Comparative proteomic analysis of *Salmonella enterica* serovar Typhimurium ppGpp-deficient mutant to identify a novel virulence protein required for intracellular survival in macrophages

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**Abstract**

**Background:** The global ppGpp-mediated stringent response in pathogenic bacteria plays an important role in the pathogenesis of bacterial infections. In *Salmonella enterica* serovar Typhimurium (S. Typhimurium), several genes, including virulence genes, are regulated by ppGpp when bacteria are under the stringent response. To understand the control of virulence genes by ppGpp in *S. Typhimurium*, agarose 2-dimensional electrophoresis (2-DE) combined with mass spectrometry was used and a comprehensive 2-DE reference map of amino acid-starved *S. Typhimurium* strain SH100, a derivative of ATCC 14028, was established.

**Results:** Of the 366 examined spots, 269 proteins were successfully identified. The comparative analysis of the wild-type and ppGpp knockout mutant strains revealed 55 proteins, the expression patterns of which were affected by ppGpp. Using a mouse infection model, we further identified a novel virulence-associated factor, STM3169, from the ppGpp-regulated and *Salmonella*-specific proteins. In addition, *Salmonella* strains carrying mutations in the gene encoding STM3169 showed growth defects and impaired growth within macrophage-like RAW264.7 cells. Furthermore, we found that expression of *stm3169* was controlled by ppGpp and SsrB, a response regulator of the two-component system located on *Salmonella* pathogenicity island 2.

**Conclusions:** A proteomic approach using a 2-DE reference map can prove a powerful tool for analyzing virulence factors and the regulatory network involved in *Salmonella* pathogenesis. Our results also provide evidence of a global response mediated by ppGpp in *S. enterica*.

**Background**

The facultative intracellular bacterium *Salmonella enterica* causes a broad spectrum of diseases, such as gastroenteritis and bacteremia, which are typically acquired by oral ingestion of contaminated food or water. *S. enterica* serovar Typhimurium (S. Typhimurium) causes enterocolitis in humans and a typhoid-like systemic infection in mice.

Several virulence genes associated with *Salmonella* pathogenicity islands (SPIs) and the virulence plasmid have been characterized in *S. Typhimurium*. Two type III secretion systems (T3SS) encoded by SPI-1 and SPI-2 play central roles in *Salmonella* pathogenesis. SPI-1 is essential for the invasion of host cells and the induction of apoptosis in infected macrophages [1,2]. SPI-2 T3SS primarily confers survival and replication on macrophages and is required for systemic infection in the mouse infection model [3,4]. Expression of SPI-2 genes is induced within a modified phagosome, called the *Salmonella*-containing vacuole (SCV), in infected macrophages [5]. Induction of SPI-2 genes depends on a two-component regulatory system, SsrA/SsrB, encoded within the SPI-2 region [6]. Expression of SsrAB is also mediated by two-component regulatory systems, OmpR/EnvZ and PhoP/PhoQ, which sense osmotic stress and cation limitation, respectively [7,8]. In addition, a global...
transcriptional regulator, SlyA, which interacts directly with the ssrA promoter region, is involved in the expression of SPI-2 T3SS [9-11].

During infection of mammalian hosts, S. Typhimurium has to rapidly adapt to different environmental conditions encountered in its passage through the gastrointestinal tract and its subsequent uptake into epithelial cells and macrophages. Thus, establishment of infection within a host requires coordinated expression of a large number of virulence genes necessary for the adaptation between extracellular and intracellular phases of infection. It has been demonstrated that the stringent response plays an important role in the expression of Salmonella virulence genes during infection [12-14].

The stringent response is mediated by the signal molecules, guanosine tetraphosphate (ppGpp) and guanosine pentaphosphate (pppGpp) (both are referred to as ppGpp in this manuscript), which accumulate in bacterial cells and exert both positive and negative effects on the transcription of many genes. ppGpp plays an important role in the virulence of pathogenic bacteria [15]. In Gram-negative bacteria, ppGpp is synthesized by two synthases, the synthase I and the synthase II, which are encoded by the relA and spoT genes, respectively [16]. These enzymes respond differently to environmental conditions. RelA is activated by the binding of uncharged tRNA to ribosomes upon amino acid starvation. SpoT is induced during the exponential growth phase and responds to other changes in environmental conditions, specifically a lack of carbon sources or energy deprivation [17]. ppGpp binds directly to the β and β’ subunits of RNA polymerase (RNAP), leading to destabilization of the RNAP-rRNA promoter open complex [18]. Moreover, the stringent response is increased by the availability of free RNAP, which gives rise to σ competition [19]. ppGpp indirectly activates the expression of many stress-induced genes by its release from RNAP σ70-dependent promoters and by facilitating the use of alternative σ factors. It has been shown that ppGpp is not only essential for surviving periods of stress but also for the interaction of bacteria with their host [20].

