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MOLECULAR PATHOGENESIS

Contribution of Asparagine Catabolism to *Salmonella* Virulence

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ABSTRACT Salmonellae are pathogenic bacteria that cause significant morbidity and mortality in humans worldwide. Salmonellae establish infection and avoid clearance by the immune system by mechanisms that are not well understood. We previously showed that L-asparaginase II produced by *Salmonella enterica* serovar Typhimurium (*S.* Typhimurium) inhibits T cell responses and mediates virulence. In addition, we previously showed that asparagine deprivation such as that mediated by L-asparaginase II of *S.* Typhimurium causes suppression of activation-induced T cell metabolic reprogramming. Here, we report that *STM3997*, which encodes a homolog of disulfide bond protein A (*dsbA*) of *Escherichia coli*, is required for L-asparaginase II stability and function. Furthermore, we report that L-asparaginase II localizes primarily to the periplasm and acts together with L-asparaginase I to provide *S.* Typhimurium the ability to catabolize asparagine and assimilate nitrogen. Importantly, we determined that, in a murine model of infection, *S.* Typhimurium lacking both L-asparaginase I and II genes competes poorly with wild-type *S.* Typhimurium for colonization of target tissues. Collectively, these results indicate that asparagine catabolism contributes to *S.* Typhimurium virulence, providing new insights into the competition for nutrients at the host-pathogen interface.

KEYWORDS asparaginase, asparagine, catabolism, host response, metabolism, nitrogen metabolism, pathogenesis, *Salmonella*, T cells, virulence

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Salmonella enterica (*S. enterica*) serovars cause significant morbidity and mortality in humans worldwide (1–5). Clinical syndromes associated with *S. enterica* serovars include typhoid and paratyphoid fever caused by *S. enterica* serovar Typhi (*S.* Typhi) and *S. enterica* serovar Paratyphi, respectively, gastroenteritis caused by nontyphoidal *Salmonella* (NTS) serovars such as *S. enterica* serovar Typhimurium (*S.* Typhimurium) in immunocompetent individuals, and bacteremia caused by NTS serovars in immunocompromised individuals (6). The different clinical syndromes associated with *S. enterica* serovars are responsible for a combined global toll of approximately 1 million deaths annually (7), which makes these pathogenic bacteria one of the leading causes of mortality in humans worldwide.

Much of what is known about the pathogenesis of and host response to infections with *Salmonella* comes from experimental infection of mice with *S.* Typhimurium, which has served as a useful model for the human disease caused by *S.* Typhi (8–10). In susceptible strains of mice, *S.* Typhimurium infection results in acute immunosuppression and delayed onset of protective immune responses (8, 9, 11–13). Immunity to *S.* Typhimurium that eventually develops requires both humoral and cell-mediated immune responses. T cells, particularly gamma interferon (IFN-γ)-producing $CD4^+$ T cells, play a critical role in the clearance of *S.* Typhimurium. However, T cell responses to *S.*

Typhimurium are dampened during infection by mechanisms that are not well understood (8, 9, 11).

We previously showed that L-asparaginase II produced by *S.* Typhimurium inhibits T cell responses and mediates virulence (14). In addition, we previously showed that asparagine deprivation such as that mediated by L-asparaginase II of *S.* Typhimurium causes inhibition of activation-induced T cell metabolic reprogramming (15).

Here, we show that *STM3997*, which encodes a putative periplasmic thiol-disulfide oxidoreductase, is required for *S.* Typhimurium to cause inhibition of T cells and, furthermore, is required for L-asparaginase II stability and function. We also show that L-asparaginase II localizes primarily to the periplasm and acts together with the cytoplasmic asparagine hydrolase L-asparaginase I to provide *S.* Typhimurium the ability to catabolize asparagine *in vitro* and during infection. These findings provide new insights into the L-asparaginase-based mechanism used by *S.* Typhimurium to cause inhibition of T cells and advance our knowledge of the competition for nutrients at the host-pathogen interface.

RESULTS

***STM3997* is required for *S.* Typhimurium to cause inhibition of T cells.** The L-asparaginase II gene *STM3106* is required for *S.* Typhimurium to inhibit T cells and mediate virulence (14). The *STM3106* gene was identified in a partial screen of an *S.* Typhimurium mutant library used to isolate mutants unable to cause down-modulation of T cell receptor beta (TCR-β) surface expression (14). Each *S.* Typhimurium mutant in the partially screened library carries a targeted deletion of multiple linked, nonessential genes (a total of 3,476 genes are deleted in the entire library [16]). To identify additional genes required for *S.* Typhimurium to cause inhibition of T cells, we screened the remainder of the targeted multigene deletion library. One mutant, Δ*STM3993–*Δ*STM3998* (Δ*STM3993–8*) *S.* Typhimurium, was unable to cause down-modulation of TCR-β surface expression (Fig. 1A and C).

