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GENETICS AND MOLECULAR BIOLOGY

glnA Truncation in *Salmonella enterica* Results in a Small Colony Variant Phenotype, Attenuated Host Cell Entry, and Reduced Expression of Flagellin and SPI-1-Associated Effector Genes

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ABSTRACT Many pathogenic bacteria use sophisticated survival strategies to overcome harsh environmental conditions. One strategy is the formation of slow-growing subpopulations termed small colony variants (SCVs). Here we characterize an SCV that spontaneously emerged from an axenic *Salmonella enterica* serovar Typhimurium water culture. We found that the SCV harbored a frameshift mutation in the glutamine synthetase gene *glnA*, leading to an ∼90% truncation of the corresponding protein. Glutamine synthetase, a central enzyme in nitrogen assimilation, converts glutamate and ammonia to glutamine. Glutamine is an important nitrogen donor that is required for the synthesis of cellular compounds. The internal glutamine pool serves as an indicator of nitrogen availability in *Salmonella*. In our study, the SCV and a constructed *glnA* knockout mutant showed reduced growth rates, compared to the wild type. Moreover, the SCV and the *glnA* mutant displayed attenuated entry into host cells and severely reduced levels of exoproteins, including flagellin and several *Salmonella* pathogenicity island 1 (SPI-1)-dependent secreted virulence factors. We found that these proteins were also depleted in cell lysates, indicating their diminished synthesis. Accordingly, the SCV and the *glnA* mutant had severely decreased expression of flagellin genes, several SPI-1 effector genes, and a class 2 motility gene (*flgB*). However, the expression of a class 1 motility gene (*flhD*) was not affected. Supplementation with glutamine or genetic reversion of the *glnA* truncation restored growth, cell entry, gene expression, and protein abundance. In summary, our data show that *glnA* is essential for the growth of *S. enterica* and controls important motility- and virulence-related traits in response to glutamine availability.

IMPORTANCE *Salmonella enterica* serovar Typhimurium is a significant pathogen causing foodborne infections. Here we describe an *S.* Typhimurium small colony variant (SCV) that spontaneously emerged from a long-term starvation experiment in water. It is important to study SCVs because (i) SCVs may arise spontaneously upon exposure to stresses, including environmental and host defense stresses, (ii) SCVs are slow growing and difficult to eradicate, and (iii) only a few descriptions of *S. enterica* SCVs are available. We clarify the genetic basis of the SCV described here as a frameshift mutation in the glutamine synthetase gene *glnA*, leading to glutamine auxotrophy. In *Salmonella*, internal glutamine limitation serves as a sign of external nitrogen deficiency and is thought to regulate cell growth. In addition to exhibiting impaired growth, the SCV showed reduced host cell entry and reduced expression of SPI-1 virulence and flagellin genes.

KEYWORDS *Salmonella enterica*, small colony variant, glutamine synthetase, motility, SPI-1

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Nontyphoidal *Salmonella* strains, such as *Salmonella enterica* serovar Typhimurium, are a leading cause of bacterial diarrhea worldwide, and the pathogens are isolated from a wide range of hosts, including humans. Infections in humans exhibit manifestations ranging from enteric symptoms to systemic disease, and they often occur as outbreaks. *S. enterica* invades host cells and proliferates intracellularly in epithelial and phagocytic cells. Among others, important virulence factors are two needle-like type III secretion systems, encoded on *Salmonella* pathogenicity island 1 (SPI-1) and SPI-2, and their individual sets of effector proteins (1–3). Furthermore, *S. enterica* possesses an extraordinary capacity to survive and to persist outside infected hosts in soil, sediments, and water, which poses a major public health concern. Water is thought to be a main reservoir, facilitating bacterial dissemination into the environment and transmission between hosts (4–6).

Natural water reservoirs often provide challenging living conditions, in terms of temperature, salinity, or nutrient availability. Such conditions lead to stress and trigger microbial phenotypic diversity (7–9). A well-known result of such diversification is the occurrence of small colony variants (SCVs), which constitute slow-growing subpopulations with unusual phenotypic traits. SCVs have been described for a range of pathogens, including staphylococci, *Escherichia coli*, pseudomonads, and *Salmonella enterica* (10). In addition to the fact that stress, including stress conferred by the immune systems of hosts, stimulates SCV formation, SCVs emerge randomly within fast-growing populations, as shown for *Staphylococcus aureus* (11). SCV formation is often caused by genetic changes that lead to growth rate reductions and auxotrophy, which can result in pleiotropic phenotypic manifestations. There is no defined core set of SCV genes. Most SCVs studied carry mutations in genes required for hemin, menadione, or thymidine biosynthesis, resulting in diminished electron transport and reduced synthesis of ATP. Importantly, specific phenotypes of auxotrophic SCVs are typically reversed by supplementation with the required metabolite (10).

Knowledge of persistence mechanisms and their reversion is of major importance for developing strategies for pathogen control. Currently, only a few descriptions of *S. enterica* SCVs are available (12–15). Cano et al. (12) isolated SCVs when fibroblasts restricting *S*. Typhimurium intracellular growth were infected with the bacteria for long periods. Several SCVs turned out to be nutrient auxotrophs, and some of them revealed a mutation in *aroD*, a gene involved in the synthesis of aromatic amino acids. The *aroD* mutants showed increased intracellular persistence rates in fibroblasts and reduced susceptibility to aminoglycoside antibiotics. Pränting and Andersson (13) described the spontaneous appearance of *S*. Typhimurium SCVs when the parental strain was treated with protamine, a cationic peptide with antimicrobial activity. The SCVs revealed mutations in genes involved in heme biosynthesis and respiration, resulting in reduced bacterial fitness but lower susceptibility to several antimicrobials. Recently, Li and colleagues (15) characterized two *S*. Typhimurium SCVs obtained with streptomycin treatment. Using a whole-genome sequencing approach, they demonstrated that frameshift mutations in the *ubiE* gene were responsible for the phenotypic switch from the wild type to the small colony variant. The *ubiE* gene product is required for coenzyme Q_8 and menaquinone biosynthesis; therefore, the resulting mutant strains are expected to have defects in the electron transport chain. Moreover, the authors uncovered two modes of genetic alterations for reversion to a fast-growing phenotype, i.e., (i) acquisition of a secondary mutation in *ubiE* or (ii) acquisition of a compensatory mutation in *pfrB*, encoding peptide chain release factor 2.

The aim of this study was to characterize a novel SCV that spontaneously emerged from a water culture inoculated with *S*. Typhimurium. Our data show that the variant harbors a loss-of-function mutation in the gene for glutamine synthetase, *glnA*. Defects in *glnA* attenuated bacterial growth and also affected motility- and virulence-related traits in *S*. Typhimurium.