In case of S. Typhimurium, a mutant strain deficient in both relA and spoT (ΔrelAΔspoT) shows marked reductions in both bacterial invasion into host cells and proliferation in macrophages [12,13]. Furthermore, the virulence of the ΔrelAΔspoT mutant is severely attenuated in mice [12,13]. ppGpp controls the expression of SPI-1 to -5 and Spv through their transcriptional regulators HilA, InvF, RtsA, SsrA, SlyA, and SpvR [12-14,21]. These observations indicate that ppGpp may play a major role in Salmonella virulence via the altered expression of regulatory genes. Because ppGpp has been shown to affect the expression of many virulence genes in S. Typhimurium, it is likely that there are additional virulence genes among the ppGpp-regulated genes.

In this study, we constructed an agarose 2-dimensional electrophoresis (2-DE) reference map of S. Typhimurium grown under amino acid starvation to identify ppGpp-regulated proteins from whole-cell preparations. By comparative proteomic analysis of ppGpp-regulated and Salmonella-specific proteins, we identified a novel virulence factor, STM3169, required for intracellular survival within macrophages.

Results and Discussion

Agarose 2-DE reference map of S. Typhimurium with induced stringent responses

Because the correlation between mRNA and protein expression levels is nonpredictive, the direct measurement of protein expression is essential for the analysis of biological processes [22]. 2-DE allows several hundred proteins to be displayed on a single gel, thus producing a direct and global view of the proteome at a given time point [23]. Agarose 2-DE takes advantage of the process of protein separation over a broad range [24,25]. In this study, to separate and identify more proteins, we applied agarose 2-DE to the bacterial proteome, and also used 12% and 15% SDS-PAGE gels for the second dimension. Whole-cell proteins were obtained from the S. Typhimurium strain SH100, a derivative of ATCC 14028, with the stringent response induced by serine hydroxamate, as described previously [26]. Agarose 2-DE was performed at least three times on independent samples. More than 350 protein spots from the strain were detected on each 2-DE gel stained with Coomassie Brilliant Blue. To identify proteins on the agarose 2-DE gels, we excised 230 spots from the 12% gel and 136 spots from the 15% gel. We finally identified a total of 360 proteins (273 proteins from the 12% gel [Figure 1A] and 87 proteins from the 15% gel [Figure 1B]) by MS/MS analysis out of 307 protein spots (232 spots from the 12% gel and 75 spots from the 15% gel) that were successfully excised (see additional file: 1). In total, 267 proteins were obtained from the gels, with 40 proteins identified as being redundant. The highest and lowest molecular masses of identified proteins were 93.4 kDa for AconB (aconitate hydrase 2, spot 188) and 7.4 kDa for CspC (cold-shock protein, spot 303), respectively. Fifty spots (35 spots from the 12% gel and 15 spots from the 15% gel) were found in a basic range. Interestingly, 78 protein spots (25.4%) were annotated as putative proteins on the genome of the S. Typhimurium LT2 strain, which is more than 98% identical in sequence to the 14028 strain [27].

We estimated the molecular weight of the protein spots on the 2-DE gels and compared them with the theoretical molecular weight of strain SH100. While
Figure 1 Agarose 2-DE reference map of the S. Typhimurium strain SH100, prepared using a 12% gel focused on high-molecular-mass proteins (A) and a 15% gel focused on low-molecular-mass proteins (B). Strain SH100 was grown under amino acid starvation as described previously [26]. Gels are stained with Coomassie Brilliant Blue. Identified spots are numbered (corresponding to the spot numbers in additional file: 1. Proteins identified on the reference map).
most of the estimated molecular weight values matched the theoretical values, we found 14 protein spots on the map that had different experimental and predicted molecular weights values (Figure 2). These proteins might be post-translationally modified by proteolytic processing, phosphorylation of multiple amino acid residues and/or an artifact caused by sample preparation. For example, the experimental molecular weight of OmpA indicated that the protein was likely processed by a proteolytic enzyme, because two different spots (spot nos. 152 and 287) were identified as OmpA, the experimental masses of which were significantly lower than the theoretical values. Similar results were described in other reports [28,29].

Next, we classified proteins identified on the map using the KEGG pathway database. While 156 proteins (45.3%) were grouped into several metabolic categories (carbohydrate, energy, lipid, nucleotide, amino acid, and other amino acids), 70 proteins (22.8%) were grouped in the no entry category, which means that these proteins do not belong to the other categories. This category contained 20 known virulence-associated proteins, including flagella and flagella biosynthesis proteins (FlaC, FlaB, FlhB, FlhD, FliM, and FliJ), SPI-1 effectors (SipD, SopB, and SopE2), an SPI-1 translocase (SipC), an iron transporter (SitA), superoxide dismutases (SodA, SodB, SodC1, and SodC2), a quorum-sensing protein (LuxS), a two-component response regulator (PhoP), peptidyl-prolyl cis-trans isomerases (FkpA and SurA), and a periplasmic disulfide isomerase (DsbA).

