A Role for the SmpB-SsrA System in *Yersinia pseudotuberculosis* Pathogenesis

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Yersinia **utilizes a sophisticated type III secretion system to enhance its chances of survival and to overcome the host immune system. SmpB (small protein B) and SsrA (small stable RNA A) are components of a unique bacterial translational control system that help maintain the bacterial translational machinery in a fully operational state. We have found that loss of the SmpB-SsrA function causes acute defects in the ability of *Yersinia pseudotuberculosis* to survive in hostile environments. Most significantly, we show that mutations in *smpB-ssrA* genes render the bacterium avirulent and unable to cause mortality in mice. Consistent with these observations, we show that the mutant strain is unable to proliferate in macrophages and exhibits delayed Yop-mediated host cell cytotoxicity. Correspondingly, we demonstrate that the *smpB-ssrA* mutant suffers severe deficiencies in expression and secretion of *Yersinia* virulence effector proteins, and that this defect is at the level of transcription. Of further interest is the finding that the SmpB-SsrA system might play a similar role in the related type III secretion system that governs flagella assembly and bacterial motility. These findings highlight the significance of the SmpB-SsrA system in bacterial pathogenesis, survival under adverse environmental conditions, and motility.**

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Introduction

Bacterial pathogens employ a variety of unique mechanisms to manipulate and/or disable their hosts. Many Gram-negative bacteria, including species of *Yersinia, Escherichia, Salmonella, Xanthomonas,* and *Erwinia,* utilize a contact-dependent type III secretion system (TTSS) to deliver virulence effector proteins directly into the host cell cytoplasm [1–6]. Three *Yersinia* species encode the type III secretion system on a 70-kb plasmid that is absolutely required for virulence [2,7]. These species include *Yersinia pestis,* the etiological agent of plague, as well as *Y. pseudotuberculosis* and *Y. enterocolitica,* which cause gastrointestinal disorders. The virulence plasmid encodes for gene products that constitute the secretion channel (the injectisome, encoded by the *ysc* genes), Yops and their chaperones [7,8]. The effector proteins, which include YopE, YopH, YopJ (YopP), YopM, YopO (YpkA), and YopT, are injected via the injectisome into the host cell cytoplasm to interfere with a number of cellular functions including rearrangement of the actin cytoskeleton, pathogen phagocytosis, and pro-inflammatory immune responses [9]. YopB, YopD, and LcrV are thought to comprise the outermost part of the translocation machinery that delivers virulence proteins across the host cell membrane [10–14]. Accordingly, both YopB and YopD have been shown to insert into the host cell membrane after secretion and are required for the formation of a transmembrane channel [15]. The TTSS is required for sustained bacterial replication and colonization in host tissues. Indeed, Yop mutants are attenuated and less efficient in colonization in mouse infection models [16,17]. The Ysc-Yop TTSS is evolutionarily related to the bacterial motility organelle, the flagellum. The basal body of a flagellum links the cytoplasm to the outside of the cell in a manner structurally analogous to the type III secretion apparatus. Assembly of a functional flagellar apparatus requires the export of protein subunits from the cytoplasm to the cell surface by a mechanism that resembles type III secretion [18,19]. Moreover, the flagellum export apparatus of *Y. enterocolitica* has been shown to function as a secretion system for several secreted proteins, including a virulence-associated phospholipase, YplA [20].

SsrA RNA (small stable RNA A), also known as tmRNA and 10Sa RNA, is the central player of a unique quality control system that deals with ribosomes stalled on mRNAs lacking stop codons. SsrA RNA functions both as tRNA and mRNA through its unique structure. The tRNA-like domain of SsrA has high similarity to $tRNA^{Ala}$ in that it can be aminoacylated with alanine by ala-tRNA synthetase [21]. SsrA activity depends on three proteins: SmpB (small protein B), EF-Tu (Elongation Factor Tu), and ribosomal protein S1 [22–24]. SmpB binds specifically to the tRNA-like domain of SsrA and, although not required, enhances the efficiency of its aminoacylation by stabilizing SsrA tertiary structure [25]. SmpB is essential for recognition and delivery of SsrA to target stalled ribosomes [22,25,26]. All known functions of SsrA require SmpB [22]. In the current model for SsrA function [27], also known as *trans*-translation, a translating ribosome becomes

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Abbreviations: ΔBA, Δ*smpB-ssrA*; CFU, colony-forming unit; MLN, mesenteric lymph node; MOI, multiplicity of infection; OD_{600}, optical density at 600 nm; TTSS, type III secretion system

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Synopsis

Bacteria have evolved sophisticated mechanisms to monitor, adapt, and respond to environmental and host-mediated assaults. Many Gram-negative pathogenic bacteria utilize a needle-like type III secretion system (TTSS) to inject a cocktail of effector proteins into host cells, disabling the host defenses against the pathogen. There is evolutionary, structural, and sequence similarity between this TTSS and the bacterial motility apparatus, the flagellum. Experiments described in this study examine the role played by the SmpB-SsrA system in *Yersinia* virulence, motility, and adaptation to adverse environments. The authors present evidence to demonstrate that an *smpB-ssrA* mutant of Yersinia pseudotuberculosis is more sensitive to adverse environmental conditions, lacks motility, exhibits severe defects in Yop secretion, and is avirulent in a mouse infection model. On the basis of these findings, they postulate that the SmpB-SsrA system, through its ribosome rescue, and protein tagging for directed degradation functions, affects the expression of the Ysc-Yop TTSS, and likely the flagellar TTSS, at the level of transcription. Their findings are consistent with a proposed regulatory role for the SmpB-SsrA system in regulation of bacterial gene expression.

stalled on a nonstop mRNA, and the alanine-charged SsrA-SmpB complex is recruited to the stalled ribosome. SsrA RNA functions initially as a tRNA to transfer its alanine to the nascent peptide. SsrA then translocates to the ribosomal P-site, displaces the damaged nonstop mRNA, and acts as a surrogate mRNA to direct the co-translational addition of a ten–amino acid tag to the C-terminus of the nascent polypeptide. Translation terminates on the SsrA encoded stop codon, recycling the stalled ribosomes and releasing the tagged polypeptide. Finally, the tagged polypeptide is recognized and degraded by C-terminal–specific cellular proteases [27].

The *smpB* and *ssrA* genes are present in all bacteria examined to date [27–29]. Recent reports suggest essential biological roles for the SmpB-SsrA system. For instance, experiments in *Synechocystis* showed that *ssrA* mutants are not as resistant as wild-type cells to low concentrations of protein synthesis inhibitors such as chloramphenicol, lincomycin, spiramycin, and streptomycin [30]. Analysis of mutants in the pathogenic bacteria *Neisseria gonorrhoeae, Mycoplasma pneumoniae,* and *M. genitalium* suggests that SsrA activity is essential for viability [31,32]. The SmpB-SsrA system may also be important for bacterial pathogenesis. It was reported that an *smpB* mutant of *Salmonella* showed a defect in survival within macrophages and an *ssrA* mutant had reduced virulence in a competitive index analysis [33,34].

In this study, we investigated the role of the SmpB-SsrA system in *Yersinia* survival and pathogenesis. We demonstrate that the SmpB-SsrA tagging and ribosome rescue system is functional in *Yersinia*. Furthermore, we show that a Δ*smpB-ssrA* mutant strain is avirulent and thus unable to cause mortality in a mouse infection model. Consistent with these observations, we show that the expression of a key TTSS transcriptional activator, VirF, and a number of other Ysc-Yop system genes is reduced in the Δ*smpB-ssrA* mutant. Finally, we provide evidence to show that the SmpB-SsrA system plays a similar but distinct role in flagella assembly and bacterial motility.

Results

The SmpB-SsrA Protein Tagging and Ribosome Rescue System Is Functional in *Y. pseudotuberculosis*

The SmpB-SsrA system is thought to play a crucial role in bacterial survival under adverse environmental conditions. To study the role of this system in *Yersinia* survival and pathogenesis, we constructed a non-polar deletion mutation of the *smpB-ssrA* genes in *Y. pseudotuberculosis*. We constructed an *ssrA* disruption strain (an *ssrA::tn5kan* disruption) and a marked *smpB-ssrA* deletion strain. Functional disruption of *smpB-ssrA* genes was confirmed by PCR, Northern blot, and Western blot analysis. These evaluations showed that neither SsrA RNA nor SmpB protein was detectable in the mutant strain (not shown). Our initial experiments indicated that the resulting strains, denoted Δ*ssrA-1* and Δ*smpB-ssrA* (abbreviated as ΔBA) have indistinguishable phenotypes; thus, we will discuss only results obtained with the ΔBA strain. The *smpB-ssrA* gene mutations demonstrate that the SmpB-SsrA system is not required for viability of *Y. pseudotuberculosis,* as is the case for *E. coli, Bacillus subtilis,* and *S. enterica* mutants [34,35,36]. The ΔBA mutants of *Y. pseudotuberculosis* have a slight growth defect at 37 °C and a moderate growth defect at 42 °C (not shown).