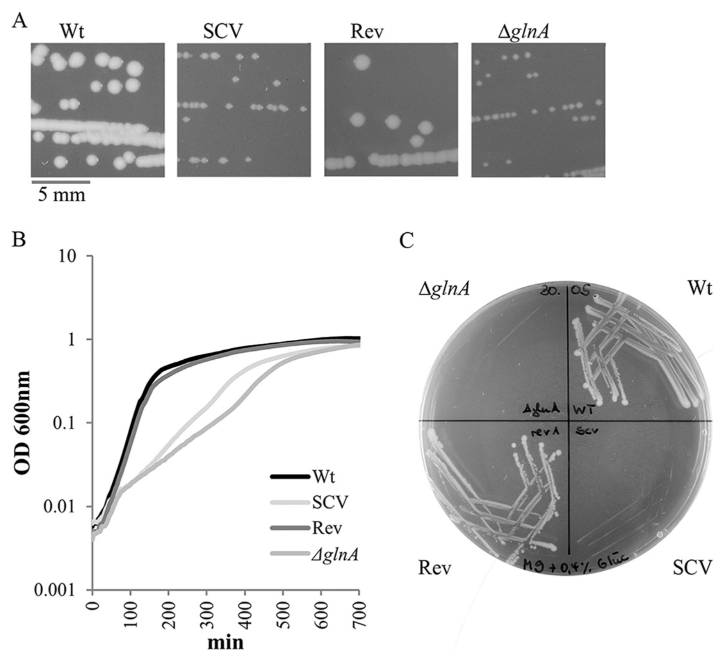

FIG 1 Attenuated growth of the *S.* Typhimurium SCV and a *glnA* knockout mutant, compared to the wild type and a revertant. (A) Colony morphology on LB agar. (B) Growth curves for growth in LB broth. (C) Growth behavior on M9G minimal medium. Data shown in panel B are means from quadruplicate cultures for which OD_{600} values were read at 15-min intervals, and all data are representative of three independent experiments. Wt, wild type; SCV, small colony variant; Rev, revertant; Δ*glnA*, *glnA* knockout mutant.

RESULTS

Isolation of an *S.* Typhimurium SCV. The SCV described in this study was isolated from an *S.* Typhimurium starvation experiment. A microcosm was set up in autoclaved distilled water with the aim of generating viable but unculturable bacteria and was stored at 23°C in the dark. The microcosm was repeatedly assayed for bacterial culturability as determined by plating aliquots on Luria-Bertani (LB) agar. Shortly after no CFU were detectable (after 913 days), a population of tiny uniform colonies emerged when the culture was plated on LB agar (day 941). To exclude contamination, we confirmed those colonies as *Salmonella* by using selective media (Oxoid *Salmonella* chromogenic medium [OSCM] and Rambach agars) and *invA* PCR (16). We colony purified one clone, termed SCV WPASI01, for further characterization.

Attenuated growth of the *S.* Typhimurium SCV, compared to the growth of the wild type and revertants. First, we examined growth of the SCV on LB agar, in LB broth, and on M9-glucose (M9G) minimal agar; the last contains glucose as the sole carbon source. As expected, the SCV showed a reduced growth rate or no growth at all, compared to the wild type (Fig. 1A to C). The SCV specifically exhibited slowed exponential growth (SCV doubling time in the exponential phase of 93 min, compared with 30 min for the wild type). Moreover, we subjected the SCV to repeated passages in rich medium in order to check whether fast-growing revertants appeared. Indeed, after two cycles, revertants showing wild-type-like colony morphology arose spontaneously (Fig. 1A). A phenotypically reverted colony, named WPASI01r (in short, Rev), was included in the growth experiments and behaved like the wild type (doubling time in the exponential phase of 31 min) (Fig. 1A to C).

Reading frame truncation in the glutamine synthetase gene *glnA* determining the SCV phenotype, which can be mimicked by *glnA* knockout. In order to clarify the genetic background of the SCV phenotype, we performed shotgun whole-genome sequencing by means of Illumina MiSeq technology. Within the genome, we identified three differences from the parental strain. First, the SCV showed an adenosine insertion

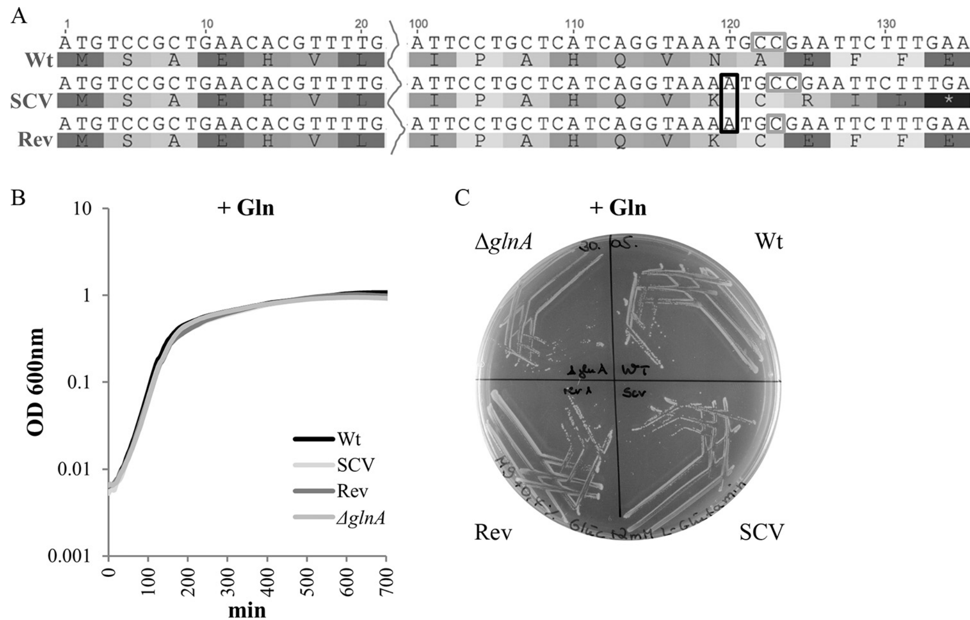

FIG 2 Reading frame truncation in the glutamine synthetase gene *glnA*, triggering the *S.* Typhimurium SCV phenotype. The addition of glutamine restores SCV and *glnA* mutant growth. (A) Genetic characteristics of the wild-type, SCV, and revertant strains with regard to the glutamine synthetase gene *glnA*. The numbering of nucleotides is relative to the translational start site. Insertion of adenosine 120 in the *glnA* gene of *S.* Typhimurium SCV (boxed in black) causes a frameshift mutation, resulting in a premature stop codon (marked by an asterisk). The revertant shows a compensatory deletion of cytosine 123 (boxed in gray), leading to restoration of the *glnA* open reading frame. (B) Growth behavior of the *S.* Typhimurium strains in liquid LB medium supplemented with 2 mM L-glutamine (+ Gln). Growth of the SCV and Δ*glnA* mutant was restored to wild-type and revertant levels, respectively, when glutamine was added to the LB broth. The results were derived from the same experiment as shown in Fig. 1B, with the difference that L-glutamine was added to the growth medium. Data shown are means from quadruplicate cultures for which OD_{600} values were read at 15-min intervals, and data are representative of three independent experiments. (C) Growth of the SCV and Δ*glnA* mutant, compared to the wild type and revertant, respectively, on 2 mM L-glutamine-supplemented M9G minimal medium (+ Gln). Wt, wild type; SCV, small colony variant; Rev, revertant; Δ*glnA*, *glnA* knockout mutant.

at position 120 in the glutamine synthetase gene *glnA*, resulting in a frame shift and therefore a premature stop codon in *glnA* at positions 133 to 135. This translated into a truncated GlnA protein (from originally 469 amino acids long to 44 amino acids), presumably with a loss of function (Fig. 2A). Second, the SCV harbored the point mutation G85T in the *hfq* gene, leading to the amino acid substitution G29C in the RNA-binding protein Hfq (17). Third, the SCV revealed the lack of an 18.9-kb genomic stretch from position 1685885 (in *srfC*) to position 1704752 (in STM1615) of the *S.* Typhimurium LT2 genome (GenBank accession no. NC_003197.1) (18).