Identification of ppGpp-regulated proteins using comparative proteomics
To identify proteins associated with the stringent response in S. Typhimurium, we compared the agarose 2-DE pattern for each total protein prepared from amino acid-starved S. Typhimurium SH100 and ArelAΔspoT strain (TM157) (Figure 3). As shown in Table 1, 24 protein spots (23 proteins) were found at higher levels in SH100 than in TM157, while 23 protein spots were found at lower levels in SH100 than in TM157. We focused on 23 proteins, which were positively regulated by ppGpp in the stringent response.

Of these proteins, six genes (treA, ugpB, ynhG, yliB, ugpB, degQ) had previously been identified as ppGpp-regulated genes in E. coli at the transcriptional level [30]. In S. Typhimurium, it has been shown that ppGpp controls the expression of known virulence-associated genes, including sipC, fliY, sopB, and sodC1, in response to growth conditions relevant to host infection [14]. Thus, to confirm the results from the comparative proteomic analysis, mRNA levels of the remaining 13 genes were assessed by qRT-PCR. As a result, mRNA expression levels of eight genes (stm3169, cpdB, tolB, ydhG, oppA, yajQ, yhbN, yffl) were significantly higher in SH100 than in TM157 under stringent conditions (Table 1).

Identification of novel virulence-associated factors regulated by ppGpp
Among 13 genes newly identified as ppGpp regulated, 12 genes were present in non-pathogenic E. coli K-12 strain. Therefore, to examine whether ppGpp-regulated putative or hypothetical proteins could contribute to the virulence of S. Typhimurium, we chose Salmonella-specific protein, STM3169, which is present in S. Typhimurium, but is absent in the E. coli K-12 strain (Figure 4 [27,31]). To determine the roles of STM3169 in virulence, a deletion mutant was constructed in the S. Typhimurium wild-type SH100 strain, and its virulence was assessed by a mouse mixed infection using a competitive index analysis. As shown in Figure 5A, mouse mixed infections showed that disruption of the stm3169 gene conferred a defect in virulence in mice, and that successful complementation was achieved for TH973 (Δstm3169:kan) by expression of intact STM3169 from a plasmid. These findings provide the first evidence that STM3169 functions as a virulence factor of S. Typhimurium in a mouse infection model.

Because it is believed that intracellular Salmonella is likely to be restricted to the acquisition of nutrient substrates from infected host cells, the stringent response
could occur in SCV. Thus, we next analyzed the contribution of STM3169 to intracellular survival of *S. Typhimurium* in macrophages. In accordance with previous data that a ppGpp\(^0\) mutant strain deficient in both *spoT* and *relA* genes resulted in a severe reduction of intracellular proliferation and survival [12]. In contrast to the wild-type level of invasion, intracellular survival of TH973 in RAW264.7 cells was reduced, compared with that of the wild-type strain. The reduced CFU of TH937 in IFN-\(\gamma\) treated-RAW264.7 cells was not more severe than that in the \(\Delta relA\Delta spoT\) double mutant, \(\Delta ssaV\) (SH113, SPI-2 T3SS component-defected mutant), and \(\Delta ssrB\) (YY1, SPI-2 regulator mutant) strain, but was equal to that in the \(\Delta sseF\) (TM548, SPI-2 effector

\[ \text{Figure 3 Comparison of the agarose 2-DE maps of *S. Typhimurium* wild-type SH100 (A) and ppGpp-deficient strain TM157 (B) during amino acid starvation. Both strains were grown under the same condition as described in Figure 1. Gels were stained with Coomassie Brilliant Blue.} \]
### Table 1 S. Typhimurium proteins regulated by ppGpp