For more detailed analysis of the role of the SmpB-SsrA system in *Y. pseudotuberculosis,* we wanted to establish that the SmpB-SsrA protein tagging and ribosome rescue system was operational in this bacterial species. To this end, we utilized the λ-*cI-N-trpAt* nonstop mRNA expression plasmid pKW540. This expression vector harbors a synthetic gene construct that encodes for the N-terminal 93 residues of the λ-cI repressor followed by a 6-His epitope and a *trpA* transcriptional terminator, but lacks an in-frame stop codon. To permit direct monitoring of the SmpB-SsrA mediated tagging process, we re-engineered the pKW540 plasmid to also encode for an $SsrA^{H6}$ variant, which mediates the addition of an altered peptide tag (A-ANDEHHHHHH). Proteins appended with this mutant tag are more resistant to proteolytic degradation and can be easily detected using antibodies to the His6-epitope. We transformed the wild-type and ΔBA strains with pKW540 and induced the expression of the λ-*cI-N-trpAt* reporter construct to evaluate the fate of the λ-cI-N protein for the presence or absence of $SsrA^{H6}$–encoded peptide tag (Figure 1). Western blot analysis revealed that the strain lacking the SmpB-SsrA function failed to accumulate tagged λ-cI-N protein (Figure 1, lane 2). Untagged λ-cI-N protein was, however, expressed in this strain, indicating that the defect is in the tagging of the λ-cI-N protein rather than its synthesis. In contrast, in the wild-type strain, we detected a protein of the mass expected of the λ-cI-N protein plus the $SsrA^{H6}$–encoded tag (Figure 1, lane 3). These results demonstrate that the SmpB-SsrA protein tagging and ribosome rescue system is operational in *Y. pseudotuberculosis* and that the absence of this system results in the accumulation of untagged protein products of nonstop mRNAs.

ΔBA Mutant of *Y. pseudotuberculosis* Is More Sensitive to Adverse Environments

The SmpB-SsrA system is crucial for tolerance to sub-lethal concentrations of translation-specific antibiotics [37], most likely through rescuing stalled ribosomes and targeting the

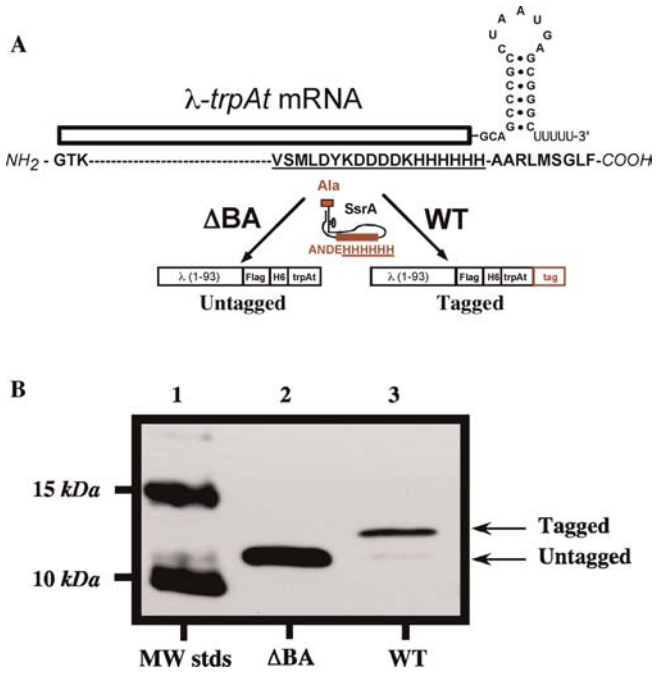

Figure 1. SmpB-SsrA Mediated Protein Tagging Activity Is Observed in *Y. pseudotuberculosis*

(A) Schematic representation of the λ-cI-N-trpAt reporter construct encoded on the pKW540 plasmid and anticipated outcomes of protein tagging in wild-type (WT) or ΔBA strains.
(B) The λ-cI-N-trpAt reporter was induced in wild-type and mutant (ΔBA) strains, and protein samples were analyzed by Western blot using HRP-conjugated anti-H6 serum.
MW stds, protein molecular weight standards.
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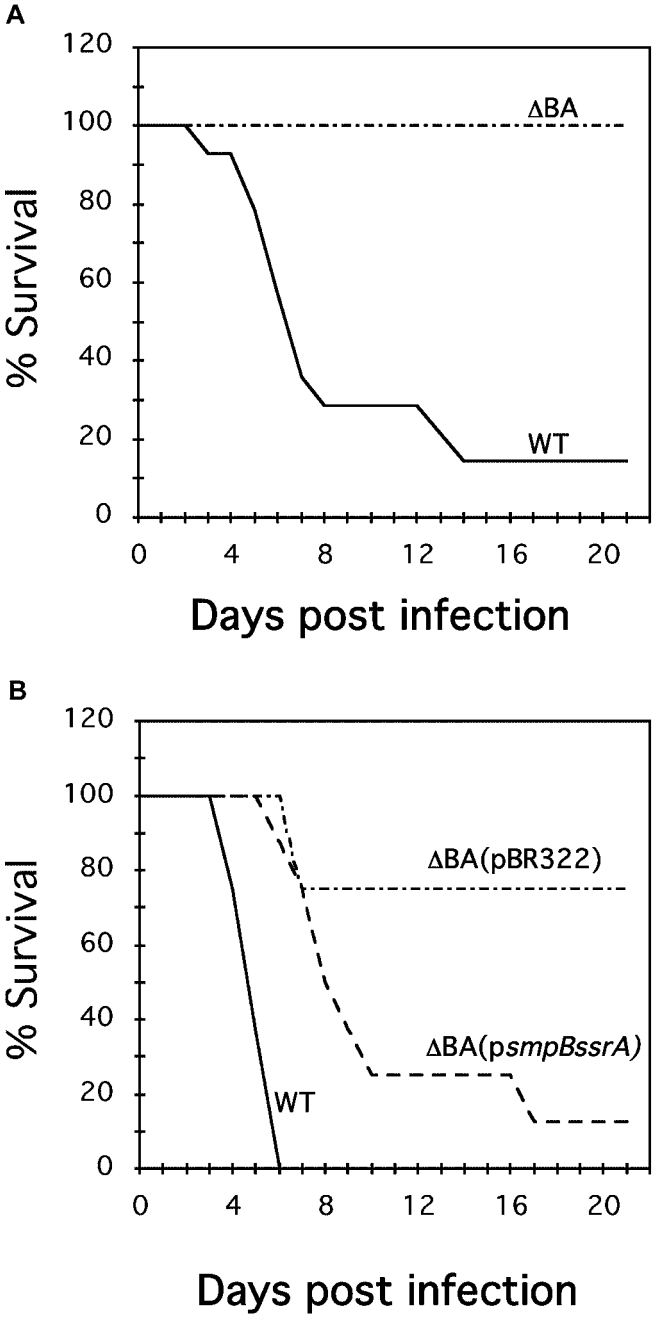

Figure 2. The *smpB-ssrA* Mutation Affects Survival of Mice Infected with *Y. pseudotuberculosis*

(A) Thirty mice, in groups of five mice per strain in three independent experiments, were challenged via the orogastric route with 2×10^9 CFU of wild-type (WT) or ΔBA strain.
(B) For complementation studies, a total of 16 mice, in groups of four mice per strain in two independent experiments, were infected via the orogastric route with wild-type, ΔBA (p*smpBssrA*), or ΔBA (pBR322) strains. Mice were monitored for at least 21 d post bacterial infection.
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associated protein fragments for proteolytic degradation. We postulated that the SmpB-SsrA system might also play an important role in bacterial survival in hostile environments, such as inside a macrophage in which the intruding bacteria are subjected to limited concentrations of iron and magnesium, changes in pH, and oxidative and nitrosative stresses. Therefore, we evaluated the ability of the *smpB-ssrA* mutant to grow under a number of adverse environmental conditions, including oxidative and nitrosative stresses, low pH, and sub-lethal concentrations of translation-specific antibiotics (for details see Protocol S1 and Figure S1). We found that compared to the isogenic parental strain, the *smpB-ssrA* mutant exhibits increased sensitivity to oxidative stress, low pH, and nitrosative stress (unpublished data). We also found that the mutant is more sensitive to sub-lethal concentrations of translation-specific antibiotics such as streptomycin, chloroamphenicol, and spectinomycin (Protocol S1 and Figure S1). Wild-type and mutant cells were equally affected by all concentrations of ampicillin, a cell wall synthesis inhibitor, indicating that the defect is specific to translation inhibitors (Figure S1).

ΔBA Mutant of *Y. pseudotuberculosis* Is Avirulent

To directly assess the role of the SmpB-SsrA system in *Y. pseudotuberculosis* virulence and pathogenesis, we evaluated the ability of the ΔBA mutant to cause lethal disease in a mouse infection model. We infected BALB/c mice via the orogastric route with 2×10^9 colony-forming units (CFU) of either wild-type or mutant bacteria and monitored for 3 wk. The vast majority of the mice (13 out of 15 mice) infected with the wild-type strain became moribund within 4–9 d of infection; the remaining mice appeared sick but survived up to 13 d post infection (Figure 2A). Most strikingly, all 15 mice infected with the mutant strain appeared normal for the duration of the 21-d observation period (Figure 2A). Addi-

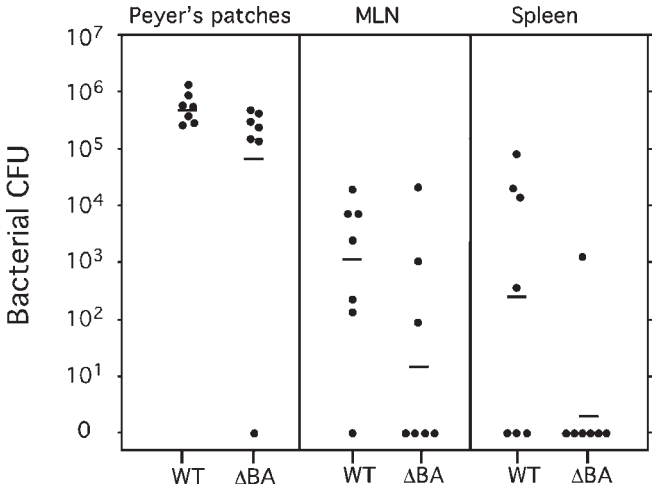

Figure 3. Wild-Type and ΔBA Strains Display Different Patterns of Tissue Colonization

Groups of seven mice per strain were infected via the orogastric route with 2×10^9 cells and sacrificed on day 4 of infection. Harvested tissues were processed for CFU determination as described in Materials and Methods. Each symbol represents the CFU contained in the indicated tissue sample from one mouse. Data were analyzed by Student *t*-test in order to determine statistical significance of CFU counts in Peyer's Patches ($p < 0.051$), MLN ($p < 0.271$), and spleen ($p < 0.044$). The *smpB-ssrA* mutant was found to be able to reach extra-intestinal sites but unable to proliferate efficiently within the MLN and spleen.

