* 14028s genomic DNA as a template. The PCR product was digested with PstI and ligated into plasmid pUHE-21–2-$lacI^q$ (6) digested with the same restriction enzyme. The ligation reaction was used to transform DH5α *E. coli* cells via heat-shock. The identity of the p_{lac1-6}-*bcsA* insert was verified by DNA sequencing.

Estimation of Phagocytosed Bacteria. The macrophage-like cell line J774A.1 was allowed to grow in cell culture plates to a density of ~1×10^5 cells/well. Macrophages were then infected with *Salmonella* strains at a multiplicity of infection of 10 as described previously (7). After 30 min of phagocytosis, monolayers were washed twice with PBS and treated with gentamycin (100 μg/mL) for 1 h. Samples were then washed three times with sterile PBS, and macrophages were lyzed as described (7). The number of internalized bacteria was estimated by spreading 100 μL of serial dilutions onto LB plates as described (7).

Immunofluorescence Techniques. The macrophage-like cell line J774A.1 was allowed to grow on microscope coverslips and was then placed in cell culture plates to a density of ~1×10^5 cells/mL These cells were then infected with *Salmonella* strains as described previously (7). After 9 h of intracellular growth, the medium was removed from samples, and monolayers were washed twice with sterile PBS. Samples were then fixed and permeabilized using the Cytofix/Cytoperm Fixation and Permeabilization Kit (BD Biosciences), and subsequently blocked overnight at 4 °C with blocking buffer (1× BD Perm/Wash Buffer, 5% BSA, and 10 μg/mL mouse IgG in PBS). After blocking, samples were incubated at room temperature for 3 h with CBM3a peptide (10 μg/mL) (8) in blocking buffer. Samples were subsequently washed three times for 15 min with blocking buffer and then incubated at room temperature for 2 h with mouse anti-His Alexa Fluor 647 conjugate antibody (Qiagen), diluted 1:500 in blocking buffer.

After binding of tagged antibody, monolayers were washed at room temperature four times for 15 min each with blocking buffer. Coverslips were then mounted on microscope glass slides using ProLong Gold antifade reagent with DAPI (Life Technologies). After the coverslips were allowed to cure overnight at room temperature, slides were stored at −20 °C before microscopy.

Microscopy. Fluorescent microscope images were acquired with a Nikon Eclipse TE2000-S microscope using DAPI, FITC, and Far-Red channels. Images were analyzed with Slidebook 5.5 software (Intelligent Imaging Innovations).