| spot no. | STM no. | Gene | Fold | Anova (p) | Average fold change determined by qRT-PCR |
|----------|---------|------|------|-----------|------------------------------------------|
| 002, 091 | STM2884 | sipC  | 0.1  | 0.006     | ND<sup>a</sup>                            |
| 005      | STM0781 | modA  | 0.3  | 0.032     | 0.67 ± 0.22                              |
| 012      | STM3169 | Stm3169| 0.3  | 0.004     | 0.18 ± 0.01<sup>c</sup>                   |
| 014, 213 | STM1796 | treA  | 0.7  | 0.002     | EC<sup>b</sup>                            |
| 015      | STM4403 | cpdB  | 0.6  | 0.011     | 0.25 ± 0.06<sup>c</sup>                   |
| 027      | STM1954 | fliY  | 0.5  | 0.033     | ND                                        |
| 028      | STM2884 | sipC  | 0.1  | 0.009     | ND                                        |
| 029      | STM3557 | uppB  | 0.4  | 0.019     | EC                                        |
| 029-2    | STM0748 | tolB  | 0.4  | 0.019     | 0.25 ± 0.03<sup>c</sup>                   |
| 037      | STM0209 | htrA  | 0.6  | 0.032     | 0.60 ± 0.35                              |
| 040      | STM2638 | rseB  | 0.3  | 0.011     | 0.88 ± 0.35                              |
| 040-2    | STM1478 | ydgH  | 0.3  | 0.011     | 0.17 ± 0.06<sup>c</sup>                   |
| 041      | STM1375 | ynhG  | 0.3  | 0.011     | EC                                        |
| 056      | STM1746 | oppA  | 0.6  | 0.001     | 0.15 ± 0.05<sup>c</sup>                   |
| 058      | STM1746 | oppA  | 0.5  | 0.006     | 0.15 ± 0.05<sup>c</sup>                   |
| 059      | STM1849 | yliB  | 0.4  | 0.027     | EC                                        |
| 060      | STM3557 | uppB  | 0.3  | 0.006     | EC                                        |
| 062      | STM1091 | scpB  | 0.2  | 0.036     | ND                                        |
| 064      | STM4319 | phoN  | 0.1  | 0.014     | 0.54 ± 0.22                              |
| 108      | STM0435 | yajQ  | 0.5  | 0.038     | 0.12 ± 0.05<sup>c</sup>                   |
| 108-2    | STM1440 | sodC1 | 0.5  | 0.038     | ND                                        |
| 153      | STM3181 | yhbN  | 0.6  | 0.047     | 0.28 ± 0.12<sup>c</sup>                   |
| 154      | STM4405 | ytfI  | 0.2  | 0.049     | 0.30 ± 0.02<sup>c</sup>                   |
| 184      | STM3348 | degQ  | 0.4  | 0.038     | EC                                        |
| 194      | STM1720 | yciO  | 0.3  | 0.028     | 14.22 ± 2.22<sup>c</sup>                 |

**Proteins expressed lower in ΔrelAΔspoT strain**

**Proteins expressed higher in ΔrelAΔspoT strain**

<sup>a</sup>ND, not determined.

<sup>b</sup>EC, already identified as a ppGpp-regulated protein in E. coli by Traxler et al. [30].

<sup>c</sup>mRNA level was significantly different between wild type and the ΔrelAΔspoT mutant.

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mutant) strain (Figure 5B and 5C). These results suggest that the expression of additional virulence factors, like STM3169, in macrophages might be affected in a highly avirulent phenotype of a ppGpp-deficient strain in mice.

**stm3169 is regulated by the SPI-2 transcriptional regulator ssrB**

It has been demonstrated that ppGpp mediates the expression of virulence-associated genes involved in bacterial invasion and intracellular growth and survival via global and/or gene-specific transcriptional regulators in *S*. Typhimurium [12,14]. Since intracellular growth and survival of *Salmonella* in macrophages is dependent upon SPI-2 function, we next confirmed whether expression of *stm3169* is regulated by the SsrAB two-component system, which positively controls the expression of SPI-2 genes as well as other genes belonging to the SsrB regulon [32]. To test this, we constructed *S*. Typhimurium strains carrying *stm3169::lacZ* transcriptional fusions on the chromosome in the wild-type (SH100) and ΔrelAΔspoT (TM157) genetic background. *Salmonella* strains carrying the *stm3169::lacZ* fusion gene (TH1162 and TH1164) were grown in defined MgM medium (pH 5.8) with 0.1% casamino acids and measured β-galactosidase activity. The transcription levels of *stm3169::lacZ* fusion were significantly decreased in TM157 (Figure 6A). The reduced level was restored to the wild-type level by the introduction of an arabinose-inducible plasmid expressing His<sub>s</sub>-tagged RelA protein. We next transduced the *ssrB* mutation (*ssrB::*cat) into a *stm3169::lacZ* fusion strain (TH1162). Strains carrying the *stm3169::lacZ* fusion gene with the *ssrB* mutation were grown in MgM medium (pH 5.8), and β-galactosidase activity was measured. Control experiments were performed with the *ssaG::lacZ* fusion gene (TM129). *ssaG* expression is strongly controlled by SsrB [33]. Similar to *ssaG::lacZ*, the transcription level of the *stm3169::lacZ* fusion gene was significantly decreased in strains carrying the *ssrB* mutation (Figure 6B). Complementation was partially achieved for TM423 by expression of SsrB (SsrB-FLAG) on a plasmid (Figure 6B), probably due to the constitutive expression of SsrB from multi-copy-number plasmid pFLAG-CTC. Collectively, these data suggest that the novel virulence-associated factor STM3169 was regulated by the SPI-2 two-component regulatory system SsrAB as well as by ppGpp.