The corresponding regions were analyzed in the revertant strain WPASI01r by means of PCR amplification and, in case of the *hfq* and *glnA* loci, PCR product sequencing. We found that, in the revertant, the mutated *hfq*G58T allele was unaffected. However, the *glnA* allele harbored a secondary mutation, which by deletion of cytosine 123 restored the original *glnA* reading frame and resulted in two amino acid substitutions, from 40N41A in wild-type GlnA to 40K41C in the revertant (Fig. 2A). Furthermore, we confirmed by means of PCR that the 18.9-kb stretch was still absent in the revertant, which indicates that deficiency of functional GlnA in the SCV determines its small colony phenotype. To test this hypothesis, we constructed an *S.* Typhimurium *glnA* knockout mutant and examined its growth behavior. Like the SCV, the *glnA* mutant formed tiny colonies on LB agar, showed a reduced exponential growth rate (130 min versus 30 min for the wild type) in LB broth, and was not able to grow on M9G minimal agar (Fig. 1A to C).

Restoration of SCV and *glnA* mutant growth with the addition of glutamine. In enteric bacteria, glutamine synthetase is the only enzyme capable of synthesizing

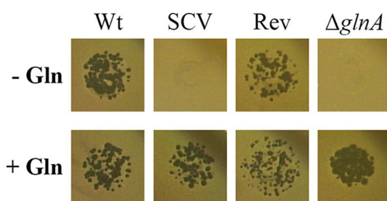

FIG 3 Felix O1 phage resistance phenotype of *S.* Typhimurium SCV and a *glnA* knockout mutant, compared to the wild type and a revertant. (Upper) In the absence of glutamine (− Gln), the wild type and revertant were susceptible to Felix O1 phage, whereas the SCV and a *glnA* knockout mutant were resistant. (Lower) In the presence of 2 mM L-glutamine (+ Gln), all strains were susceptible to Felix O1 phage. Data are representative of at least three independent experiments. Wt, wild type; SCV, small colony variant; Rev, revertant; Δ*glnA*, *glnA* knockout mutant.

glutamine (19). In order to test whether possible glutamine auxotrophy caused by GlnA truncation was responsible for the slow growth of the SCV, we analyzed the growth of wild-type, SCV, *glnA* mutant, and revertant colonies with glutamine supplementation in LB broth and M9G minimal medium. Indeed, the SCV and the *glnA* mutant grew comparably to the wild type and the revertant in glutamine-supplemented LB broth and M9G agar (Fig. 2B and C). These findings show that limitation of glutamine availability, triggered by a lack of functional GlnA, accounts for the SCV phenotype.

Increased *Salmonella* O1 phage resistance of the SCV. For further characterization of the SCV, we performed phage typing according to the scheme described by Anderson et al. (20). Mainly used for epidemiological purposes, the method uses a panel of lytic bacteriophages to subtype *S.* Typhimurium (20, 21). Despite slower growth, the phage type, determined as definitive type 4 (DT4), was not altered in the SCV or the *glnA* mutant, compared to that of the parental strain or the revertant, respectively. However, we observed a difference when susceptibility to *Salmonella* phage Felix O1 was tested (22–24). Specifically, the SCV and the *glnA* mutant showed resistance to Felix O1, whereas the wild type and the revertant were lysed. Addition of glutamine to the growth medium caused reversion of the phenotype of the SCV and the *glnA* knockout mutant to that of the wild type (Fig. 3).

Reduced host cell entry of the SCV and a *glnA* knockout mutant. Glutamine is one of only two central intermediates in nitrogen assimilation and functions as an essential amide group donor in many biosynthetic reactions (19). Thus, the defect in glutamine biosynthesis, and potential associated impairment of nitrogen metabolism, may affect the bacterium-host interplay (25). Therefore, we performed infection experiments with differentiated U937 macrophage-like cells permissive for *Salmonella* intracellular growth (26). We did not observe a difference in intracellular growth, but we noticed that SCV host cell entry was only ~30% of the wild-type level (Fig. 4A). When the SCV was grown in glutamine-supplemented broth, entry was restored to 70% of the wild-type level. The revertant showed wild-type-like behavior (Fig. 4A). As with the SCV, the number of intracellular Δ*glnA* bacteria was reduced to ~25% of the wild-type level, but the Δ*glnA* strain grown with glutamine behaved like the wild type (Fig. 4B). Our finding suggests that establishment of functional traits facilitating host cell entry in *S.* Typhimurium is linked to the glutamine pool.

Severely reduced exoprotein levels in the SCV and a *glnA* knockout mutant. Since *S.* Typhimurium invasion is driven by the SPI-1-associated type III secretion system and its effector proteins (27, 28), we compared the exoprotein pattern of early-stationary-phase SCV bacteria to that of the wild type. Indeed, we found a dramatic reduction in protein quantity in the SCV (Fig. 5A, left). However, when the SCV was grown in the presence of glutamine, the exoproteome was like that of the wild type (Fig. 5A). Moreover, *glnA* mutants derived from wild-type strains LT2 and ATCC 14028s showed SCV-like phenotypes with respect to their exoproteins (Fig. 5A and B). The exoprotein levels of a mutant with a nonfunctional SPI-1-associated secretion system (Δ*invC* mutant) were less severely reduced than in the SCV or the *glnA* mutant, suggesting a more general effect of glutamine deficiency on protein export or synthesis