1. García Véscovi E, Soncini FC, Groisman EA (1996) Mg^{2+} as an extracellular signal: Environmental regulation of *Salmonella* virulence. *Cell* 84(1):165–174.
2. Zorraquino V, et al. (2013) Coordinated cyclic-di-GMP repression of *Salmonella* motility through YcgR and cellulose. *J Bacteriol* 195(3):417–428.
3. Gerlach RG, Jäckel D, Hölzer SU, Hensel M (2009) Rapid oligonucleotide-based recombineering of the chromosome of *Salmonella enterica*. *Appl Environ Microbiol* 75(6):1575–1580.
4. Datsenko KA, Wanner BL (2000) One-step inactivation of chromosomal genes in *Escherichia coli* K-12 using PCR products. *Proc Natl Acad Sci USA* 97(12):6640–6645.
5. McKenzie GJ, Craig NL (2006) Fast, easy and efficient: Site-specific insertion of transgenes into enterobacterial chromosomes using Tn*7* without need for selection of the insertion event. *BMC Microbiol* 6:39.
6. Soncini FC, Véscovi EG, Groisman EA (1995) Transcriptional autoregulation of the *Salmonella typhimurium phoPQ* operon. *J Bacteriol* 177(15):4364–4371.
7. Lee EJ, Pontes MH, Groisman EA (2013) A bacterial virulence protein promotes pathogenicity by inhibiting the bacterium's own F_1F_o ATP synthase. *Cell* 154(1):146–156.
8. Blake AW, et al. (2006) Understanding the biological rationale for the diversity of cellulose-directed carbohydrate-binding modules in prokaryotic enzymes. *J Biol Chem* 281(39):29321–29329.
9. Hanahan D (1985) in *DNA Cloning: A Practical Approach*, ed Glover DM (IRL, Oxford, UK), Vol. 1, pp 109–135.
10. Fields PI, Swanson RV, Haidaris CG, Heffron F (1986) Mutants of *Salmonella typhimurium* that cannot survive within the macrophage are avirulent. *Proc Natl Acad Sci USA* 83(14):5189–5193.
11. Soncini FC, García Véscovi E, Solomon F, Groisman EA (1996) Molecular basis of the magnesium deprivation response in *Salmonella typhimurium*: Identification of PhoP-regulated genes. *J Bacteriol* 178(17):5092–5099.
12. Lee EJ, Choi J, Groisman EA (2014) Control of a *Salmonella* virulence operon by proline-charged $tRNA^{(Pro)}$. *Proc Natl Acad Sci USA* 111(8):3140–3145.
13. May JF, Groisman EA (2013) Conflicting roles for a cell surface modification in *Salmonella*. *Mol Microbiol* 88(5):970–983.
14. Chamnongpol S, Groisman EA (2002) Mg^{2+} homeostasis and avoidance of metal toxicity. *Mol Microbiol* 44(2):561–571.
15. Koebmann BJ, Westerhoff HV, Snoep JL, Nilsson D, Jensen PR (2002) The glycolytic flux in *Escherichia coli* is controlled by the demand for ATP. *J Bacteriol* 184(14): 3909–3916.
16. Sudarsan N, et al. (2008) Riboswitches in eubacteria sense the second messenger cyclic di-GMP. *Science* 321(5887):411–413.
17. Valdivia RH, Falkow S (1996) Bacterial genetics by flow cytometry: Rapid isolation of *Salmonella typhimurium* acid-inducible promoters by differential fluorescence induction. *Mol Microbiol* 22(2):367–378.