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tionally, we monitored a group of five mice infected with the mutant bacteria, for an extended period of greater than 45 d to ascertain whether there was a delay in the onset of infection and mortality. All five mice appeared healthy and did not display any telltale signs of *Yersinia* infection throughout this extended observation period, indicating that the mutant bacteria were unable to overcome the host defense mechanisms and, thus, were avirulent.

Next, we sought to determine if the phenotype of the ΔBA strain could be complemented by a plasmid encoding the *smpB-ssrA* genes. To this end, we infected groups of mice via the orogastric route with 2×10^9 CFU of either wild-type or mutant bacteria carrying p*smpB-ssrA* or a control pBR322 plasmid. Similar to previous results, we found that all of the mice infected with the wild-type strain succumbed to infection within 6 d (Figure 2B). The majority of mice (seven out of eight) infected with the ΔBA strain harboring the p*smpB-ssrA* plasmid became moribund within 17 d of infection, whereas only two out of eight mice infected with the ΔBA strain harboring the control pBR322 plasmid succumbed to infection within this time period. The surviving mice, monitored for an additional 2 wk, showed no signs of infection. Taken together, these data clearly demonstrate that the SmpB-SsrA system plays a crucial role in bacterial survival under adverse environmental conditions and a vital role in *Yersinia* pathogenesis.

To gain further insight, we evaluated the ability of the mutant bacteria to invade and colonize target tissues and organs. To this end, we infected mice with wild-type and mutant cells, and sacrificed them on day 4 of infection to determine the bacterial CFU in Peyer's patches, mesenteric lymph nodes (MLN), and spleen. We found that the colonization level of the mutant strain in Peyer's patches was comparable to that of the wild-type strain (Figure 3). However, the mutant cells were less efficient in colonizing deeper tissues such as MLN, and severely defective in colonizing the spleen (Figure 3). We continued to monitor the *smpB-ssrA* mutant–infected mice for up to 45 d. A group of the infected mice was sacrificed after 21 d to determine tissue colonization levels and potential bacterial clearance. We did not find any bacterial cells in Peyer's patches, MLN, or spleen, indicating that mutant bacteria were efficiently cleared from the host tissue and organs. These data are consistent with our earlier observation that mice infected with the mutant bacteria do not succumb to the infection and appear healthy for an extended period of 40–45 d.

ΔBA Mutant Cells Show Defects in Survival and Replication in Macrophages

Having established a clear requirement for the SmpB-SsrA system in *Yersinia* pathogenesis, we endeavored to determine the exact nature of the defect in the mutant strains. It is thought that survival and replication of *Y. pestis* in macrophages is crucial in early stages of the infection, and recent results indicate that survival of *Y. pseudotuberculosis* in macrophages is important for virulence [38]. Therefore, we assessed the ability of the mutant cells to survive and replicate in macrophages. J774A.1 macrophage-like cells were infected at a multiplicity of infection (MOI) of 5 with the wild-type and ΔBA bacteria. It should be noted that the bacteria were grown at 26 °C in the presence of calcium prior to infection to keep the Ysc-Yop TTSS repressed and minimize the potential cytotoxic effects of Yops on the macrophages [39]. Infected macrophages were washed and treated with gentamycin to remove extracellular bacteria. Intracellular bacteria were enumerated at 1 h and at 25 h post infection. Analysis of the data revealed that the J774A.1 cells internalized the same number of wild-type and ΔBA mutant bacteria after 1 h of infection. Wild-type bacteria survived and replicated within the macrophages as the number of wild-type bacteria increased by 5-fold after 25 h of infection (Figure 4). In contrast, the number of ΔBA mutant cells decreased by approximately 2-fold, indicating that the mutants suffer defects in their ability to survive and replicate in macrophages. These results support the conclusion that the SmpB-SsrA system plays an important role in the ability of *Y. pseudotuberculosis* to proliferate within host cells.

The ΔBA Mutant Exhibits Delayed Host Cell Cytotoxicity

Pathogenic *Yersinia* spp. enhance their capacity to survive within the host tissue by employing a TTSS to inject virulence proteins into the host cell cytoplasm. The injected effector proteins in turn disrupt not only the target cell's ability to mount a defense response but also the integrity of its actin cytoskeleton, thus hampering the capacity of phagocytes to engulf the pathogen. To further elucidate the role of the SmpB-SsrA system in *Y. pseudotuberculosis* virulence, we sought to determine the propensity of the mutants to cause host cell actin disruption and subsequent cytotoxicity. To this end, HeLa cells were infected with either wild-type *Y. pseudotuberculosis* or the isogenic ΔBA mutant strain and monitored at 30-min intervals for 5 h. 1 h post infection, cells infected with the wild-type strain began to show characteristic changes in morphology, indicating actin microfilaments were being disrupted, most likely as a consequence of YopE action. By

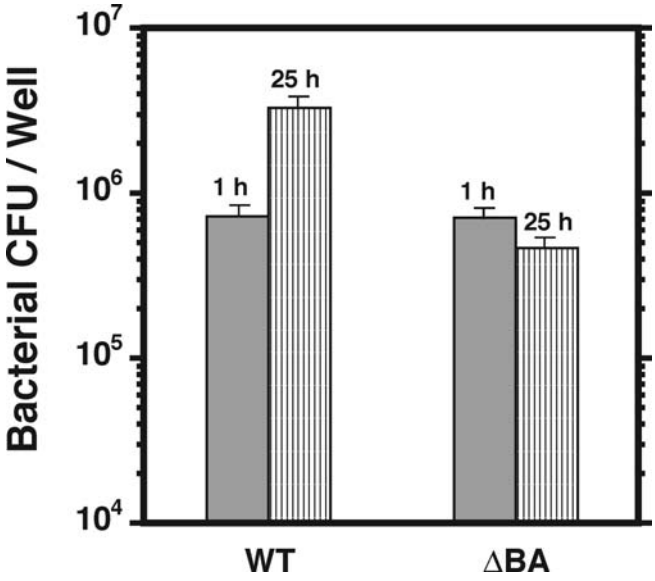

Figure 4. The ΔBA Mutant Is Defective in Survival and Replication within Macrophages

The J774A.1 cell line was infected with an equal number of 28 °C–grown log-phase wild-type (WT) or ΔBA strain of *Y. pseudotuberculosis*. Extracellular bacteria were removed and intracellular bacteria were enumerated at 1 h and 25 h post infection as described in Materials and Methods. Error bars indicate standard deviations of triplicate samples.
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2.5 h post infection, the majority of HeLa cells infected by the wild-type strain displayed significant cytotoxic effects and appeared detached from the monolayer (Figure 5). In contrast, in the initial 2.5 h of the assay, cells infected with the ΔBA or the *yopE* mutant strain [40] behaved like the uninfected control cells and did not display any significant cell rounding/cytotoxic effect (Figure 5A, 5C, and 5D), suggesting that the mutant strains might have defects in delivering YopE, and most likely other virulence factors, into host cells.

The HeLa cell cytotoxicity induced by the ΔBA strain

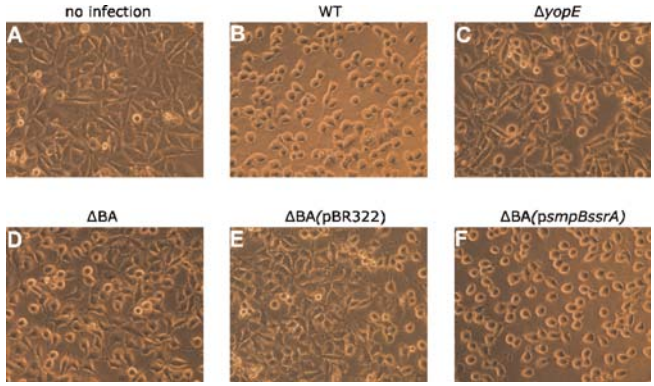

Figure 5. The ΔBA Mutant Shows Delayed Host Cell Cytotoxicity

HeLa cells (A) were infected at an MOI of 20 with wild-type *Y. pseudotuberculosis* (B), Δ*yopE* strain used as a control (C), the ΔBA mutant (D), and the mutant strain complemented with the p*smpBssrA* plasmid (E) or control plasmid (F). Morphology of the infected HeLa cells was monitored at 30-min intervals under a phase-contrast microscope. Images were captured 2.5-h post infection.
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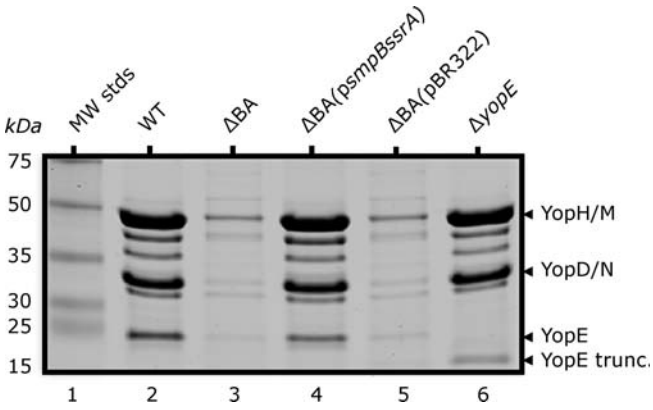

Figure 6. The ΔBA Mutant Is Defective in Secretion of Yops

Bacterial cultures of indicated strains of *Y. pseudotuberculosis* were grown in low-calcium medium, and cells were harvested 3.5 h after temperature shift to 37 °C. Culture supernatants were precipitated with TCA, and the protein pellets were resolved by electrophoresis on 10%-polyacrylamide Tris-tricine gel, and stained with Coomassie Brilliant Blue. The Δ*yopE* strain was used as a reference.
MW stds, protein molecular weight standards; WT, wild type.
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appeared delayed relative to the wild-type-infected cells. Longer incubations (>5 h) produced some cytotoxic effects, indicating that the mutant is capable of injecting low levels of Yops into the host cells (not shown). Complementation experiments showed that the ΔBA strain that were transformed with a plasmid encoding *smpB-ssrA* genes regained the ability to induce HeLa cell cytotoxicity in a manner identical to wild-type cells (Figure 5E and 5F).