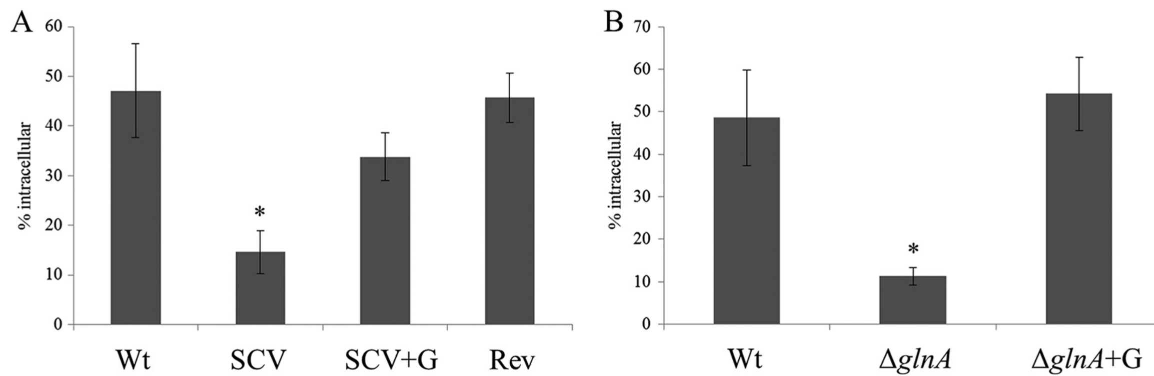

FIG 4 Reduced entry of the *S.* Typhimurium SCV and a *glnA* knockout mutant in U937 cells. Entry is restored in the revertant strain or when strains are grown in culture medium with glutamine before infection. CFU were determined after a 30-min entry period and 90 min of gentamicin killing of extracellular bacteria. (A) Entry of the wild type, SCV, and revertant grown without glutamine and the SCV grown with 2 mM L-glutamine (+G) in U937 cells. (B) Entry of the *S.* Typhimurium wild type, a *glnA* knockout mutant grown without glutamine, and a *glnA* knockout mutant grown in the presence of 2 mM L-glutamine (+G) in U937 cells. Data are representative of at least three independent experiments. *, significantly reduced number of intracellular bacteria, compared to the wild type ($P < 0.02$, Student's two-tailed *t* test, type 2). Wt, wild type; SCV, small colony variant; Rev, revertant; Δ*glnA*, *glnA* knockout mutant.

than a sole impact on SPI-1-dependent secretion (Fig. 5B). A mutant with a defect in the SPI-2-associated secretion system (Δ*ssaV* mutant) did not show visible exoprotein changes under the conditions tested (Fig. 5B). Interestingly, the whole-cell fractions of the wild-type, SCV, and *glnA* mutant strains did not show major differences in the total protein amounts, as judged by SDS-PAGE (Fig. 5A, right), implying that glutamine might specifically trigger protein export or production of exported proteins in *S.* Typhimurium. Nevertheless, some differences in the whole-cell protein patterns of the SCV and *glnA* mutant were observed; a prominent band of ~50 kDa was absent and several band size shifts of >100 kDa occurred (Fig. 5A, right). All differences reverted to the wild-type pattern with the addition of glutamine (Fig. 5A, right).

Reduced amounts of flagellin and SPI-1-associated effectors in the SCV and a *glnA* knockout mutant. Flagellin is a highly abundant protein in flagellated bacteria. Therefore, the lack of a prominent ~50-kDa protein band in the SCV cell lysates suggested reduced amounts of the flagellin proteins FliC (51.6 kDa) and/or FljB (52.5 kDa). By means of Western blotting using a *Salmonella* group flagellar antibody, we established the absence of flagellin in SCV or *glnA* mutant cell lysates and extracellular fractions. The phenotype was reversed when strains were grown with glutamine supplementation (Fig. 5C). To test whether other exported proteins were absent under low-glutamine conditions, we analyzed the known SPI-1-associated effectors SopA (87 kDa), SopB (62 kDa), and SopD (36 kDa) (29–31). We tested these effectors in particular due to missing protein bands in the extracellular fractions of the SCV, *glnA* mutant, and *invC* mutant strains that corresponded to their respective molecular sizes (3). SopA, SopB, and SopD were absent or were only weakly present in cell lysates of the SCV and *glnA* mutant strains, and levels were reduced in the corresponding extracellular fractions, suggesting decreased production, similar to findings for flagellin (Fig. 5D). The presence of effectors was restored to wild-type levels when glutamine was added for bacterial growth (Fig. 5D). These findings indicate reduced production of flagellin and the SPI-1-associated effectors in the SCV and the *glnA* mutant when glutamine levels are low; their production is again induced when glutamine levels are replenished.

Reduced expression of flagellin genes, the class 2 motility gene *flgB*, and several SPI-1-associated effector genes in the SCV and a *glnA* mutant. Next, we used semiquantitative reverse transcription (RT)-PCR to test whether decreased quantities of flagellin proteins and SPI-1-associated effectors were due to decreased mRNA levels of the respective genes, implying their reduced expression. Indeed, as shown in Fig. 6A and B, expression of the flagellin genes *fliC* and *fljB* and the SPI-1-associated effector genes *sopA*, *sopB*, and (to a lesser extent) *sopD* was reduced in the SCV or the