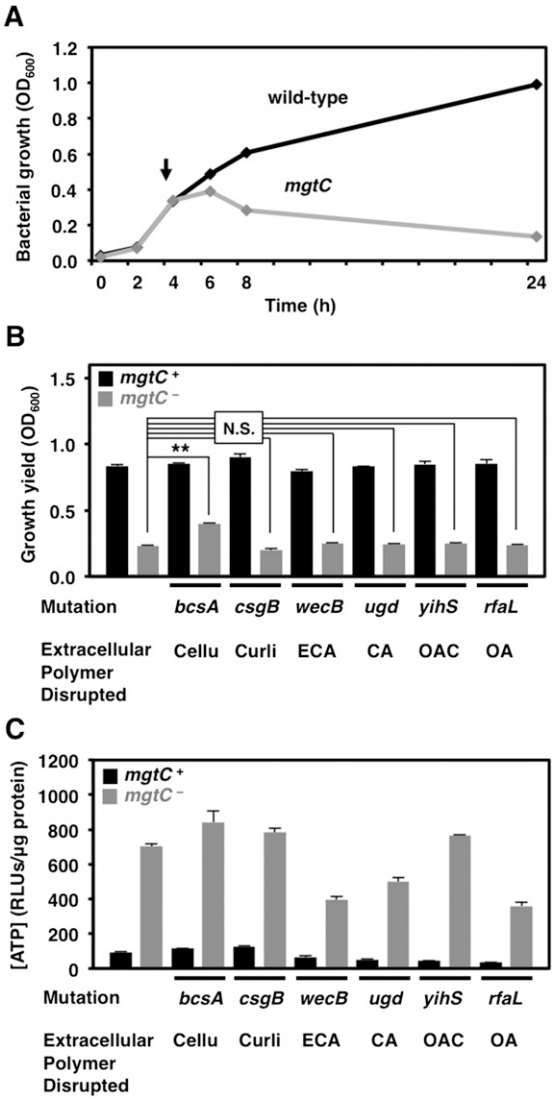

Fig. S1. Inactivation of the cellulose synthase gene *bcsA*, but not of genes specifying the synthesis of other surface components, partially restores growth of the *mgtC* mutant in low-Mg^{2+} (10 μM) media without correcting ATP levels. (*A*) Growth curves of WT (14028s) and *mgtC* mutant (EL4) *Salmonella* strains in low-Mg^{2+} liquid medium. The arrow indicates the time at which the MgtC protein is normally produced. (*B*) Growth yield of isogenic $mgtC^+$ and $mgtC^-$ *Salmonella* strains harboring mutations in genes that disrupt the biosynthesis of extracellular polymers (MP140, MP142, MP362, MP363, MP118, MP122, EG9524, MP125, MP116, MP120, MP70, and MP72). Cellu, cellulose; ECA, enterobacterial common antigen; CA, colanic acid; OAC, O-antigen capsule; OA, O-antigen. Error bars represent SDs. $^{**}P < 0.01$, two-tailed *t* test; N.S., not significant. (*C*) ATP levels in the strains listed in *B* after 8 h of growth in low-Mg^{2+} medium. Results are representative of at least four independent experiments.