To identify the gene(s) deleted in the mutant required for this phenotype, we generated Δ*STM3993–8 S.* Typhimurium strains with a plasmid carrying *STM3993*, *STM3994*, *STM3995*, *STM3996*, *STM3997*, or *STM3998*. Only the plasmid carrying *STM3997* (p*STM3997*) complemented the phenotype of Δ*STM3993–8 S.* Typhimurium (Fig. 1B). Consistent with these results, *S.* Typhimurium carrying a targeted deletion of *STM3997* (Δ*STM3997*) was unable to cause down-modulation of TCR-β surface expression (Fig. 1D). Similar results were obtained when we measured the ability of Δ*STM3997 S.* Typhimurium to cause inhibition of T cell blastogenesis (Fig. 1E) and interleukin-2 (IL-2) secretion (Fig. 1F). Furthermore, the presence of *STM3997* in *trans* reversed the phenotype of Δ*STM3997 S.* Typhimurium (Fig. 1D to F). Collectively, these results indicate that *STM3997*, like *STM3106* (14), is required for *S.* Typhimurium to cause inhibition of T cells.

***STM3997* encodes a periplasmic thiol-disulfide oxidoreductase required to maintain the steady-state level of L-asparaginase II.** The *STM3997* gene of *S.* Typhimurium encodes a homolog of disulfide bond protein A (DsbA) of *Escherichia coli*. DsbA is a periplasmic thiol-disulfide oxidoreductase involved in the formation of structural disulfide bonds that are essential for the stability and function of many proteins, particularly those that are secreted (17). Given the function of DsbA and our published results indicating that L-asparaginase II produced by *S.* Typhimurium is necessary and sufficient to cause inhibition of T cells (14), we investigated whether *STM3997* is required to maintain the steady-state level of L-asparaginase II. Consistent with the hypothesis that DsbA is involved in the production of functional L-asparaginase II, the steady-state level of L-asparaginase II was reduced significantly in Δ*STM3997 S.* Typhimurium (Fig. 2A and B). Furthermore, the presence of *STM3997* in *trans* restored the steady-state level of L-asparaginase II in Δ*STM3997 S.* Typhimurium (Fig. 2A and B). As expected, L-asparaginase II gene expression was not affected in Δ*STM3997 S.* Typhimurium (see Fig. S1 in the supplemental material). These results indicate that *STM3997* is required to maintain the steady-state level of L-asparaginase II.