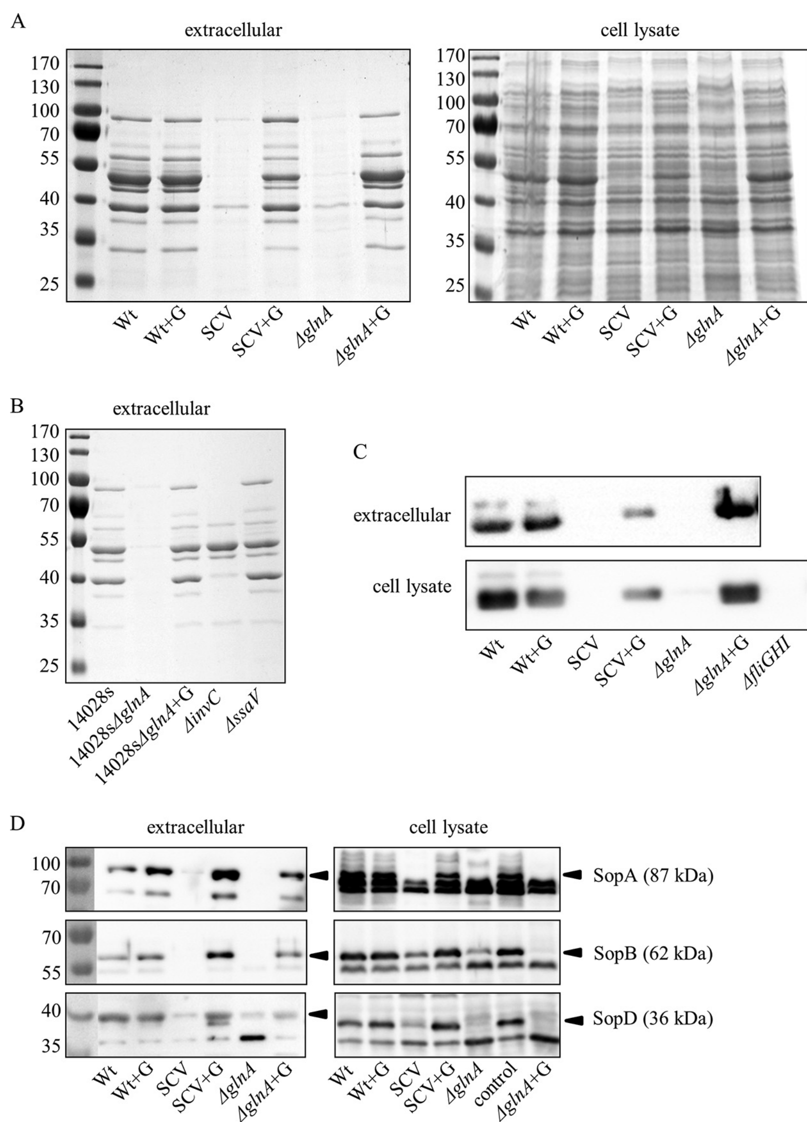

FIG 5 Severely reduced levels of flagellin and SPI-1-associated effectors in the SCV and a *glnA* knockout mutant. Protein levels are restored when strains are grown in culture medium with glutamine. (A and B) Coomassie blue-stained SDS-PAGE gels of early-stationary-phase bacteria grown in LB broth. (A) Extracellular (left) and whole-cell (right) proteins of wild-type, SCV, and *glnA* knockout mutant strains grown without or with (+G) L-glutamine supplementation. (B) *S.* Typhimurium ATCC 14028s wild-type and isogenic *glnA*, *invC* (nonfunctional SPI-1-dependent secretion system), and *ssaV* (nonfunctional SPI-2-dependent secretion system) knockout mutant strains grown without glutamine and a *glnA* knockout mutant strain grown with the addition of 2 mM L-glutamine (+G). (C and D) Western blot analysis of the extracellular and whole-cell protein fractions, as described above, with flagellin-specific antibodies (C) and SopA-, SopB-, and SopD-specific antibodies (D). Negative controls were the Δ*fliGHI* strain for antiflagellin blots, strain M712 for anti-SopA/anti-SopB blots, and strain MvP1895 for anti-SopD blots. Lanes on the left show prestained protein ladders; the values at the left indicate kilodaltons. Wt, wild type; SCV, small colony variant; Δ*glnA*, *glnA* knockout mutant; 14028s, ATCC 14028s; control, negative control.

glnA mutant, compared to the wild type or the revertant (Fig. 6A and B). This shows that both flagellin and SPI-1 effector gene transcription is linked to intact *glnA* and presumably glutamine availability. Next, we asked the question of which stage of the regulatory network controlling flagellum biosynthesis is affected. In *E. coli* and *S. enterica*, flagellar genes are organized into a transcriptional hierarchy of three promoter classes. Class 3 promoters drive expression of late motility genes, such as *fliC* and *fljB*, and depend on the flagellum-specific alternative sigma factor σ^{28}, coded by *fliA*. The *fliA* gene belongs to one of several class 2 operons producing proteins required for the

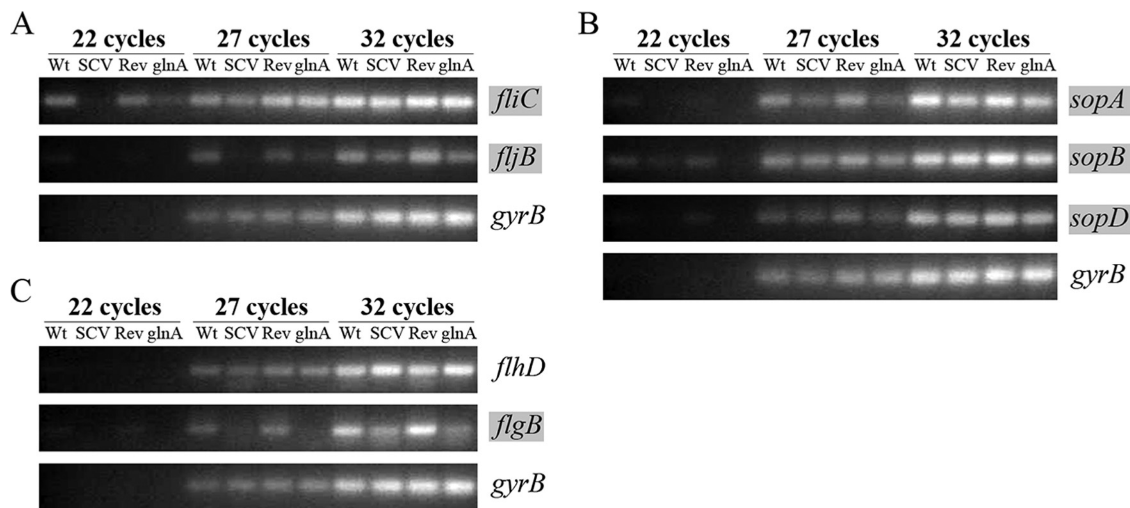

FIG 6 Reduced expression of flagellin genes, the class 2 motility gene *flgB*, and several SPI-1-associated effector genes in the SCV and the *glnA* mutant, compared to the wild type and a genetic revertant. Analysis of transcript levels in different *S.* Typhimurium strain backgrounds was performed by semiquantitative RT-PCR, using gene-specific primers for the flagellin-encoding genes *fliC* and *fljB* (class 3 motility genes) (A), the SPI-1 effector genes *sopA*, *sopB*, and *sopD* (B), and the class 2 motility gene *flgB* and the class 1 motility gene *flhD* (C). Transcript levels of *S.* Typhimurium *gyrB*, the constitutively expressed gene encoding gyrase subunit B, were used as the control. Images represent agarose gels of ethidium bromide-stained PCR products after different rounds of amplification. Genes whose transcript levels differed markedly in the different strain backgrounds are marked in gray. Data are representative of three independent experiments. Wt, wild type; SCV, small colony variant; Rev, revertant; *glnA*, *glnA* knockout mutant.

structure and assembly of the flagellar hook basal body. Transcription of class 2 promoters depends, in turn, on the transcriptional activator complex $FlhD_4C_2$, which is encoded by the class 1 *flhDC* operon (32, 33). Therefore, we analyzed expression of representative class 2 and class 1 promoter genes, i.e., *flgB*, encoding a component of the flagellar basal body, and *flhD*, respectively. We observed that *flgB* but not *flhD* expression was reduced in the SCV and the *glnA* mutant, compared to the wild type and the revertant (Fig. 6C). This finding reveals that reduced expression of class 2 and class 3 motility genes is not linked to decreased expression of the class 1 flagellar master operon *flhDC*. The regulation circuit hindered upstream of class 2 flagellar genes remains to be determined.

DISCUSSION

In this study, we analyzed the genetic basis and the phenotype of an *S.* Typhimurium SCV that emerged under nutrient limitation. It is important to elucidate the genetic alterations leading to SCV formation because (i) SCVs may arise spontaneously upon stress exposure, (ii) SCVs are slow growing and difficult to eradicate, and (iii) only a few descriptions of *S. enterica* SCVs are available (34).