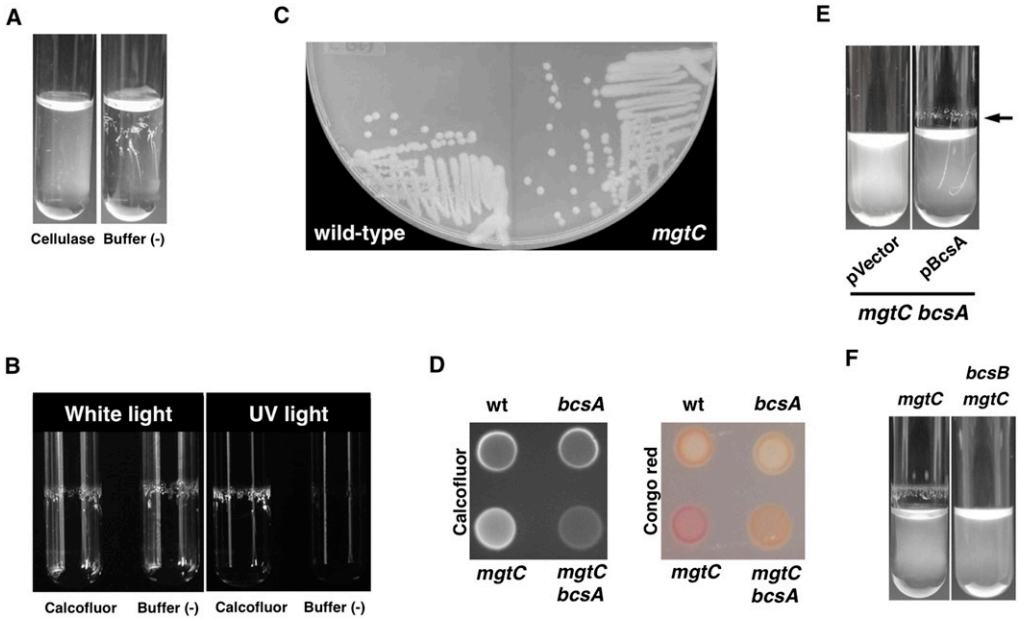

Fig. S2. MgtC represses cellulose production. (*A*) Bacterial cultures of *mgtC* mutant *Salmonella* (EL4) grown in low-Mg^{2+} (10 μM) liquid medium after overnight incubation with cellulase (*Left*) or buffer (*Right*). (*B*) Visualization of aggregates formed by the *mgtC* mutant under 360-nm UV light following staining with the cellulose-binding dye calcofluor. (*C*) WT (14028s) and *mgtC* (EL4) *Salmonella* form similar colonies on low-Mg^{2+} solid medium. (*D*) Growth of WT (14028s), *mgtC* (EL4), *bcsA* (MP140), and *bcsA mgtC* (MP142) *Salmonella* strains on low-Mg^{2+} solid medium containing the cellulose-binding dye calcofluor (10 μg/mL) or Congo red (20 μg/mL). The *mgtC* mutant accumulates these dyes in a *bcsA*-dependent fashion. (*E*) The *bcsA*-expressing plasmid pUH-BcsA, but not the plasmid vector pUHE21-2-*lacI*^{q}, restores cellulose production to the *mgtC bcsA* double mutant (MP142) after growth in low-Mg^{2+} liquid medium. Arrow indicates pellicle. (*F*) Formation of cellular aggregates at the liquid–air interface in *mgtC* (EL4) and *mgtC bcsB* (MP655) following overnight growth in low-Mg^{2+} liquid medium. Results are representative of at least four independent experiments.

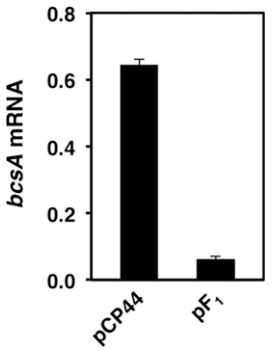

Fig. S3. mRNA levels of the *bcsA* gene increase in response to elevated cytosolic ATP levels. mRNA levels of the *bcsA* gene in *mgtC* (EL4) strains harboring the plasmid expressing the F_1 subunits of the ATP synthase (pF1) or the vector control (pCP44) after 8 h in low-Mg^{2+} liquid medium. The *bcsA* gene is located within the *bcsABZC* operon, which specifies *Salmonella*'s cellulose synthase complex. Values were normalized to those of the *dnaK* gene.

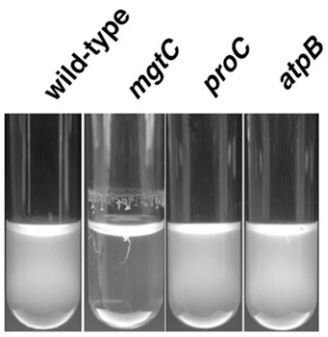

Fig. S4. Growth in low-Mg^{2+} medium of *Salmonella* strains attenuated for virulence. Pellicle formation and culture turbidity after 24 h of growth in low-Mg^{2+} liquid medium by WT (14028s), *mgtC* (EL4), *proC* (EL605), and *atpB* (MP24) *Salmonella* strains.