It has been reported that ppGpp regulates SPI-2-encoded genes under aerobic condition [14]. To further characterize the transcriptional regulation of *stm3169* by
ppGpp and SsrB, we constructed a ΔrelAΔspoTΔssrB triple mutant strain (YY2), and examined the effect of the transcriptional activity on _stm3169::lacZ_ fusion gene. While the transcriptional activity of _stm3169::lacZ_ fusion in the triple mutant strain was significantly reduced at the same level of ΔrelAΔspoT double mutant strain, it could be restored by introducing plasmid pSsrB expressing SsrB-FLAG but not pRelA expressing His-tagged RelA (Figure 6C). These results indicate that ppGpp is controlled the expression of _stm3169_ through SsrB.

STM3169 is homologous to DctP in _Rhodobacter capsulatus_ with a 31% identity and a 73% similarity. DctP, along with DctQ and DctM, constitutes a tripartite ATP-independent periplasmic transporter (TRAP-T) system involved in sucinate utilization, and DctP plays a role as an extracytoplasmic solute receptor in this transporter [34]. STM3170 and STM3171, which are located immediately downstream from STM3169, have a 66% and an 80% similarity with DctQ and DctM, respectively. These suggest that the TRAP-T in _S. Typhimurium_ is composed of _stm3169, stm3170_, and _stm3171_ genes. In addition, two hypothetical operons, _yiaOMN_ and _stm4052-4054_, are annotated as TRAP-T in the _S. Typhimurium_ strain LT2 [31]. Recently, it has been reported that the TRAP-T (SiaPQM) in _Haemophilus influenzae_ is essential for LPS sialylation and virulence [35]. Further research is necessary to determine the role of these transporters in _S. Typhimurium_ virulence.

**Conclusions**

We constructed an agarose 2-DE reference map of amino-acid starved _S. Typhimurium_ and identified a novel virulence-associated factor, STM3169, regulated by ppGpp by applying the map to comparative proteomics. _stm3169_ is also regulated by an SPI-2 two-component regulator, SsrB. Recently, it has been reported that the lack of ppGpp synthesis in _Salmonella_ strains attenuates virulence and induces immune responses in mice [36]. Thus, further analysis of proteins regulated by ppGpp may lead to the development of new vaccines.

**Methods**

**Bacterial strains, primers, and culture conditions**

The bacterial strains and plasmids used in this study are listed in Table 2. The oligonucleotide primers used are listed in Table 3. Bacteria were grown in Luria-Bertani (LB) medium or on LB agar under conditions suitable for selection for resistance to ampicillin (100 μg/mL), chloramphenicol (25 μg/mL), nalidixic acid (50 μg/mL), or spectinomycin (50 μg/mL), as appropriate. To induce the bacterial stringent response, serine hydroxamate (Sigma; 0.005%), an inhibitor of serine tRNA synthetase, was added to a 12 h culture in LB broth, and the bacteria were further incubated for 1 h [26]. Magnesium minimal medium (MM, pH 5.8) was used to induce SPI-2 gene expression [6].

**Construction of mutants**

Nonpolar mutants of _relA_ and _spoT_ were constructed by allele exchange using the temperature- and sucrose-sensitive suicide vector pCACTUS [37]. The _relA_ and _spoT_ genes were amplified by PCR with the following primers: (1) _relA-FW_ and _relA-RV_ for _relA_ and (2) _spoT-FW_ and _spoT-RV_ for _spoT_. _S. Typhimurium_ strain SH100 genomic DNA was used as the template. The PCR products were cloned into TA cloning vector pGEM-T Easy (Promega) generating plasmid pGEM-relA and pGEM-spoT, respectively. A disruption mutation of _relA_ was created by the insertion of the HincII-digested promoterless _cat_ gene into a unique _NruI_ site in the coding sequence of _relA_ on pGEM-relA.
Table 2 Bacterial strains and plasmids used