We found that the characterized SCV harbors ~90% truncation of the *glnA* gene, encoding glutamine synthetase (Fig. 2A). Glutamine synthetase, a major enzyme in nitrogen assimilation, converts glutamate and ammonia to glutamine in an ATP-driven reaction (35). Glutamine is a central intermediate of nitrogen metabolism and serves as an indicator of nitrogen availability in *Salmonella*. Specifically, when external nitrogen sources are limited, the internal glutamine pool decreases, whereas the glutamate pool remains stable (36, 37). Thus, *S.* Typhimurium *glnA* mutants simulate nitrogen limitation internally even when excess nitrogen is available externally. Furthermore, it is thought that a reduced glutamine pool under nitrogen-limiting conditions is responsible for slow growth (36). This is in accordance with our observation that strains with impaired glutamine synthetase (due to loss-of-function gene truncation or *glnA* knockout) display small colony morphology and reduced growth rates even if cultivated in rich medium, such as LB medium (Fig. 1). Glutamine supplementation of growth media causes reversion of this phenotype (Fig. 2B and C), indicating that the glutamine

transporter encoded by g*lnHPQ* is expressed in the SCV and the *glnA* knockout mutant at sufficient levels to replenish the internal glutamine pool (38, 39).

The amide group in glutamine is required as a nitrogen source in early steps of the synthesis of monomeric building blocks of most cellular macromolecules, such as proteins, nucleic acids, and surface polymers (25). Therefore, it can be expected that the phenotypes of the SCV and a *glnA* mutant might strongly differ from the wild-type phenotype. We found that the SCV and *glnA* mutant were severely attenuated in their entry into macrophages (Fig. 4). Earlier studies addressed the fate of *glnA* mutants in alfalfa seed exudates or intraperitoneal BALB/c mouse infection models. Slight attenuation of mutant growth in the alfalfa exudate but not in the mouse model was observed (39, 40). However, combined knockout of *glnA* and the glutamine transporter genes *glnH* and *glnQ* or genes of the nitrogen regulatory system (*ntr*), which is responsible for transcription of the glutamine transporter genes, led to severe growth attenuation in the mouse model and impaired intracellular survival in J774 macrophages, showing that glutamine uptake from the host plays an essential role in infection (39). It is important to note that studies with intraperitoneal instead of oral infection of mice might overlook important entry-associated effects.

Invasion into nonphagocytic cells and phagocytes is promoted by effectors of the SPI-1-encoded type III secretion system and by flagellin (2, 3, 28, 41). Accordingly, we found that both the SCV and a *glnA* mutant lacked flagellin and SPI-1 effector proteins, due to reduced expression of the corresponding genes. Expression of both virulence and motility regulons is under tight control, as these processes require substantial resources from the bacterial cell (42). More than 60 genes contribute to the synthesis, assembly, and functioning of the flagellar system in *Salmonella* (43). Flagellar operons are divided into three classes, based on the promoters that drive their transcription (33, 44). The class 1 operon *flhDC* produces the multimeric master regulator of flagellar synthesis, $FlhD_4C_2$, which is essential for the expression of all downstream motility genes (32). $FlhD_4C_2$ activates transcription of class 2 operons encoding structural genes of the hook basal body, a flagellum-specific type III secretion system, and the alternative sigma factor σ^{28}, which is required for class 3 promoter transcription. Upon completion of the hook basal body substructure, the σ^{28} anti-sigma factor FlgM is secreted; hence, free σ^{28} is able to transcribe class 3 promoters, resulting in synthesis of filament and motor proteins and the chemotaxis machinery (Fig. 7). In *S. enterica*, flagellum and SPI-1 gene expression is interconnected (45–49). The regulatory interlink occurs via the FliZ protein, which is part of the *fliAZ* operon that can be transcribed from class 2 and class 3 promoters (Fig. 7). In addition to being a positive regulator of $FlhD_4C_2$ activity (50), FliZ posttranslationally activates the regulatory protein HilD, which, in turn, positively regulates the expression of *hilA*, the gene for a key regulator of the SPI-1 system (45, 51, 52). Furthermore, HilD acts as a direct activator of *flhDC* expression, strengthening the transcriptional cross talk between the flagellar and SPI-1 regulons in *Salmonella* (52). When measuring mRNA levels in the SCV and a *glnA* knockout mutant, in comparison to the wild type, we found reduced transcript levels for SPI-1-associated effector genes as well as class 3 and class 2 motility genes (Fig. 6). Reduced expression of class 2 genes such as *fliZ* would give a plausible explanation for the observed reductions in both flagellin and SPI-1 effector protein levels. Here, *flgB* mRNA levels were analyzed as an example of class 2 genes (Fig. 6). Interestingly, we did not observe changes in *flhD* expression in the SCV or *glnA* mutant, which implies that impairment of flagellar synthesis takes place at a step subsequent to class 1 transcription (Fig. 7). Examples of possible factors involved include the flagellar protein FliT (an anti-$FlhD_4C_2$ factor fine-tuning flagellar expression to the state of flagellar assembly [53, 54]), the molecular chaperone DnaK (55), and the EAL-like protein YdiV (56–58). The last is of special interest because YdiV inhibits $FlhD_4C_2$ activity when nutrients are scarce (57, 59).

Finally, the SCV showed reduced susceptibility to the *Salmonella* phage Felix O1, whereas the wild-type and revertant strains revealed the lysis pattern observed for the vast majority of *Salmonella* strains (Fig. 3) (23). This demonstrates that, upon exposure