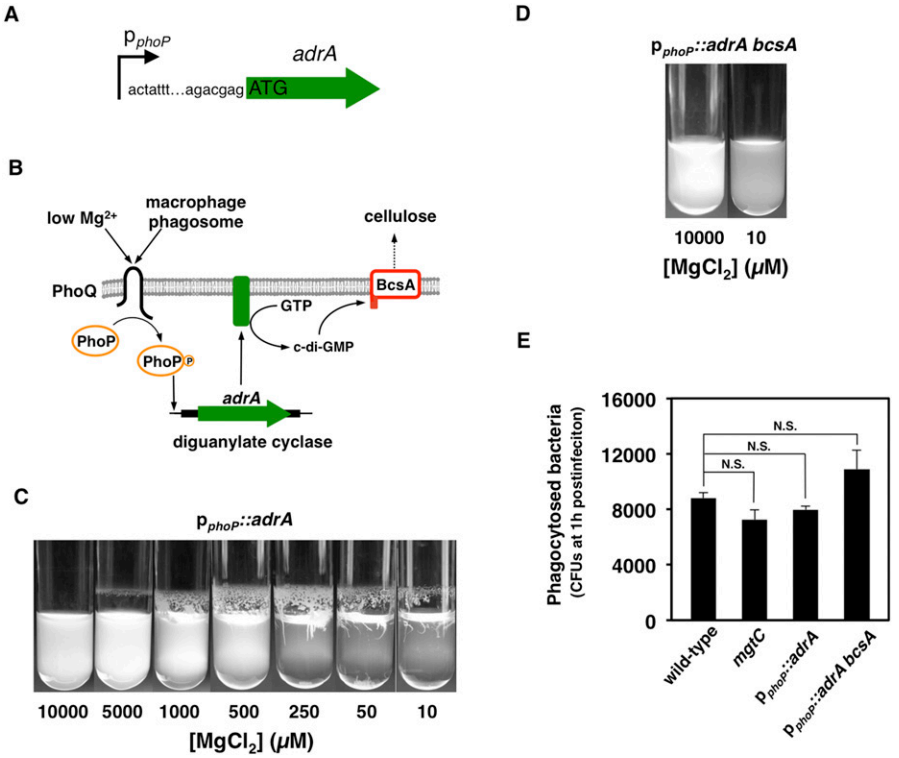

Fig. S5. A strain that produces cellulose in a *phoP*-dependent fashion. (*A*) Schematic of the gene fusion between the *S. enterica* serovar Typhimurium *phoP* promoter region to a promoterless *adrA* gene. (*B*) Schematic depicting the regulatory circuit engineered to promote cellulose biosynthesis in response to the signals activating the *phoP* promoter. (*C*) Cellulose production by p_{phoP}::*adrA att*Tn*7* *Salmonella* strain (MP269) after overnight growth at the indicated Mg^{2+} concentrations. (*D*) Same as in *C*, but for an isogenic *bcsA* mutant (MP291). (*E*) CFU of phagocytosed WT (14028s), *mgtC* mutant (EL4), p_{phoP}::*adrA att*Tn*7* (MP269), and *bcsA* p_{phoP}::*adrA att*Tn*7* (MP291) *Salmonella* strains after a 1.5-h incubation with 1×10^5 J774A.1 macrophages at a multiplicity of infection of 10. Error bars represent SDs. Results are representative of three independent experiments. *P*, not significant (N.S.), two-tailed *t* test.

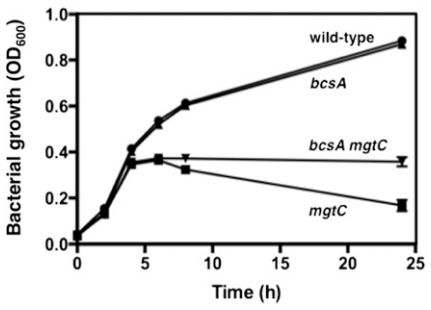

Fig. S6. Growth (OD_{600}) of WT (14028s) isogenic *bcsA* and *mgtC* *Salmonella* strains *mgtC* (EL4), *bcsA* (MP140), and *bcsA mgtC* (MP142) in low-Mg^{2+} liquid medium.