| Bacterial strains   | Relevant characteristics                           | Source/Ref. |
|---------------------|---------------------------------------------------|-------------|
| **S. Typhimurium**  |                                                   |             |
| 14028               | wild-type                                         | ATCC        |
| SH100               | Spontaneous nalidixic acid resistant derivative of wild-type 14028 | [44]        |
| TM157               | SH100 ΔrelA::cat ΔspoT::kan                       | this study  |
| YY2                 | SH100 ΔrelA::cat ΔspoT::kan ΔssrB::tet             | this study  |
| TH973               | SH100 Δstm3169-kan                                 | this study  |
| TH1162              | SH100 Δstm3169- lacZ                               | this study  |
| TH1164              | TM157 Δstm3169- lacZ                               | this study  |
| YY3                 | TH1164 ΔssrB::tet                                  | this study  |
| TM129               | SH100 ΔssaG- lacZ                                  | this study  |
| YY1                 | SH100 ΔssrB::tet                                   | this study  |
| SH113               | SH100 ΔssaV::cat                                   | [11]        |
| THM548              | SH100 ΔsseF::kan                                   | this study  |
| **E. coli**         |                                                   |             |
| DH5α                | K-12 recA1 endA1 gyrA96 thi-1 hisD17 supE44 (lacX74-argR1U169 deoR (80 dlac lacZ)M15) | Invitrogen |
| SM108,pir           | thi-1 thr leu tonA lacY supE recA::RP4-2-Tc::Mu λpir | [45]        |

**Plasmids**

| Name               | Nucleotide sequence (5’ to 3’)^a                |
|--------------------|-------------------------------------------------|
| pGEM-Teasy         | TA cloning vector                                |
| pMW118             | pSC101-based low copy number plasmid             |
| pACYC184           | p15A-based low copy number plasmid, tet template |
| pFLAG-CTC          | FLAG tag expression vector                       |
| pLD-lacZQ          | Integrational plasmid with promoterless lacZ gene |
| pBAD-HisA          | Expression vector for His6 fusion protein        |
| pMW-Strm3169       | stm3169 gene in pMW118                           |
| pLD-strm3169Z      | stm3169 lacZ operon fusion in pLD-lacZQ          |
| pLD-ssaGZ          | ssaG::lacZ operon fusion in pLD-lacZQ            |
| pRelA              | pBAD-HisA expressing relA gene                   |
| pSsrB              | pFLAG-CTC expressing ssrB gene                   |
| pKD46              | Red recombinase expression plasmid               |
| pKD4               | kan cassette used for gene deletion              |

Table 3 Primers

| Name               | Nucleotide sequence (5’ to 3’)^a                |
|--------------------|-------------------------------------------------|
| **Construction of the deletion mutants** |
| relA-FW            | CGCCATCCGGCAATGTTTACATAAA                        |
| relA-RV            | TCATTGTCCTGACATAAACG                            |
| spoT-FW            | CTTGAAATCCATCAATCCGGCTGAAACG                    |
| spoT-RV            | TCCTGCGGATCGAATGATGTGCAAAACG                    |
| strm3169-red-FW    | ACAGGCTTCGGAATCAGCGGTATTACTGCGGCGCTGAACTGGACTGAGCCTTCTCC |
| strm3169-red-RV    | ACATATCTAGATGATTTACCAATCCGCGGTATTACTGCGGCGCTGAACTGGACTGAGCCTTCTCC |
| sseF-red-FW        | AACGACCGCAATATGAAATTTGACGTAGCAAGTTGACGTACGCTGAGCCTTCTCC |
| sseF-red-RW        | TGTCATTAATGCAAGGCTTCTGACTGACGTACGCTGAGCCTTCTCC |
| pAC-tet-FW         | TTAGATCGTACGAGAATCCCTCGGAACCCCG                 |
| pAC-tet-RV         | TCGCTGCGTACGAGAATCCCTCGGAACCCCG                 |

**Construction of plasmids for the complementations**

| relA-FW2           | AGGCTCGAGGTCGGCGGTAAAGGTGCTGAGGTTAC             |
| relA-RV2           | ACAAGCCTACTGCTGGCGGTAC                        |
| ssrB-FW            | GGCGCTGAGGAAATATAGACTCTTATAGTA                 |
resulting plasmid pGEM-*relA::cat* was digested with BglII and then self-ligated, yielding plasmid pGEM-*ΔrelA::cat*. In contrast, the *spoT* gene was disrupted by the insertion of a SmaI-digested Km r-encoding gene (*kan*) cassette from pUC18K [38] into NruI sites in the coding sequence of *spoT* on pGEM-*spoT*, thus generating pGEM-*ΔspoT::kan*. The disrupted gene was then subcloned using SalI and SphI into similarly digested pCACTUS, and the resulting plasmid was introduced into strain SH100 by electroporation for allele exchange mutagenesis, which was carried out as described previously [39]. ΔrelAΔspoT mutant strain was created by phage P22-mediated transduction [40].