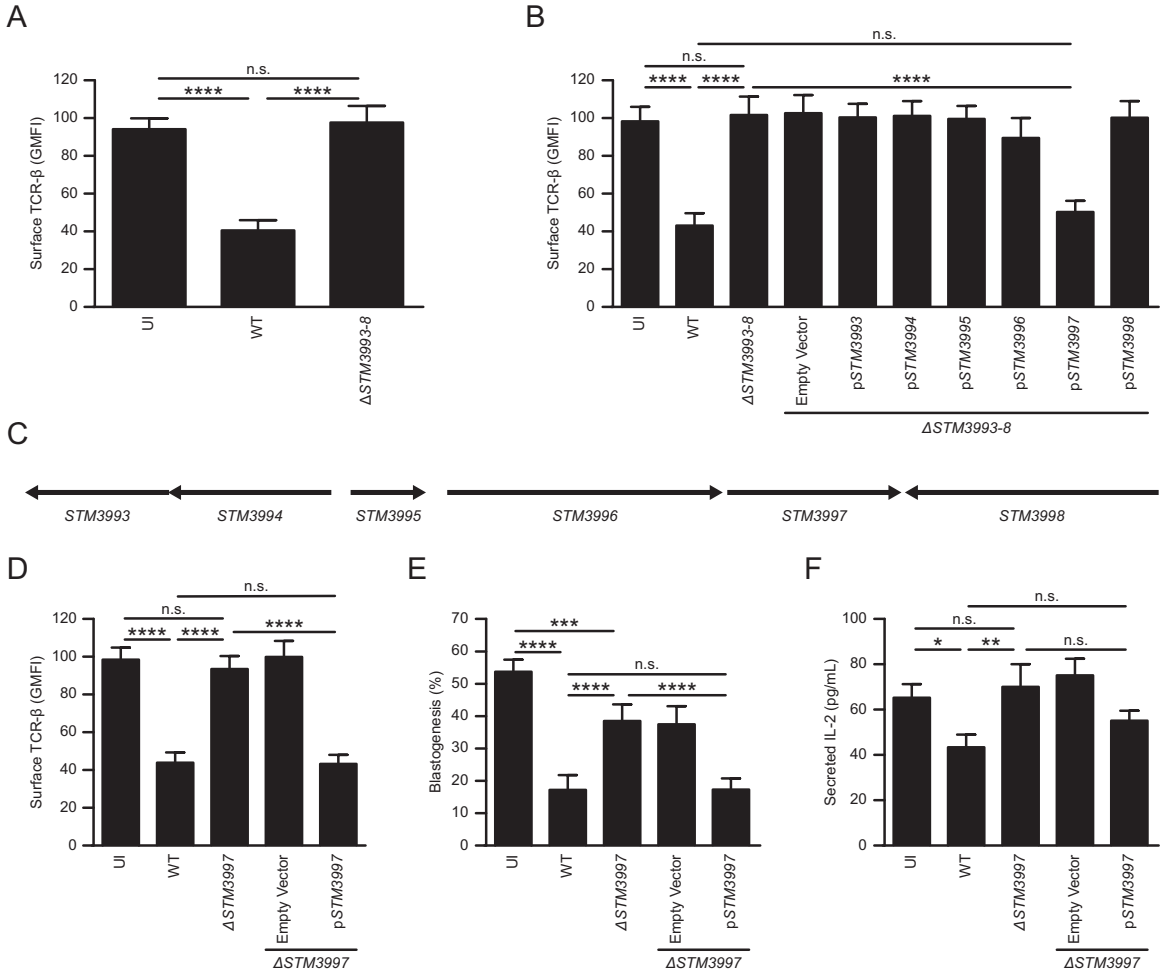

FIG 1 *STM3997* is required for *S.* Typhimurium to cause inhibition of T cells. (A) Expression of surface TCR-β by anti-CD3ε/CD28-stimulated T cells left uninfected (UI) or cultured with wild-type *S.* Typhimurium (WT) or Δ*STM3993–8 S.* Typhimurium (Δ*STM3993–8*). (B) Expression of surface TCR-β by anti-CD3ε/CD28-stimulated T cells left uninfected (UI) or cultured with wild-type *S.* Typhimurium (WT), Δ*STM3993–8 S.* Typhimurium (Δ*STM3993–8*), Δ*STM3993–8 S.* Typhimurium carrying plasmid pBAD18-Cm (Δ*STM3993–8*/Empty Vector) or Δ*STM3993–8 S.* Typhimurium carrying derivatives of pBAD18-Cm expressing *STM3993* (Δ*STM3993–8*/p*STM3993*), *STM3994* (Δ*STM3993–8*/p*STM3994*), *STM3995* (Δ*STM3993–8*/p*STM3995*), *STM3996* (Δ*STM3993–8*/p*STM3996*), *STM3997* (Δ*STM3993–8*/p*STM3997*), or *STM3998* (Δ*STM3993–8*/p*STM3998*). (C) Genetic organization of the chromosomal region deleted in Δ*STM3993–8 S.* Typhimurium. (D) Expression of surface TCR-β by anti-CD3ε/CD28-stimulated T cells left uninfected (UI) or cultured with wild-type *S.* Typhimurium (WT), Δ*STM3997 S.* Typhimurium (Δ*STM3997*), Δ*STM3997 S.* Typhimurium carrying pBAD18-Cm (Δ*STM3997*/Empty Vector) or Δ*STM3997 S.* Typhimurium carrying a derivative of pBAD18-Cm bearing *STM3997* (Δ*STM3997*/p*STM3997*). (E) Blastogenesis of anti-CD3ε/CD28-stimulated T cells treated as described for panel D. (F) IL-2 secretion by anti-CD3ε/CD28-stimulated T cells treated as described for panel D. Data show means with standard errors of the means obtained from at least five independent experiments. Data were analyzed by repeated measures one-way ANOVA with Tukey's multiple comparisons posttest; *P* values of <0.05 were considered to be statistically significant. Asterisks indicate statistically significant differences for designated posttest comparisons (****, $P < 0.0001$; ***, $P < 0.001$; **, $P < 0.01$; *, $P < 0.05$; n.s. not significant). GMFI, geometric mean fluorescent intensity.

Cysteine residues at positions 99 and 127 are essential for L-asparaginase II stability and function. The structure of L-asparaginase II of *E. coli* has been solved, revealing the existence of an intramolecular disulfide bond between the thiol groups of cysteine residues at positions 99 and 127 (18). These cysteine residues are conserved in L-asparaginase II of *S.* Typhimurium (Fig. S2). To determine the importance of these cysteine residues with respect to L-asparaginase II stability and function, we introduced site-specific mutations into a plasmid carrying *STM3106* that resulted in alanine substitutions for cysteine residues at positions 99 and 127. The resulting constructs expressing mutant L-asparaginase II proteins with alanine substitutions for cysteine residues at positions 99 (p*STM3106*_C99A), 127 (p*STM3106*_C127A), or both 99 and 127 (p*STM3106*_C99A/C127A) were transformed into Δ*STM3106 S.* Typhimurium.