ΔBA Mutant Is Defective in Secretion of Yops

Yersinia outer proteins are directionally secreted virulence factors that enable this bacterium to immobilize target cells [5]. It has been previously shown that defects in expression or delivery of Yops into host cells cause an overall decrease in virulence [41]. Since the ΔBA strain exhibited severe delays in host cell cytotoxicity, we reasoned that these mutants might have defects in Yop secretion or synthesis. To examine this possibility, we evaluated the secretion of Yops by wild-type and mutant strains in vitro. *Yersinia* grown at 37 °C in the absence of calcium can be induced to express the Ysc-Yop TTSS and secrete effector proteins into the culture medium. Hence, we performed Yop secretion analysis of cell culture supernatant fractions of wild-type and mutant bacteria grown at 37 °C for 3.5 h in the absence of calcium. This analysis revealed that the wild-type strain was fully capable of secreting all effector proteins into the culture medium (Figure 6, lane 2). Conversely, the ΔBA cells exhibited severe defects in secretion of Yops in the absence of calcium (Figure 6, lane 3), suggesting that the SmpB-SsrA system plays an important role in affecting the expression of the *Yersinia* TTSS. Although the levels of Yops secreted by the *smpB-ssrA* cells are extremely low, their accumulation over time in the host cell might be sufficient to account for the delayed ability of the mutant to induce the toxicity observed earlier (Figure 5).

These results suggest that the ΔBA strain possesses a compromised TTSS that may function suboptimally. A compromised/less efficient TTSS could account for the decreased Yop secretion phenotype of the ΔBA strain

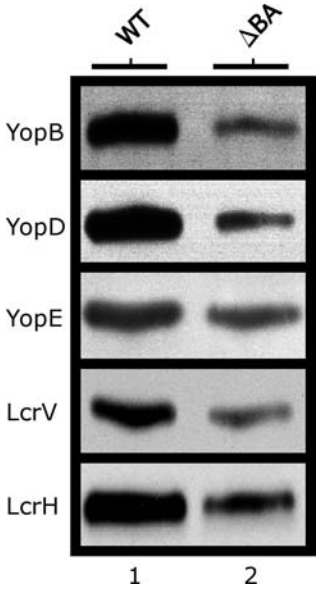

Figure 7. The ΔBA Mutant Strain Has Reduced Endogenous Levels of Effector Proteins

Cultures of wild-type (WT) or ΔBA strains of *Y. pseudotuberculosis* were grown in low-calcium medium at 37 °C for 3.5 h. Bacteria were harvested and analyzed for endogenous levels of YopB, YopD, LcrV, LcrH, and YopE proteins by Western blot analysis using Yop-specific polyclonal antibodies.

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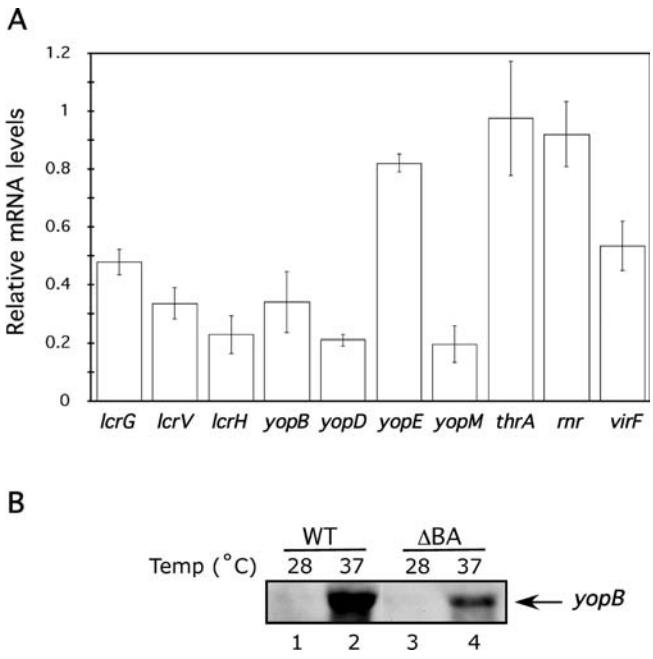

Figure 8. The *smpB-ssrA* Mutation Affects Levels of Yop Transcripts

(A) Relative mRNA levels of the indicated Yops were determined by quantitative real-time PCR as described in Materials and Methods. Each value represents the average of three independent experiments. Standard deviation bars are indicated on each column.

(B) Quantitative real-time PCR data were independently confirmed by Northern blot analysis of *yopB* mRNA. Total RNA samples were resolved on a 1% agarose-formaldehyde gel, transferred to nylon membranes, and probed with a biotin-labeled *yopB* specific probe.

WT, wild type.

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observed in the infection assays. The secretion defect of ΔBA strain could be fully complemented by a plasmid (p*smpB-ssrA*) expressing the *smpB-ssrA* genes under their native promoters (Figure 6, lane 4). These results support the conclusion that the observed Yop secretion defects are due solely to the absence of functional *smpB* and *ssrA* gene products and not to other, non-related, mutations.

Mutations in *smpB-ssrA* Affect the Endogenous Levels of Effector Proteins

One possible explanation for the drastic reduction in the secretion of Yops in the ΔBA cells might be a decrease in endogenous levels of Ysc-Yop system proteins. To test this hypothesis, we used Western blot analysis to examine the endogenous levels of TTSS components in whole-cell lysates of wild-type and mutant strains grown under inducing conditions, at 37 °C in the absence of calcium. This analysis revealed a decrease in endogenous protein levels of several TTSS components, most notably YopD, YopB, LcrH, and LcrV (Figure 7). The intracellular level of YopE was only slightly affected in the mutant strain. The decrease in protein expression levels of YopB, YopD, and LcrV could partially explain the overall decrease in secretion of the effector Yops by the ΔBA mutant.

The SmpB-SsrA System Affects Transcription of Yop mRNAs

There are a number of possible reasons for the observed reduction in the intracellular levels of YopB, YopD, and LcrV in the ΔBA mutant, including reduced transcription, enhanced mRNA degradation, reduced translation, or enhanced proteolysis. To further investigate the reason for this decrease, total RNA from the wild-type and isogenic ΔBA mutant strains were isolated after 1 h of cell growth at 37 °C in the absence of calcium and subjected to quantitative real-time PCR. This analysis showed that the levels of *yopB, yopD, yopM, lcrH,* and *lcrV* transcripts in the mutant were substantially lower (3- to 5-fold) compared to the wild-type strain (Figure 8A). However, the levels of *yopE* mRNA was only slightly reduced (1.2-fold). Consistent with the real-time PCR data, Northern blot analysis of total cellular mRNA from wild-type and mutant bacteria revealed reduced levels of *yopB* mRNA in ΔBA cells (Figure 8B). The reduction in Yop mRNA levels appears to be restricted to the virulence plasmid–encoded genes, as we did not observe any substantial reduction in the transcript levels of two chromosomally encoded genes, *rnr* and *thrA* (Figure 8A). An alternative explanation for the observed decrease in protein level of the Ysc-Yop system could be enhanced mRNA turnover. To further explore this possibility, we performed Northern blot analysis and determined the turnover rate of *yopB* mRNA. We observed only a slight difference in turnover rates of *yopB* mRNA between wild-type and mutant strains (not shown). These results mirror the observed Yop synthesis and secretion phenotypes of the ΔBA mutant cells described thus far and provide further evidence for an important role played by the SmpB-SsrA system in expression of the Ysc-Yop TTSS in *Y. pseudotuberculosis*.