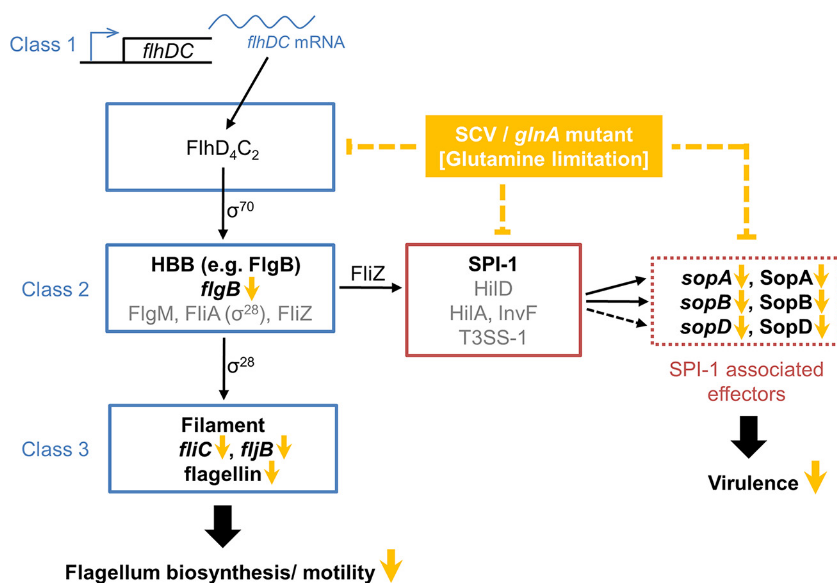

FIG 7 Model of the flagellar gene network and SPI-1-associated effector expression in the *S.* Typhimurium SCV and a *glnA* mutant. Functional impairment of *glnA* in the SCV or a *glnA* knockout mutant results in glutamine limitation and negatively affects flagellum biosynthesis and SPI-1-mediated virulence. Specifically, reduced expression of class 2 (*flgB*) and class 3 (*fliC* and *fljB*) motility genes and SPI-1-associated effector genes was detected, accompanied by reduced abundance of flagellin and effector proteins (depicted by orange arrows). The dashed orange lines outline the different possible ways of influencing flagellar and SPI-1-associated effector expression upstream of the class 2 motility genes. It is established that the flagellar and SPI-1 regulons are interconnected by the class 2 motility gene product FliZ, which activates SPI-1 via the HilD regulatory protein (45, 51). Thus, reduced levels of FliZ are expected to lead to reduced levels of the transcriptional regulators HilA (controlled by HilD) and InvF (controlled by HilA), both of which are known to activate the expression of effector genes located inside and outside (indicated by a dotted box) SPI-1 (77–79). While transcriptional regulation of *sopA* and *sopB* by HilA/InvF has been described in the literature (78, 80, 81), *sopD* regulation is less well understood (indicated by a dashed black arrow). HBB, hook basal body; SCV, small colony variant; SPI-1, *Salmonella* pathogenicity island 1; T3SS-1, SPI-1-encoded type III secretion system; σ^{70}, housekeeping sigma factor; σ^{28}, flagellum-specific alternative sigma factor (FliA).

to stress, mutations conferring phage resistance may easily arise in *Salmonella*. The occurrence of phage-resistant strains is of major importance because phage therapy has been studied as a promising alternative to combat zoonotic pathogens, such as *Salmonella*, in animals, in the food chain, and in human infections (60–65). Bacteriophage Felix O1 in particular, which has a broad host range within the genus *Salmonella* and historically was used for the identification of *Salmonella* (66), proved successful in reducing *Salmonella* colonizing broiler chickens (63) and on the surface of meat (67). However, *Salmonella* strains resistant to Felix O1 phage have been reported (68, 69). The resistance phenotype has been linked to alterations in the structure of the lipopolysaccharide core, a site that is important for successful adsorption of Felix O1 to its bacterial host (68, 70, 71).

Interestingly, in additional independent starvation experiments, we sporadically detected and isolated other *Salmonella* strains showing an SCV phenotype within long-term starvation cultures. None of those SCVs, genetically or in terms of auxotrophy, resembled the SCV described here, suggesting that starvation in distilled water does not by itself select for the specific mutations found in SCV WPASI01.

SCVs of *S. enterica* or other bacterial pathogens might emerge spontaneously upon stress exposure. As their presence can be correlated with recurrent and persistent infections in a number of diseases, it is important to understand the underlying genetic changes and phenotypic consequences (10, 34). In our case, the SCV under investigation showed a loss-of-function mutation in *glnA*, leading to slow growth, attenuated virulence, and reduced virulence gene expression.

TABLE 1 Strains used in this study

S. Typhimurium strain	Characteristics	Source or reference
LT2 strains		
Wild type		18
SCV WPASI01	*glnA*120insA, *hfq*G85T, del_*srfC*-STM1615	This study
Rev WPASI01r	*glnA*120insA123delC, *hfq*G85T, del_*srfC*-STM1615	This study
Δ*glnA* mutant	*glnA*::KmFRT	This study
ATCC 14028s strains		
Wild type	Isogenic to wild-type NCTC 12023	82
ATCC 14028s Δ*glnA*	*glnA*::KmFRT	This study
NCTC 12023 P2D6	*ssaV*::mTn*5*	83
NCTC 12023 MvP818	*invC*::FRT	84
NCTC 12023 MvP1895	Δ*sipA* Δ*sopA* Δ*sopB* Δ*sopD* Δ*sopE2*	Unpublished strain provided by M. Hensel (University of Osnabrück)
SL1344 strains		
SL1344 M712	Δ*sipA* Δ*sopA* Δ*sopB* Δ*sopE* Δ*sopE2*	85
SL1344 M913	*fliGHI*::Tn*10*	86

MATERIALS AND METHODS

Bacterial strains and growth conditions. Unless otherwise stated, all experiments were performed with the *S*. Typhimurium strain LT2, which is designated the wild type. An overview of strains used in this study is given in Table 1. All strains were grown at 37°C in LB medium supplemented with 50 μg/ml kanamycin or 2 mM L-glutamine (product no. 3772.1; Carl Roth), when appropriate, or on M9 supplemented with 0.4% glucose (M9G) agar plates. CFU of plate-grown bacteria were routinely counted after overnight incubation at 37°C. During stress experiments, plates were inspected after overnight incubation and were reinspected after 2 more days of incubation, taking into account bacteria with a prolonged lag phase. A Bioscreen C analyzer (Oy Growth Curves AB Ltd.) was used to determine bacterial growth rates. Strains were cultivated overnight in LB medium and diluted in fresh LB medium to an optical density at 600 nm (OD_{600}) of 0.05, and 200-μl samples were transferred into four replica wells of a Bioscreen plate. Plates were incubated at 37°C with continuous shaking, and absorbance at 600 nm was measured automatically every 15 min for a period of 24 h.

Preparation of axenic water cultures. For starvation microcosms, bacteria were grown overnight on LB plates and subsequently adjusted in distilled water to an OD_{600} of 0.3 (corresponding to 2×10^8 CFU/ml). Afterwards, 1 ml was added to 200 ml of autoclaved distilled water, and the suspension was stored in 250-ml Erlenmeyer flasks at 23°C in the dark. Each microcosm was set up in duplicate. At the time of inoculation and then routinely, i.e., on a weekly basis for the first year and subsequently twice a year, the microcosms were assessed for CFU by plating of serial dilutions on LB agar.

Mutant generation. The Δ*glnA* mutants were constructed by λ Red-mediated mutagenesis (72). The primers for deleting *glnA* by introducing the pKD4-derived gene for the kanamycin resistance cassette into the chromosome were ABp0_glnA (5′-GAAGTGAAGTTTGTCGATCTGCGCTTCACCGATACCAAAGTGTGTAGGCTGGAGCTGCTT-3′) and ABp2_glnA (5′-CGAAATTTGTCGGATTTTTAATATACGATTAAACGCTGTACATATGAATATCCTCCTTAGTTCC-3′). Deletion of *glnA* was confirmed by PCR with the primers glnAFL_f (5′-GCGCGTGAGATCAGATTG-3′) and glnAFL_r (5′-GTGGCTTGCGTTCTGTTG-3′), followed by gel electrophoresis and subsequent sequencing of the PCR product.