Table S1. Bacterial strains and plasmids used in this study

Strain	Relevant characteristics	Source
Escherichia coli		
DH5α	Host strain used for generation and propagation of plasmid constructs	(9)
Salmonella enterica serovar Typhimurium		
14028s	WT	(10)
EG9524	*ugd*::MudJ, Km^R	(11)
EL1	Δ*mgtC*::Km^R	This study
EL4	Δ*mgtC*	(7)
EL515	Δ*atpB*	(7)
EL605	Δ*proC*::Cm^R	(12)
MP24	Δ*atpB*::Km^R	(7)
MP70	Δ*rfaL*::Cm^R	(13)
MP72	Δ*mgtC* Δ*rfaL*::Cm^R	This study
MP116	Δ*yihS*::Km^R	(13)
MP120	Δ*mgtC* Δ*yihS*::Km^R	This study
MP118	Δ*wecB*::Cm^R	(13)
MP122	Δ*mgtC* Δ*wecB*::Cm^R	This study
MP125	Δ*mgtC ugd*::MudJ, Km^R	This study
MP133	Δ*bcsA*::Cm^R	This study
MP140	Δ*bcsA*	This study
MP142	Δ*mgtC* Δ*bcsA*	This study
MP269	p_{phoP}::*adrA attTn7*	This study
MP291	p_{phoP}::*adrA attTn7* Δ*bcsA*::Cm^R	This study
MP358	Δ*bcsA proC*::Cm^R	This study
MP362	Δ*csgB*::Km^R	This study
MP363	Δ*mgtC* Δ*csgB*::Km^R	This study
MP518	$bcsA_{R700D}$	This study
MP520	Δ*mgtC* $bcsA_{R700D}$	This study
MP654	Δ*bcsB*::Cm^R	This study
MP655	Δ*mgtC* Δ*bcsB*::Cm^R	This study
MP715	Δ*bcsA*::Cm^R Δ*atpB*:: Km^R	This study
Plasmids		
pCP20	$rep_{pSC101}{}^{ts}$ λ cI857 FLP Amp^R Cm^R	(4)
pKD3	rep_{R6Kg} Amp^R FRT Cm^R FRT	(4)
pKD4	rep_{R6Kg} Amp^R FRT Km^R FRT	(4)
pKD46	$rep_{pSC101}{}^{ts}$ Amp^R P_{araBAD}-γβexo	(4)
pGRG36	$rep_{pSC101}{}^{ts}$ Amp^R mTn*7*::MCS	(5)
pCR-XL-TOPO	rep_{pUC} Kan^R, general cloning vector	Life Technologies
pUHE-21–2-*lacI*^{q}	rep_{pMB1} *lacI*^{q} Amp^R	(6)
pUHE-MgtC	rep_{pMB1} *lacI*^{q} Amp^R P_{lac}-*mgtC*	(14)
pUHE-BcsA	rep_{pMB1} *lacI*^{q} Amp^R $P_{lac1\text{-}6}$-*bcsA*	This study
pCP44	rep_{pMB1} *lacI*^{q} Erm^R pCP44-*lacLM*, pF_1 vector control	(15)
pF_1	pCP34-AtpAGD	(15)
pCP34-AtpAGD	rep_{pMB1} *lacI*^{q} Erm^R pCP34- *atpAGD*, pF_1	(15)
Vc2-pRS414	rep_{pMB1} Amp^R P_{Vc2}-*lacZY*	(16)
Vc2m3-pRS414 M3	rep_{pMB1} Amp^R P_{Vc2_m3}-*lacZY*	(16)
pFPV25.1	rep_{ColE1} Amp^R *rpsM*::*gfp*mut3	(17)