Transcription of the Ysc-Yop TTSS is regulated by both temperature and extracellular calcium [42–45], and requires the VirF (LcrF) protein, a member of the AraC family of

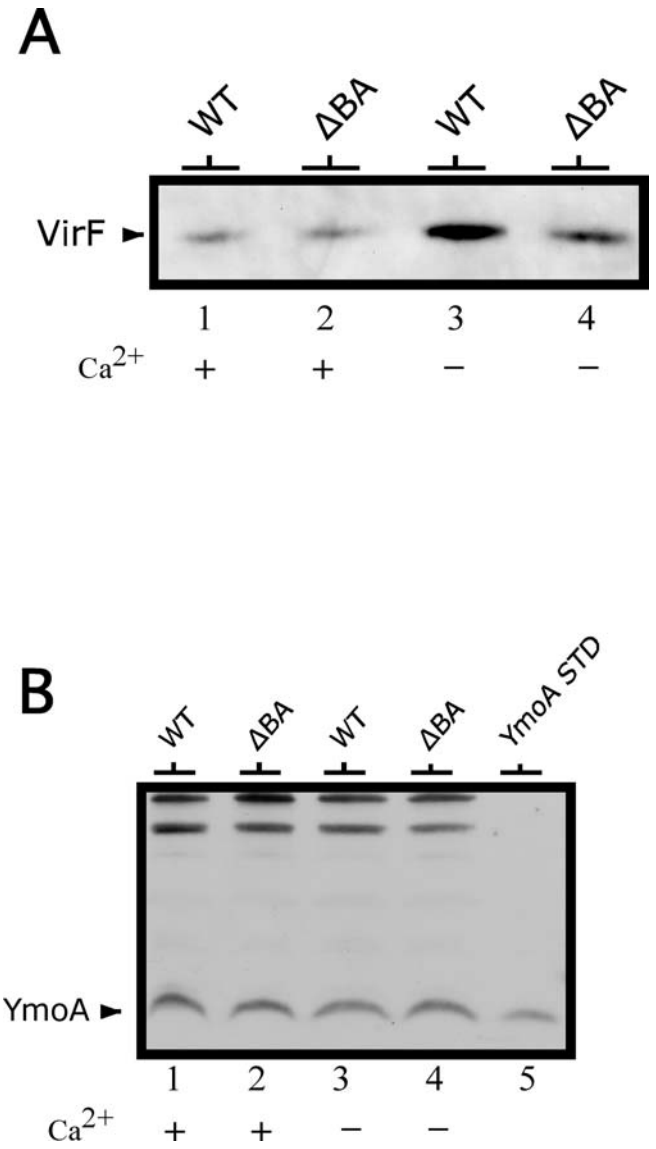

Figure 9. Affect of *smpB-ssrA* Mutation on VirF and YmoA Protein Levels
Cultures of wild-type (WT) or ΔBA strains of *Y. pseudotuberculosis* were grown in secretion non-permissive (lanes 1 and 2) or permissive medium (lanes 3 and 4) at 37 °C for 3.5 h as described in Materials and Methods. Bacteria were harvested and analyzed for VirF (A) and YmoA (B) proteins by Western blot analysis using specific polyclonal antibodies. Purified YmoA protein was used as a control (lane 5).
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transcriptional activators [46]. Therefore, we analyzed the mRNA levels of VirF by quantitative RT-PCR. This analysis revealed a 45%–50% reduction in VirF transcript levels (Figure 8A). To gain further insight, we examined VirF protein levels in wild-type and *smpB-ssrA* mutant cells under secretion non-permissive and permissive conditions. This analysis showed that in the wild-type strain, VirF protein level increases dramatically upon induction of the TTSS (Figure 9A, lanes 1 and 3). In contrast, in the *smpB-ssrA* mutant strain there is only a modest increase in VirF protein level upon induction (Figure 9A, lanes 2 and 4). These results are consistent with our quantitative RT-PCR data, showing a 45%–50% reduction in virF mRNA levels. These results support the conclusion that the reduction in VirF protein levels could in part explain the observed reduction in steady-state mRNA levels of the Ysc-Yop system components in the mutant strain. Furthermore, these results indicate that in the *smpB-ssrA* mutant strain, the Ysc-Yop TTSS remains in a transcriptionally repressed state under both secretion non-permissive and permissive conditions.

The expression of the VirF transcription activator is regulated, in a temperature-dependent manner, by changes in DNA topology and stability of YmoA, a small histone-like protein. YmoA has been shown to have repressor-like activity on Ysc-Yop expression [42,47–51]. Therefore, we reasoned that an increase in YmoA protein levels could perhaps explain the observed repressed state of the Ysc-Yop TTSS. To directly examine the intracellular levels of YmoA protein, we cloned, expressed, and purified the YmoA gene product and raised polyclonal antibodies to it. We then analyzed YmoA protein levels in wild-type and mutant strains under secretion non-permissive (Figure 9B, lanes 1 and 2) and permissive conditions (Figure 9B, lanes 3 and 4). Our analysis showed that comparable levels of YmoA protein were present in wild-type and *smpB-ssrA* cells, indicating that the absence of the SmpB-SssrA system did not substantially affect the intracellular levels of YmoA protein.

The ΔBA Mutant Has Impaired Motility

The Ysc-Yop and flagellar TTSSs are thought to be evolutionarily related [52,53]. Therefore, we wanted to investigate whether the *smpB-ssrA* mutation affected *Y. pseudotuberculosis* motility and flagellum assembly. To this end, we inoculated wild-type and mutant cells onto soft agar plates and evaluated their ability to migrate away from the point of inoculation. Wild-type cells were motile and formed concentric motility rings around the point of inoculation. In stark contrast, the ΔBA mutant cells had impaired motility and thus were unable to move from the point of inoculation (Figure 10A). We did not observe any motility upon prolonged incubation, although some cell growth was observed. Transformation with p*smpBssrA* plasmid rescued the motility defect of the mutant cells (Figure 10A).

The lack of motility could be due to a defective flagellar apparatus or the inability to assemble a flagellum. To gain further insight into these possibilities, wild-type and mutant strains grown in motility agar were isolated, negatively stained, and examined under a transmission electron microscope. As expected, the majority of the wild-type cells carried flagella; however, most, if not all, of the ΔBA cells lacked intact flagella (Figure 10B). To determine the percentage of cells carrying a functional flagellum, ΔBA cells carrying an empty vector as a control, and ΔBA cells carrying p*smpBssrA* were grown in motility agar, and the bacteria were prepared for transmission electron microscopy analysis. After staining, 300 cells from each strain were scored for the presence or absence of flagella. Fifty-one percent of the ΔBA cells complemented with p*smpBssrA* had a flagellum. In marked contrast, only 1% of the pBR322-complemented mutant cells carried flagella. These data suggest an important role for the SmpB-SssrA system in the assembly of the flagellar apparatus in *Y. pseudotuberculosis*.

Complementation with SsrA-Tagging Variants

The SmpB-SssrA system performs two key functions in *trans*-translation: (1) ribosome rescue by acting as a surrogate

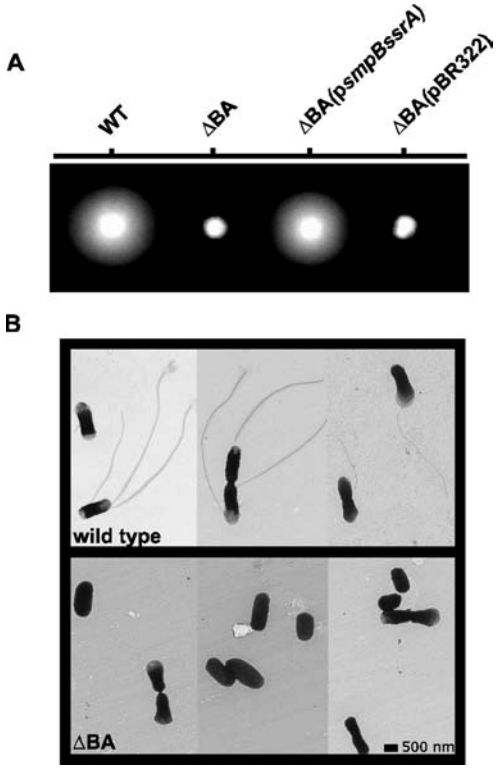

Figure 10. The ΔBA Mutant Has Impaired Motility

(A) An equal number of cells from each bacterial strain were inoculated onto 0.25% agar-T medium plates and incubated for 48 h at room temperature.

(B) Wild-type and mutant cells were immobilized on grids, negatively stained with phosphotungstic acid, and analyzed using a JEOL JEM-1200EX II transmission electron microscope. Magnification = 12,000×; scale bar = 500 nm.

WT, wild type.

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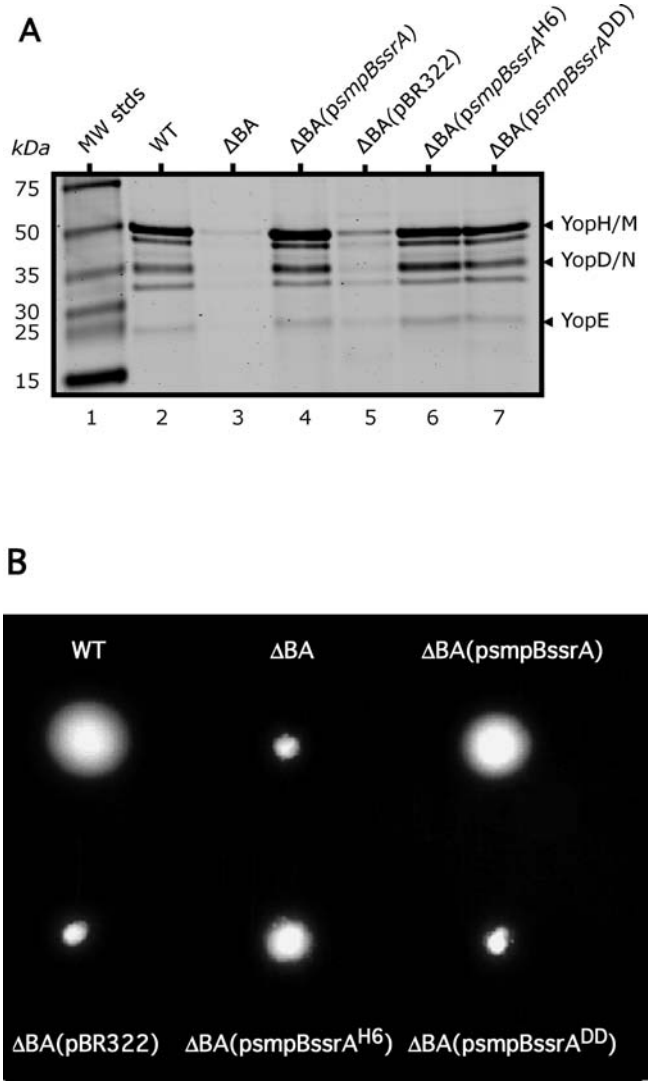

Figure 11. SsrA-Tagging Variants Complement the ΔBA Mutant to Different Degrees

(A) Cultures of wild-type (WT) or the ΔBA mutant strains were grown in low-calcium medium at 37 °C. Bacteria were removed by centrifugation and the secreted proteins were precipitated with TCA, resolved by electrophoresis on a 10%-polyacrylamide Tris-tricine gel, and stained with Coomassie Brilliant Blue.