Phage typing. O1 phage experiments were performed on Oxoid no. 3 plates, as described previously (21). Briefly, 10 μl of 10-fold serial dilutions (routine test dilutions of 1:10^{-4} to 1:10^{-10}) of phage suspension was spotted onto bacterial lawns. The plates were incubated overnight at 37°C, and the lytic activity, expressed as the maximal dilution at which no lysis was observed, was determined by monitoring areas of lysis on the bacterial lawns. The routine phage typing of *S*. Typhimurium was performed according to the Anderson typing system (20, 21).

Gentamicin protection assay. For determination of invasion/entry in U937 monocytes (CRL-1593.2; American Type Culture Collection, Manassas, VA), a human cell line that differentiates into macrophage-like cells upon treatment with phorbol esters (80 nm phorbol 12-mystrate 13-acetate [product no. P-8139; Sigma]) and incubation for 36 to 48 h, was used. Cells were routinely cultured in RPMI 1640 medium containing 10% fetal bovine serum (FBS), in a humidified atmosphere (37°C in 5% CO_2). Infection experiments were performed as described elsewhere (73, 74). Briefly $\sim 1 \times 10^6$ differentiated U937 cells were added to the wells of 24-well plates and allowed to adhere for 2 to 4 h. Bacteria were grown overnight in LB medium under static conditions at 37°C. Infections were performed at a multiplicity of infection of 10. Bacteria were centrifuged onto the cells for 5 min at $800 \times g$ and subsequently incubated for 25 min. Incubation mixtures were then washed twice with phosphate-buffered saline (PBS), and medium containing 100 μg/ml gentamicin was added for 90 min. Subsequently, cells were washed twice and then lysed with PBS containing 0.1% Triton X-100. After repeated pipetting, serial dilutions of lysates were plated onto LB agar to determine the number of internalized bacteria.

Genome sequencing. Nextera XT sequencing libraries (Illumina) were prepared from 1 ng bacterial DNA and sequenced on a MiSeq instrument, to generate 250-bp paired-end reads for >30-fold average coverage. Illumina reads of the SCV and its parental LT2 wild-type strain were mapped to the

TABLE 2 Primers used for semiquantitative RT-PCRs

Primer name	Sequence
JD_cpxR_qPCR_F1	CATTTAACGACCGCGAGCTG
JD_cpxR_qPCR_R1	ACCCGGATTAAGGCTTAGCG
JD_flgB_qPCR_F1	TTATCGCGTACCCGATCAGC
JD_flgB_qPCR_R1	CTACCCAGAACGGTAAGCCC
JD_flhD_qPCR_F2	AGCGTTTGATCGTCCAGGAC
JD_flhD_qPCR_R2	ACTAACTGGTTCGTCTCCGC
JD_fliC_qPCR_F2	CTCGGCTACTGGTCTTGGTG
JD_fliC_qPCR_R2	CCGTAACGGTAACTTTGGCG
JD_fljB_qPCR_F1	ATGGTACTACACTGGATGTATCG
JD_fljB_qPCR_R1	GTAAAGCCACCAATAGTAAC
JD_gyrB_qPCR_F1	ATGGTACGTTTCTGGCCGAG
JD_gyrB_qPCR_R1	GAATGACAGTTCACGCAGGC
JD_rpoE_qPCR_F1	CACCTTACGGGAGCTGGATG
JD_rpoE_qPCR_R1	GAAGATACGTGAACGCACCG
JD_sopA_qPCR_F1	ATCCCCCTCCCTCTCACTTC
JD_sopA_qPCR_R1	AGGGTTTCTCCGGCTTTCTG
JD_sopB_qPCR_F1	CGCTCGCCCGGAAATTATTG
JD_sopB_qPCR_R1	AGAGGTTATGCAGCGAGTGG
JD_sopD_qPCR_F2	TTCGAAGATGACCTGGCACC
JD_sopD_qPCR_R2	ACCGCCGTTTTGATTTGTGC
JD_ydiV_qPCR_F1	TGAAAACGCTCGGCTTGTTG
JD_ydiV_qPCR_R1	GAGGCGCTACCAACTCAGTC

Salmonella enterica serovar Typhimurium LT2 reference genome sequence (18) (GenBank accession no. NC_003197.1) (99.5% coverage) by using the map-to-reference tool of Geneious v7.1 software, with default settings. Contigs of the mapped SCV and wild-type genomes were aligned with the reference genome using the Mauve alignment tool implemented in Geneious. Alignments were manually inspected for differences from the reference sequence.

SDS-PAGE and Western blot analysis of cellular and extracellular proteins. For comparison of extracellular protein patterns, bacteria were grown at 37°C in LB broth, with shaking at 250 rpm, until early stationary phase was reached. For total cellular proteins, 500 μl culture was pelleted, the supernatant was removed, and the pellet was boiled in reducing SDS-PAGE sample buffer (75). For extracellular proteins, 1 ml cell-free culture supernatant (from centrifugation at $8,000 \times g$ for 5 min) was precipitated with trichloroacetic acid, washed with cold acetone, and finally suspended and boiled in SDS-PAGE sample buffer. All samples were run on 10% SDS-PAGE gels and stained with colloidal Coomassie blue (Carl Roth). Antibodies for detection of flagellin (clone 4H2; Biotrend GmbH) or SopA, SopB, and SopD proteins (76) were used at 1,000-fold or 500-fold dilutions and were detected with horseradish peroxidase conjugates of anti-mouse IgG or anti-rabbit IgG (both Sigma-Aldrich), respectively, which were used at 10,000-fold dilutions.

RNA extraction and semiquantitative RT-PCR. For total RNA extraction, stationary-phase bacteria were diluted in fresh LB medium to an OD_{600} of 0.05 and were grown to early stationary phase (OD_{600} of 1.8 to 2.0). Aliquots of 7.5×10^8 bacteria were treated with RNAprotect (Qiagen) as recommended by the manufacturer, and RNA was isolated using the RNeasy minikit (Qiagen), following the standard protocol. Possible DNA contamination was removed by using the Turbo DNA-free kit from Ambion, and then 1 μg RNA was reverse transcribed to cDNA using the Superscript IV enzyme (Invitrogen) as recommended. Subsequently, 2 μl of cDNA diluted 1:5 was used in a semiquantitative PCR with gene-specific primer pairs, as listed in Table 2, and the *Taq* polymerase (New England BioLabs GmbH). The following cycle conditions were used for all primer combinations: 95°C for 2 min and then 24 to 33 cycles of 95°C for 30 s, 59°C for 30 s, and 68°C for 15 s. Product formation was analyzed by agarose gel electrophoresis and ethidium bromide staining after different rounds of amplification. All semiquantitative RT-PCRs were performed with at least three independent RNA/cDNA preparations. Primers amplifying a part of *S*. Typhimurium *gyrB*, the constitutively expressed gene coding for gyrase subunit B, were used as an internal control. cDNA reactions performed without the addition of Superscript IV served as negative controls, to prove that RNA preparations were free of DNA.

Accession number(s). Sequencing reads were submitted to the European Nucleotide Archive (ENA) and assigned study accession number PRJEB12856.

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We declare we have no competing interests.

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