The PCR-based λ Red recombinase system using pKD46 and pKD4 was performed to disrupt *stm3169* or *sseF* [41]. The growth rate of these mutant strains in LB

### Table 3 Primers (Continued)

| Primer         | Sequence          |
|----------------|-------------------|
| ssrB-RV        | CCCGGATCCATACTCTATTAACCTCATTCT |
| ssrB-FW        | CCCGGATCCATACTCTATTAACCTCATTCT |
| stm3169-FW     | GGAAGATCTTTTATGTCGATGATCTCGTC |
| stm3169-RV     | GGAAGATCTTTTATGTCGATGATCTCGTC |
| Construction of the *lacZ* fusions
| ssAG-Pro-FW    | AAAAAGCTCTTACCTGCGATTATGC |
| ssAG-Pro-RV    | AAAAAGCTCTTACCTGCGATTATGC |
| stm3169-Pro-FW | AGGGCGGATCCGAGATGATGAGGAG |
| stm3169-Pro-RV | AGGGCGGATCCGAGATGATGAGGAG |

### Confirmation of gene expression by qRT-PCR

| Primer         | Sequence          |
|----------------|-------------------|
| gyrA-FW        | AAGAGGTGTTTCACCATGTTACT |
| gyrA-RV        | TATTATGCTAAGGTCAGCAAC |
| relA-FW        | ATGGTCCCATATCATACATAGTT |
| relA-RV        | GATATTGTTGTCAGCATGTCGTC |
| invF-FW        | ATCCGCTGTAATGATGTAAGAG |
| invF-RV        | CATTTTGTCCCGAATAATGAAAT |
| stm0209-FW     | GTCAGGTTTTTCACCATGTTACT |
| stm0209-RV     | GTCAGGTTTTTCACCATGTTACT |
| stm0343-FW     | GCTAATCATGTCGATGATCTCG |
| stm0343-RV     | GCTAATCATGTCGATGATCTCG |
| stm0748-FW     | TGAACCTTGTAGGTATTAGTTCGTC |
| stm0748-RV     | TGAACCTTGTAGGTATTAGTTCGTC |
| stm0781-FW     | GAAAGGCGGATCCGAGATGAGGAG |
| stm0781-RV     | GAAAGGCGGATCCGAGATGAGGAG |
| stm1478-FW     | ACAAAAGGTAGAGAGGAGATGAGGAG |
| stm1478-RV     | ACAAAAGGTAGAGAGGAGATGAGGAG |
| stm1720-FW     | TGGTTTGGAAATGAAAGAAGAAG |
| stm1720-RV     | TGGTTTGGAAATGAAAGAAGAAG |
| stm1764-FW     | GCAATTGCTAAGGTCAGCAAC |
| stm1764-RV     | GCAATTGCTAAGGTCAGCAAC |
| stm2638-FW     | GTACTGTGCTGATGATGATGATGTC |
| stm2638-RV     | GTACTGTGCTGATGATGATGATGTC |
| stm3169-FW     | GCAACTGACTGACCCGCGAATAATGTCGTC |
| stm3169-RV     | GCAACTGACTGACCCGCGAATAATGTCGTC |
| stm3318-FW     | CAAAATCTGACCTTAATCCTATGC |
| stm3318-RV     | CAAAATCTGACCTTAATCCTATGC |
| stm3318-FW     | CAAAATCTGACCTTAATCCTATGC |
| stm3318-RV     | CAAAATCTGACCTTAATCCTATGC |
| stm3419-FW     | ATTGATATATGCTGAGGCAGGAGAC |
| stm3419-RV     | ATTGATATATGCTGAGGCAGGAGAC |
| stm4403-FW     | AGGTAGATATAGCTGAGGCAGGAGAC |
| stm4403-RV     | AGGTAGATATAGCTGAGGCAGGAGAC |
| stm4405-FW     | GAAACCTGACCCGCGAATAATGTCGTC |
| stm4405-RV     | GAAACCTGACCCGCGAATAATGTCGTC |

*Underlined part indicates P1 or P2 site for pKD4, or restriction sites.*
and MgM (pH 5.8) broth showed the same levels to wild-type strain.

To construct $\Delta relA\Delta spoT\Delta ssrB$ mutant strain, the cloned ssrB gene was disrupted by the insertion of a Tet'-encoding gene (tet) cassette, which was amplified with pAC-tet-FW and pAC-tet-RV primers using pACYC184 (New England Biolabs) as template. The $\Delta ssrB::tet$ fragment was amplified by PCR using ssrB-FW and ssrB-RV primers, and the resulting PCR product was introduced into S. Typhimurium SH100 carrying pKD46. The disrupted genes were transferred by phage P22 transduction into $\Delta relA\Delta spoT$ mutant strain TM157.

To construct ssaG::lacZ and $stm3169::lacZ$ transcriptional fusions, pLD-ssaGZ and pLD-stm3169Z were transferred from Escherichia coli SM10λpir to S. Typhimurium SH100 by conjugation. The fusions were introduced into SH100, $\Delta relA\Delta spoT$ (TM157), $\Delta ssrB::tet$ (YY3), and $\Delta ssaV$ (SH113) mutant strains by phage P22-mediated transduction. All constructs were verified by PCR or DNA sequencing.