(B) To measure motility, equal numbers of bacteria from each strain were inoculated onto 0.25% agar-T medium plates and images were obtained after incubation for 48 h at room temperature.

MW stds, protein molecular weight standards.

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mRNA, providing a stop codon to facilitate normal translation termination, and (2) tagging of the incomplete ribosome-associated protein fragments for subsequent degradation. Although both functions of the system are thought to be important, we sought to decipher if one of the two functions has a greater impact on the observed phenotypes of the *smpB-ssrA* mutant. To aid us in this analysis, we re-engineered the SsrA mRNA segment to produce two biologically active variants, $SsrA^{H6}$ and $SsrA^{DD}$. These SsrA variants are both capable of rescuing stalled ribosomes and tagging target proteins, yet they impart varying degrees of stability on the tagged proteins [54–56]. The $SsrA^{H6}$ variant is thought to add a partially stabilizing proteolytic tag, while the $SsrA^{DD}$ variant is thought to add a fully stabilizing tag, as most C-terminal specific proteases do not efficiently degrade proteins with terminal aspartic acid residues. We transformed the ΔBA mutant with plasmids p*smpBssrA*^{H6}, p*smpBssrA*^{DD}, or p*smpBssrA* (wild-type) as a control and assessed the effect of these variants on both the secretion and motility phenotypes of the ΔBA mutant. It should be noted that the ΔBA mutants carrying the p*smpBssrA*^{DD} or p*smpBssrA* plasmids have indistinguishable growth rates.

The Yops secretion phenotype of the mutants was fully complemented by wild-type SsrA, and partially complemented by both $SsrA^{H6}$ and $SsrA^{DD}$ variants (Figure 11A). The motility phenotype of the mutant strains was fully complemented by SsrA, weakly complemented by the $SsrA^{H6}$ variant, and not complemented by the $SsrA^{DD}$ variant (Figure 11B). Since all three SsrA constructs are capable of ribosome rescue and the only known difference between these constructs is the stability of the tagged proteins, we conclude that the protein tagging function of the SmpB-SsrA system is not essential for Yop secretion, whereas both ribosome rescue and protein tagging functions of the system are required for motility.

Discussion

In this report, we have clearly documented a crucial role for the SmpB-SsrA system in *Yersinia* pathogenesis. We have shown that the *Y. pseudotuberculosis* SmpB-SsrA system is critically important for fitness of the bacteria to withstand adverse growth conditions and to survive within its host. We have also learned that this system plays a key role in the expression Ysc-Yop TTSS.

Most significantly, we found the *Y. pseudotuberculosis* ΔBA mutant to be non-pathogenic and unable to cause lethal disease in a mouse infection model (Figure 2). According to a recent report by Pujol and Bliska [57], *Yersinia* spp. may exploit one, or both, of two distinct pathways to survive and proliferate inside the host: (1) The bacterium is phagocytosed by a macrophage, survives and proliferates within a phagosome, and is transported to deeper lymphoid tissue where it eventually escapes the macrophage to establish infection, or (2) the bacterium remains extracellular, avoids phagocytosis (most likely through the action of its TTSS effector proteins), and survives and replicates in the extracellular milieu to establish infection. Since the ΔBA mutant was non-pathogenic, we investigated both proposed colonization pathways to determine if one or both were affected in the mutant cells. Our investigation revealed that although equal numbers of wild-type and mutant bacteria were phagocytosed by macrophages, the ΔBA mutant was compromised in its ability to survive and proliferate within these host cells (Figure 4). Furthermore, in an in vitro host cell cytotoxicity assay, the ΔBA mutant exhibited delayed host cell toxicity, a strong indication that they might possess a compromised TTSS (Figure 5). Indeed, the ΔBA mutants had severely compromised capacity to secrete Yops (Figure 6). A closer examination of the defect revealed that the intracellular levels of the YopB, YopD, and LcrV translocator proteins were substantially reduced. These results suggest that both pathways normally exploited by *Y. pseudotuberculosis* to survive and proliferate within the host are compromised in the *smpB-ssrA* mutant.

Bacterial gene expression is frequently regulated at the level of transcription. Activators and repressors exert their control at the level of transcription initiation, whereas transcript elongation is controlled via intricate termination/anti-termination mechanisms [58]. There are at least three possible explanations for the reduced intracellular levels of some effector proteins in the ΔBA strain: (1) a reduction in gene activation/transcription of *yop* mRNAs, (2) an increase in the rate of mRNA degradation, or (3) a decline in synthesis of some essential regulatory factors. To differentiate among these possibilities, we examined the mRNA levels of several Yop effectors. Consistent with the first possibility, and in keeping with the immunoblot analysis of intracellular effector proteins, we found that the ΔBA mutant had substantially reduced levels of *yopB, yopD,* and *lcrV* mRNAs. On the other hand, *yopE* transcript level was reduced by only 20%. Furthermore, the mRNA level of *lcrH* was reduced by 80% in mutant cells, suggesting that the *lcrGVHyopBD* operon might be particularly affected. The reduction in the level of this poly-cistronic mRNA could be due to either reduced transcription from the operon or a change in mRNA stability. Northern blot analysis showed that although lower levels of *yopB* mRNA were present in the mutant cells, its rate of decay was not substantially affected, strongly indicating a change in rate of transcription from the virulence plasmid.

Expression of the Ysc-Yop TTSS is regulated in a temperature-dependent manner by an AraC-type transcriptional activator (VirF), the transcriptional repressor (YmoA), and an intact secretion apparatus. The secretion apparatus is required as a post-transcriptional regulatory mechanism that involves the LcrH chaperone and its cognate secretion substrate, YopD. When LcrH is complexed with YopD, it represses expression of type III secretion–associated mRNAs, as the chaperone–substrate complex binds target mRNAs to prevent their expression [59]. Assembly of the TTSS and secretion of YopD relieve this repression. Our analysis of *smpB-ssrA* mutant shows that both YopD and LcrH protein and mRNA levels are reduced, suggesting that the SmpB-SsrA effect is upstream of the LcrH-YopD control, likely at the level of transcriptional activation. The intracellular levels of the YmoA repressor do not appear to be affected in *smpB-ssrA* cells (Figure 9B). These observations pointed to the VirF transcriptional activator as the likely candidate whose levels might be reduced in the *smpB-ssrA* strain. Consistent with this conclusion, we observed substantial reduction in VirF mRNA and protein levels (approximately 50% and approximately 70%, respectively). Given that VirF is the sole known transcriptional activator of the Ysc-Yop TTSS, the large reduction in its cellular level in *smpB-ssrA* cells could explain the non-pathogenic (avirulent) phenotype of this mutant.

One possible explanation for the observed repressed Ysc-Yop phenotype of *smpB-ssrA* cells could be that perhaps in the absence of competing SsrA-tagged polypeptides, other substrates of the ClpXP protease system might be degraded faster. An alternative, yet related, explanation could be that the stability, and hence intracellular level, of a repressor of the Ysc-Yop system might be higher in the *smpB-ssrA* deletion strain. The YmoA protein is a temperature-dependent transcriptional repressor of the Ysc-Yop system and a known natural substrate of the ClpXP and Lon protease [50]. However, our analysis of the intracellular level of YmoA showed that it was not affected substantially by the absence of the SmpB-SsrA system.

A number of recent reports have suggested that the SmpB-SsrA system plays a regulatory role, through its ribosome rescue and tagging for directed degradation functions, in modulating gene expression by maintaining the requisite intracellular concentrations of some regulatory factors. These factors might include transcriptional activators and repressors [27,28]. Experiments aimed at identifying endogenous proteins that carry SsrA-encoded tags have revealed the identity of a number of regulatory proteins including the LacI repressor [60,61], λ-cI repressor, YbeL, GalE, and RbsK [61]. In the case of the Lac repressor, it was proposed that an increase in its intracellular concentration results in the binding of LacI protein to secondary *lac* operator sequences located within the *lacI* gene. This binding is thought to lead to premature transcription termination and generation of truncated nonstop *lacI* mRNAs that, in turn, cause ribosome stalling and recognition by the SmpB-SsrA system [60,61]. The SsrA-tagged LacI protein is proposed to undergo rapid degradation by cellular proteases. This type of negative feedback regulation is thought to maintain the desired concentration of LacI protein for sensitive tuning of physiological responses to the metabolic state of the cell.

Indeed, *ssrA*$^-$ cells show a marked delay in induction of the *lac* operon, consistent with the notion that truncated yet functional LacI repressor molecules accumulated in these cells, leading to enhanced repression of the *lac* operon.