Table S2. Oligonucleotides sequences used in this study

Name	Sequence (5′ → 3′)	Purpose	Source
1908	ATATAGCACGTACTTATTCTTCCAGAAAAAGTGTAGGCTGGAGCTGCTTC	*mgtC* inactivation	This study
1909	TGGCGTAATGTTGCAATTGAATAAAAAACTACATATGAATATCCTCCTTA	*mgtC* inactivation	This study
12241	ATGAAAAACAAATTGTTATTTATGATGTTGACAATAGTGTAGGCTGGAGCTGCTTC	*csgB* inactivation	This study
12242	GCGTTGGGTGACGCGAATAGCCATATGCGACTGTTTCTGCATATGAATATCCTCCTTA	*csgB* inactivation	This study
12244	ATTAGCACTTTGGTATGAG	Δ*csgB*::Km^R verification	This study
12589	ATGCTAACCACATCATTAAC	Δ*rfaL*::Cm^R verification	This study
12696	CAAAACGTCGTTAATACGAGG	Δ*yihS*::Km^R verification	This study
12699	AGGGGGCTGGGCCCCTACTG	Δ*wecB*::Cm^R verification	This study
12888	CCGTTTAGCGCAGCGCTCGGCTGCCTGTGGACGATTCTGCATATGAATATCCTCCTTA	*bcsA* inactivation	This study
12889	TCATTGTTGAGCCTGAGCCATAACCCGATCCGACGGCTGTGTAGGCTGGAGCTGCTTC	*bcsA* inactivation	This study
12887	ATGAGCGCCCTTTCCCGGTG	Δ*bcsA*::Cm^R verification	This study
W1509	ATTGTCCTGGATGTGTGCGGCGGTAATAGGATTAAGCGCGTGTGTAGGCTGGAGCTGCTTC	*bcsB* inactivation	This study
W1510	TACTCATGGTCAGGGTCGAGACGACGGCGACTGAGAATACGCATATGAATATCCTCCTTA	*bcsB* inactivation	This study
W1511	ATGGCTCAGGCTCAACAATG	Δ*bcsB*::Cm^R verification	This study
12190	TCATTGCCATACGTAATTC	Verification of insertions containing Cm^R markers	This study
12243	GTACGTGCTCGCTCGATG	Verification of insertions containing Km^R markers	This study
W885	ACTGGTAACCTGCAGCTTTACACTTTAAGCTTTTTATGTTTATGTTGTGTGGACCGCCGGGAGC CTGCGATGAGCGCCCTTTCCCGGTGGCTGC	Cloning of $P_{lac1\text{-}6}$-*bcsA*	This study
W885	ACTGGTAACCTGCAGTCATTGTTGAGCCTGAGCCATA	Cloning of $P_{lac1\text{-}6}$-*bcsA*	This study
W1048	TTGCGGTTTCGGTAGAGAGTAAACAGGTCAGGCGCGCGCATTTAAGACCCACTTTCACATT	Generation of *bcsA*::Tet^R	This study
W1049	TCCATCTTCGCGGGCGATGGCCCCCGGCATGGCAATCTCGACCCCTAAGCACTTGTCTCCTG	Generation of *bcsA*::Tet^R	This study
W1050	TGCGGTTTCGGTAGAGAGTAAACAGGTCAGGCGCGCGCATGATGTCGAGATTGCCATGCCGGGGGGCCATCGCCCGCGAAGATG	Generation of $bcsA_{R700D}$	This study
W1051	CATCTTCGCGGGCGATGGCCCCCGGCATGGCAATCTCGACATCATGCGCGCGCCTGACCTGTTTACTCTCTACCGAAACCGCA	Generation of $bcsA_{R700D}$	This study
W1052	TAACGTCACGGCGAAAGGC	Verification of $bcsA_{R700D}$	This study
W1053	AAGCGCCCACGTATCGGCGC	Verification of $bcsA_{R700D}$	This study
W1110	GCTCACGCGGCCGCACTATTTGTCTGGTTTATTAACTGTTTATCCCCAAAGCACCATAATCAACGCTAGACTGTTCTTATTGTTAACACAAGGGAGAAGAGATGTTCCCAAAAATAATGA	p_{phoP}::*adrA* construction	This study
W1111	ATGCTGCTCGAGTCATGCCGCCACTTCGGTGC	p_{phoP}::*adrA* construction	This study
9454	GATGCTGGTGGCGAAACTGTC	Verification of Tn*7* insertion	This study
9455	GAAGAGTGGAACGTCGGTAC	Verification of Tn*7* insertion	This study
W890	TGTGGCGATCTTCGATTGC	*bcsA* mRNA quantification	This study
W891	GCCCATTGTCATTTGCAGAA	*bcsA* mRNA quantification	This study
W892	CGCCGCGCGGTATG	*dnaK* mRNA quantification	This study
W893	GATACCGTCAGCATCGATATCG	*dnaK* mRNA quantification	This study
W1127	TGATGGTGCTGCGTTGGA	*lacZ* mRNA quantification	This study
W1128	CCGCCACATATCCTGATCTTC	*lacZ* mRNA quantification	This study