**Construction of plasmids**

For construction of the complementing plasmid, pMW-Stm3169, $stm3169$ gene was amplified by PCR with $stm3169$-FW and $stm3169$-RV primers. S. Typhimurium SH100 genomic DNA was used as the template. The PCR products were digested with BglII and XhoI, and cloned into the same sites on pFLAG-CTC (Sigma). pRelA and pSsrB were amplified using ssaG-FW and ssaG-RV for $relA$ and ssrB-FW and ssrB-RV for $ssrB$. The PCR product containing $relA$ was digested with XhoI-HindIII and cloned into the same sites on pACYC184 (New England Biolabs) according to the manufacturer’s instructions.

To construct lacZ transcriptional fusions, the DNA fragments containing (predicted) promoter regions of $ssaG$ were amplified by PCR using the primers $ssaG$-Pro-FW and $ssaG$-Pro-RV, and those containing promoter regions of $stm3169$ were amplified using $stm3169$-Pro-FW and $stm3169$-Pro-RV. The PCR products digested with Sall and BamHI were ligated into the same sites of pLD-lacZΩ [39].

**Sample preparation for agarose 2-DE**

Agarose 2-DE samples were prepared from amino-acid starved S. Typhimurium strain SH100, as well as $relA$ and $spoT$ double knockout strain TM157 ($\Delta relA\Delta spoT$). The cell pellets were washed twice with cold phosphate-buffered saline (PBS) and dissolved in lysis buffer containing 5 M urea, 1 M thiourea, 0.05% w/v β-mercaptoethanol, and one tablet of protein inhibitor (Complete Mini EDTA-free; Roche Diagnostics, Mannheim, Germany), which was dissolved in 10 mL of the solution. The lysates were centrifuged (10,400 x g, 20 min, 4°C) and the clear supernatant was used.

**Proteome analysis**

We performed proteome analysis according to the procedures of Oh-Ishi et al. [25] and Kuruma et al. [42]. An aliquot of 200-300 μL (containing 500 μg of protein) of sample solution was subjected to first-dimension IEF at 667 V for 18 h at 4°C, followed by second-dimension SDS-PAGE. The slab gel was stained with CBB R-350 (PhastGel Blue R; GE Healthcare).

Protein spots were excised from a destained gel with 50% (v/v) ACN and dried under vacuum. The proteins were digested in the gel with trypsin. Digested fragments of 15 pmol were loaded on a Liquid Chromatography-Mass Spectrometry/Mass Spectrometry (LC-MS/MS), which consisted of Nanospace SL-2 (Shiseido Fine Chemicals), an HPLC (LCQ Deca), and an ion trap mass spectrometer (Thermo Finnigan). We identified a protein from measured masses of the tryptic peptides and their MS/MS fragments using the SEQUEST program. When the top-ranked candidates had SEQUEST scores lower than 90, we inspected the raw MS and MS/MS spectra of peptides to judge their qualities. We classified identified proteins according to the Kyoto Encyclopedia of Genes and Genomes (KEGG) PATHWAY database http://www.genome.ad.jp/kegg/pathway.html.

Gel-to-gel comparisons between SH100 and TM153 were performed for two separately prepared samples. Each scanned 2-DE gel image was analyzed with the gel image analysis software SameSpots (Progenesis).

**RNA extraction and quantitative real-time PCR**

S. Typhimurium strains were grown in LB and ppGpp expression was induced as described above. Total RNA was isolated from the bacterial culture using RNAprotect Bacteria Reagent and the RNeasy Protect Bacteria Mini Kit with the gDNA Eliminator spin column (Qiagen) according to the manufacturer’s instructions. cDNA was synthesized using the QuantiTect Reverse Transcription Kit (Qiagen). Real-time PCR was performed with the primer pairs listed in Table 3 using QuantiTect SYBR Green and the 7900HT Sequence Detection System (Applied Biosystems). The data were analyzed using the comparative Ct method (Applied Biosystems). Transcription of the target gene was normalized to the levels of gyrA mRNA.
Mouse infections
For the competitive index assay, female BALB/c mice (5-6 weeks old) were used for the mouse infection study and were housed at Kitasato University according to the standard Laboratory Animal Care Advisory Committee guidelines. Mice were inoculated by intraperitoneal injection with 100 μL of inoculum containing a total of 1 × 10^8 bacteria (each strain at 5 × 10^5), consisting of an equal number of wild-type and mutant strains. At 48 h after infection, the mice were sacrificed by carbon dioxide inhalation. The spleens were homogenized in cold PBS by mechanical disruption. The number of each strain in the spleen was determined by plating a dilution series of the lysate onto LB agar alone and LB agar with appropriate antibiotics.

Additional material

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Additional file 1: Table S1. Proteins identified on the reference map.

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