A similar regulatory role is ascribed to the SmpB-SsrA system in the bacteria phage Mu life cycle, in which the SmpB-SsrA system is thought to play a role in maintaining the repressor population in a responsive state such that the cell can undergo de-repression [22,62]. In an *ssrA* knockout strain, truncated but functional repressor molecules accumulate and render the cell unresponsive to conditions that trigger de-repression. Such a regulatory role of the SmpB-SsrA system implies that tagging of some regulatory factors is a normal, constitutive process in the cell. Additionally, tagging of GalE and RbsK suggests involvement of the SmpB-SsrA system in regulation of carbon source metabolism, which is consistent with our finding that the ΔBA mutant exhibits slow recovery from carbon starvation. Further support for a potential regulatory role for the SmpB-SsrA system comes from recent studies in *Caulobacter crescentus,* whereby the optimal timing between the degradation of a specific response regulator, CtrA, and initiation of DNA replication is influenced by specific tagging and targeted degradation of the response regulator [63].

The SmpB-SsrA system is thought to also affect gene expression solely through its ribosome rescue function. Although no direct evidence links the SmpB-SsrA system to the expression/translation of a specific transcriptional activator, a number of reports point to the potential presence of such regulatory factors whose cellular concentrations are particularly sensitive to the balance between its proteolysis (perhaps by Clp proteases in an SsrA tagging–independent manner) and translational efficiency of the cell [31,62,64]. It is proposed that ribosome stalling, in the absence SsrA RNA, results in substantial reduction in the synthesis of these factors. Such protein synthesis reduction coupled with their proteolytic sensitivity is proposed to reduce the intracellular concentrations of these factors to a nonresponsive level. This proposal is supported by the observation that deletion of Clp proteases compensate, to some extent, for the λimmP22 phenotype of an *E. coli ssrA* mutant [64].

Interestingly, in the course of our studies, we also discovered that the ΔBA mutant strain suffered severe impairment in motility, due mainly to a lack of flagella. Motility is demonstrated to be essential for virulence of a number of bacterial pathogens [65,66], and is thought to be required for *Y. enterocolitica* pathogenesis as it provides migration capability to contact host cells [67]. There is evolutionary, structural, and sequence similarity between the TTSS and the bacterial motility apparatus, the flagellum [68]. Previous studies have shown that mutations in *fliZ* significantly reduce expression of the SPI-1 TTSS components in *S. enterica* [69,70]. Given the evolutionary, structural, and sequence similarity between the *Y. pseudotuberculosis* type III system and the flagellar apparatus, and knowing that the two systems are subject to alternative expression control, it is plausible that there is a link between the expression and regulation of both systems. Indeed, Bleves et al. have shown that the Ysc-Yop TTSS of *Y. enterocolitica* is deregulated in an *flhDC* mutant in such a manner that the Yop effector proteins are efficiently secreted at non-permissive temperatures [71]. Our results therefore support the conclusion that there is a link between regulatory mechanisms that control the flagellar and the Ysc-Yop TTSSs in *Y. pseudotuberculosis*. In agreement with this conclusion, Petersen and Young have recently presented evidence to show that mutation in *cya* and *crp,* which encode adenylate cyclase and the cyclic AMP (cAMP) receptor protein (CRP, also called CAP), have a negative affect on the expression of both the Ysc-Yop and flagellar TTSS of *Y. enterocolitica* [72]. We have not examined the status of *cya* and/or CRP in our *smpB-ssrA* mutant.

At present, the molecular mechanism by which the SmpB-SsrA system directly or indirectly affects the expression of the *Y. pseudotuberculosis* Ysc-Yop, and perhaps flagellar type III secretion systems remains unknown. The simplest model is that the SmpB-SsrA system exerts its control via one or both of its core functions: ribosome rescue and protein tagging for subsequent degradation. If the SmpB-SsrA system exerts its effect through tagging of a key transcription repressor, thereby controlling its intra-cellular concentrations, then loss of this regulation in a ΔBA strain should result in the accumulation of the regulatory factor. This imbalanced accumulation will in turn prevent activation of genes under the control of the regulatory factor. However, if the SmpB-SsrA system exerts its effect through the ribosome rescue function, then the nature of the C-terminal tag added should be of little consequence. Our results with the SsrA-tagging variants, which do not affect ribosome rescue but impart varying degrees of stability to the tagged protein, suggest that the ribosome rescue function of the SmpB-SsrA system is sufficient for expression of the Ysc-Yop TTSS. In contrast, both ribosome rescue and tagging/targeted degradation of key regulator(s) are required for expression of the flagellar TTSS. Therefore, we reason that for some regulatory pathways, the ribosome rescue function is necessary and sufficient to achieve the desired regulation, whereas for other regulatory pathways, ribosome rescue without tagging and directed degradation of the target proteins might not be sufficient to achieve the desired regulatory outcome.

Materials and Methods

***smpB-ssrA* mutation.** The *smpB* and *ssrA* genes are located adjacent to each other in the *Y. pseudotuberculosis* genome, separated by only 48 nucleotides. The DNA fragment encompassing the linked *smpB* and *ssrA* genes was PCR amplified and cloned into the EcoRI and PstI restriction sites of the pUC18 plasmid. The resulting pUC*smpB-ssrA* plasmid was subjected to random in vitro mutagenesis using the EZ::TN<*kan*> transposon (Epicenter Technologies, Madison, Wisconsin, United States) in accordance with the manufacturer's instructions. Kanamycin-resistant colonies were screened by PCR for transposon insertions in the *ssrA* gene. The resulting plasmid was digested with SalI (a unique site present in the *smpB* gene and the 5′ upstream region of the EZ::TN<*kan*> transposon) and self-ligated to delete a region of both genes. In this process, half of the 3′ end of the *smpB* gene sequence, the entire intragenic region, and 250 nucleotides of the 5′ end of the *ssrA* gene were replaced by the transposon. The DNA segment containing the transposon-disrupted *smpB-ssrA* genes was amplified by PCR and cloned into SmaI-digested suicide vector pSB890 [40]. The final construct, pSB890Δ*smpB-ssrA*, was transformed into *E. coli* S17 λ*pir* cells. This strain was used for conjugal transfer of the suicide plasmid into *Y. pseudotuberculosis*. Tetracyclin-resistant trans-conjugants were selected on *Yersinia*-selective medium (Oxoid, Basingstoke, United Kingdom). Recombinant colonies were selected by growing the cells on LB agar plates lacking NaCl and supplemented with 5% sucrose to select against the *sacB* gene carried on the pSB890 plasmid. Sucrose-resistant colonies were tested for Tet^s phenotype, and mutation was confirmed by using colony PCR and Northern blot analysis. The resulting *Y. pseudotu-*

berculosis IP2666 *smpBssrA::kan* strains are hereafter referred to as Δ*smpB-ssrA* (abbreviated as ΔBA).

Construction of p*smpBssrA* and SsrA-tagging variants. To create a complementation plasmid that harbors *smpB-ssrA* genes under their native promoters, a DNA fragment including *smpB, ssrA,* and a 500-nucleotide region upstream of *smpB* was PCR amplified from genomic DNA and cloned into PstI and EcoRI sites of pBR322. Genes on the plasmid were verified by DNA sequencing and the confirmed plasmid was denoted p*smpBssrA*. Two SsrA tag sequence variants: (1) $SsrA^{H6}$, which instead of a wild-type tag (ANDENYALAA) encodes for a six-histidine tag variant (ANDEHHHHHH), and (2) $SsrA^{DD}$, which encodes a tag with two stabilizing aspartic acid residues (ANDENYALDD), were generated by site-directed mutagenesis using p*smpBssrA*. The resulting SsrA-tagging variant plasmids were confirmed by DNA sequencing. Plasmid pKW540, a pPW500 derivative [61], expresses the λ-*cI-N-trpAt* nonstop mRNA under the control of a lac-promoter and the $ssrA^{H6}$ variant under the native SsrA promoter.

SsrA-mediated tagging of λ-cI-N-trpAt. Wild-type and ΔBA strains were transformed with the pKW540 plasmid. Cultures were grown in LB broth to OD_{600} of approximately 0.5, and expression of the λ-cI-N protein was induced by the addition of 1 mM isopropyl-1-thio-ß-D-galactopyranoside (IPTG) to the culture medium. After 2 h of induction, cell pellets were collected and resuspended in SDS-PAGE loading buffer, heat denatured, and resolved by electrophoresis on 20%-polyacrylamide Tris-tricine gels. Resolved proteins were transferred to polyvinylidene difluoride (PVDF) membrane and probed with horseradish peroxidase (HRP) conjugated anti-H6 antibodies (Santa Cruz Biotechnology, Santa Cruz, California, United States) to detect the H6 epitope present in the λ-cI-N protein.

Mouse infection assays. Female BALB/c mice 6–8 wk old (Taconic, Germantown, New York, United States) were used for infections. All animal experimentation was performed in accordance with institutional guidelines. Mice were subjected to fasting for 20 h prior to infection, while bacteria were grown overnight in LB broth. Overnight cultures were diluted to an optical density at 600 nm (OD_{600}) of 0.1 and grown at 26 °C to an OD_{600} of approximately 0.8. Harvested bacterial pellets were washed once and suspended in PBS (phosphate-buffered saline). Mice were challenged via the orogastric route with 0.2-ml aliquots of each culture, containing 2×10^9 bacteria. Animals were monitored twice daily over a 3-wk period. Mice exhibiting severe signs of disease (hunched posture and immobility) were deemed incapable of survival and humanely euthanized. For tissue colonization experiments, mice were challenged as above and sacrificed by CO_2 asphyxiation 4 d after infection. Three Peyer's patches, the entire MLN, and the spleen were aseptically removed and individually homogenized in 5 ml of PBS. Serial dilutions of tissue homogenates were spread on LB plates and bacterial CFU were enumerated for each tissue.

Macrophage infection assay. The murine macrophage-like cell line J774A.1 was cultured in Dulbecco's modified Eagle's medium (DMEM; Invitrogen, Carlsbad, California, United States) supplemented with 10% heat-inactivated fetal bovine serum (FBS; HyClone, Logan, Utah, United States), 2 mM L-glutamine and 1 mM sodium pyruvate in a humidified incubator with 5% CO_2 at 37 °C. J774A.1 cells were seeded into 24-well tissue culture plates (2×10^5 cells/well) 24 h prior to infection. Meanwhile, wild-type and ΔBA mutant cells were grown overnight in HI medium at 26 °C. The day of infection, J774A.1 cells were inoculated at an MOI of 5 and plates were centrifuged at $50 \times g$ for 4 min to facilitate interactions between bacteria and macrophages [39]. After centrifugation, plates were incubated for 20 min at 37 °C in 5% CO_2, and wells were washed twice with PBS. To kill extracellular bacteria, the macrophage cultures were incubated with fresh medium containing 8 μg/ml gentamycin. After 1 h at 37 °C, medium was removed, the J774A.1 cells were washed twice with PBS, and fresh medium containing 4.5 μg/ml gentamycin was added. Samples were taken for analysis at this time point (1 h post infection), and 25 h post infection. CFU counts were carried out to evaluate the survival and replication of intracellular bacteria [39]. Briefly, at 1-h and 25-h time points, infected cells were washed three times with PBS and lysed by 0.1% Triton X-100 in PBS. The lysates were collected in 2-ml tubes, and wells were rinsed with HI medium. The lysates and rinse from each well were combined and gently sonicated to disperse the bacteria. Serial dilutions of the samples were spread onto LB-Agar plates and grown at 28 °C for 2–3 d. CFU were enumerated for each sample.

Cytotoxicity assay. HeLa cells were cultured in DMEM supplemented with 10% heat-inactivated FBS, 2 mM L-glutamine, and 1 mM sodium pyruvate in a 5% CO_2–humidified incubator at 37 °C. HeLa cells (2×10^5) were seeded into wells of a 24-well tissue culture plate 24 h prior to assay. Meanwhile, bacteria were grown overnight in LB broth at 26 °C. Saturated cultures were diluted 1:40 in LB broth containing 2.5 mM $CaCl_2$ and grown at 37 °C for 2 h. HeLa cells were infected at an MOI of 20, and plates were centrifuged at $50 \times g$ for 4 min. After centrifugation, plates were incubated in a humidified incubator, at 37 °C in 5% CO_2. Cells were monitored every 30 min under a phase-contrast microscope for characteristic Yop-mediated cytotoxic rounding. Images were captured using a digital camera.

Yop secretion assay. Overnight cultures of *Yersinia* were diluted to an OD_{600} of 0.1 in low-calcium medium (LB supplemented with 20 mM $MgCl_2$ and 20 mM sodium oxalate for chelating Ca^{++}). Cultures were incubated at 26 °C for 1 h and then switched to 37 °C and incubated for additional 3.5 h. Before harvesting the cultures, OD_{600} values of each culture were measured. Bacterial cultures, containing equal number of cells, were centrifuged at $6{,}000 \times g$ for 10 min and secreted proteins in supernatants were precipitated with ice-cold 10% TCA (trichloroacetic acid) for 1 h at −20 °C. Samples were centrifuged at $18{,}000 \times g$ for 30 min, and the protein pellets were washed with ice-cold acetone. Dried pellets were resuspended in urea sample buffer (100 mM Tris [pH 7.5], 4.2 M urea, 2 mM DTT, 1× SDS-PAGE loading buffer), heat denatured, and resolved by electrophoresis on 10%-polyacrylamide Tris-tricine gels. Protein bands were visualized by staining with Coomassie Brilliant Blue R250 dye.

Immunoblot analysis. In order to determine the endogenous protein levels of Yops, wild-type and ΔBA mutant cells were grown in low-calcium medium and harvested. For the analysis of VirF and YmoA, overnight cultures of cells were diluted to OD_{600} of 0.1 in secretion permissive (low-calcium) or non-permissive medium (LB + 2.5 mM $CaCl_2$). Before harvesting, bacterial cultures were grown for 1 h at 26 °C and then for 3.5 h at 37 °C. All bacterial pellets, which contained the same number of cells, were resuspended in 1× SDS-PAGE loading buffer, and protein samples were boiled for 10 min and resolved by electrophoresis on 10%-polyacrylamide Tris-tricine gels. Immunoblot analysis was performed using individual polyclonal antibodies to YopB, YopD, YopE, LcrH, LcrV, VirF, and YmoA proteins. Protein levels were quantified by densitometry. Specific anti- sera to YopB, YopD, YopE, LcrH, and LcrV were a kind gift from Dr. Bliska's laboratory. Anti-VirF serum was a kind gift from Dr. Plano's laboratory.

Quantitative real-time PCR. Wild-type and ΔBA mutant cells were grown in low-calcium medium as described above and harvested with RNA Protect (Qiagen, Valencia, California, United States) 1 h after temperature shift. Total RNA was isolated using the RNeasy kit (Qiagen), according to the manufacturer's recommendations. Genomic DNA was removed by incubating the samples with 10 units of DNase I (Roche Diagnostics, Basel, Switzerland) at 37°C for 30 min in a mixture of 50 mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$, and 50 μg/ml BSA (20 μl volume). One microgram of RNA was used to synthesize cDNA using the SuperScript III first-strand synthesis system (Invitrogen). Quantitative analysis of cDNAs was performed with a Light Cycler instrument using SYBER Green I DNA binding dye (Roche Applied Sciences, Indianapolis, Indiana, United States) to detect PCR products. The PCR mixture was prepared using 2.5 mM $MgCl_2$, 10 pmol/L of each primer (Table S1), and 2.5 units of FastStart Taq DNA polymerase. Parameters for the amplification were: initial denaturation at 95 °C for 10 min, followed by 35 cycles each consisting of 10 sec at 95 °C, 5-s annealing at 57 °C, 12 s of extension at 72 °C, and 3 s of fluorescence measurement at indicated temperatures (Table S1). The temperature at which the fluorescence was measured was determined by melting-curve analysis. A standard curve was generated from 3-fold and 9-fold dilutions of the wild-type sample, via which the relative ratios were calculated, and 16S rRNA was used as an internal normalization control.

Northern blot analysis. Wild-type and ΔBA mutant cells were grown in low-calcium medium as described above, and Rifampin (200 μg/ml) was added 1 h after temperature shift to prevent new rounds of transcription. Cells were harvested with RNA Protect (Qiagen) at 0, 2, 4, 6, 8, 10, and 12-min time points. Total RNA was isolated using an RNeasy kit (Qiagen), and 5 μg of each sample was resolved on a 1% agarose-formaldehyde gel. Upon transfer, samples were crosslinked to a positively charged nylon membrane (Ambion, Austin, Texas, United States) by UV Stratalinker 1800 (Stratagene, La Jolla, California, United States). A 1.2-kb fragment of *yopB* gene was PCR amplified and labeled with a Brightstar Psoralin-Biotin labeling kit (Ambion) for use as a probe in Northern blot analysis. Hybridization was followed by detection with a BM-Chemiluminescence Biotin/Streptavidin blotting kit (Roche Applied Sciences) according to the manufacturer's recommendations.

Motility assay and transmission electron microscopy. *Yersinia* strains were grown in LB media containing the appropriate antibiotics and diluted to an OD_{600} of 0.1. Five microliter aliquots of each culture were spotted onto motility T-medium plates (1%

tryptone, 0.25% Difco agar) containing appropriate antibiotics when necessary [73]. The plates were incubated for 48 h at room temperature, and examined for motility.

The flagellar apparatus was visualized by whole-cell negative staining. Bacteria from motility assay were scooped off the plates with a wire loop and suspended in PBS. After a brief spin, 50 μl of sample was allowed to adsorb to polyvinyl formal-coated grids (Ernest F. Fullam, Latham, New York, United States) for 2 min. The grids were fixed in 1% glutaraldehyde (Sigma-Aldrich, St. Louis, Missouri, United States), washed twice with PBS, twice with water, and stained with 0.5% phosphotungstic acid (Ted Pella, Inc, Redding, California, United States). Samples were examined with a JEOL JEM-1200EX II transmission electron microscope (JEOL USA, Peabody, Massachusetts, United States) at an accelerating voltage of 80 kV.

Supporting Information

Figure S1. The ΔBA Mutant of *Y. pseudotuberculosis* Shows High Sensitivity to Translation-Specific Antibiotics

Equal number of bacterial cells from saturated cultures of wild-type and ΔBA strains were serially diluted and spotted onto plates containing various concentrations of the indicated antibiotic. Plates were incubated at 26 °C for 3 d.

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Protocol S1. Bacterial Strains, Growth Conditions, and Sensitivity to Antibiotics

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Table S1. Oligonucleotide Primers Used in This Work

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Accession Numbers

The GenBank (http://www.ncbi.nlm.nih.gov/Genbank/) accession numbers for genes and gene products discussed in this paper are LcrG (pYV0058), LcrH (pYV0056), LcrV (pYV0057), Rnr (YPTB0432), SmpB (YPTB1135), SsrA (YPTB__RNA__88), ThrA (YPTB0602), VirF (pYV0076), YmoA (YPTB0978); YopB (pYV0055), YopD (pYV0054), YopE (pYV0025), and YopM (pYV0047) .

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Author contributions. NAO and AWK conceived and designed the experiments. NAO, JBB, and AWK performed the experiments. NAO, JBB, and AWK analyzed the data. NAO and AWK contributed reagents/materials/analysis tools. NAO and AWK wrote the paper.

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