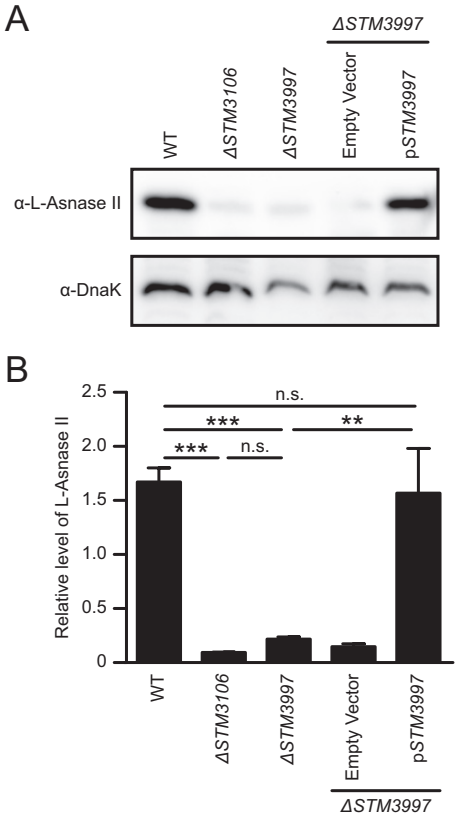

FIG 2 *STM3997* encodes a periplasmic thiol-disulfide oxidoreductase required to maintain the steady-state level of L-asparaginase II. (A) Western blot analysis of whole-cell lysates of wild-type *S.* Typhimurium (WT), *S.* Typhimurium lacking the L-asparaginase II gene *STM3106* (Δ*STM3106*), *S.* Typhimurium lacking the DsbA gene *STM3997* (Δ*STM3997*), Δ*STM3997 S.* Typhimurium carrying plasmid pBAD18-Cm (Δ*STM3997*/Empty Vector), or Δ*STM3997 S.* Typhimurium carrying a derivative of pBAD18-Cm expressing *STM3997* (Δ*STM3997*/p*STM3997*) using anti-L-asparaginase II antibody (α-L-Asnase-II) or anti-DnaK antibody (α-DnaK). (B) Quantification of Western blot data shown in panel A. DnaK was used as a loading control for Western blotting. Electrophoretic densitometry was used to quantify the relative levels of L-asparaginase II normalized to DnaK. Data are representative of (A) or show means with standard errors of the means obtained from (B) four independent experiments. Data in panel B were analyzed by repeated measures one-way ANOVA with Tukey's multiple comparisons posttest; *P* values of <0.05 were considered to be statistically significant. Asterisks indicate statistically significant differences for designated posttest comparisons (***, $P < 0.001$; **, $P < 0.01$; n.s. not significant).

Consistent with the hypothesis that the cysteine residues at positions 99 and 127 are required for the formation of a stabilizing structural disulfide bond, steady-state levels of L-asparaginase II were reduced significantly in Δ*STM3106 S.* Typhimurium carrying p*STM3106*_C99A, p*STM3106*_C127A, or p*STM3106*_C99A/C127A compared to the level in Δ*STM3106 S.* Typhimurium carrying p*STM3106* (Fig. 3A and B). Furthermore, Δ*STM3106 S.* Typhimurium carrying p*STM3106*_C99A, p*STM3106*_C127A, or p*STM3106*_C99A/C127A was unable to cause down-modulation of TCR-β surface expression relative to the level of Δ*STM3106 S.* Typhimurium carrying p*STM3106* (Fig. 3C and data not shown). Collectively, these results indicate that the cysteine residues at positions 99 and 127 are essential for the stability and function of L-asparaginase II of *S.* Typhimurium.

L-Asparaginase II localizes primarily to the periplasm and is used by *S.* Typhimurium to catabolize asparagine and assimilate nitrogen. The primary structure of L-asparaginase II of *S.* Typhimurium contains a predicted signal peptide and signal peptide cleavage site (Fig. S2), suggesting that L-asparaginase II is exported across the inner membrane by use of the general secretory pathway. To determine the subcellular localization of L-asparaginase II of *S.* Typhimurium, we obtained whole-cell lysates, cytoplasmic and periplasmic fractions, and precipitated proteins from cell-free culture

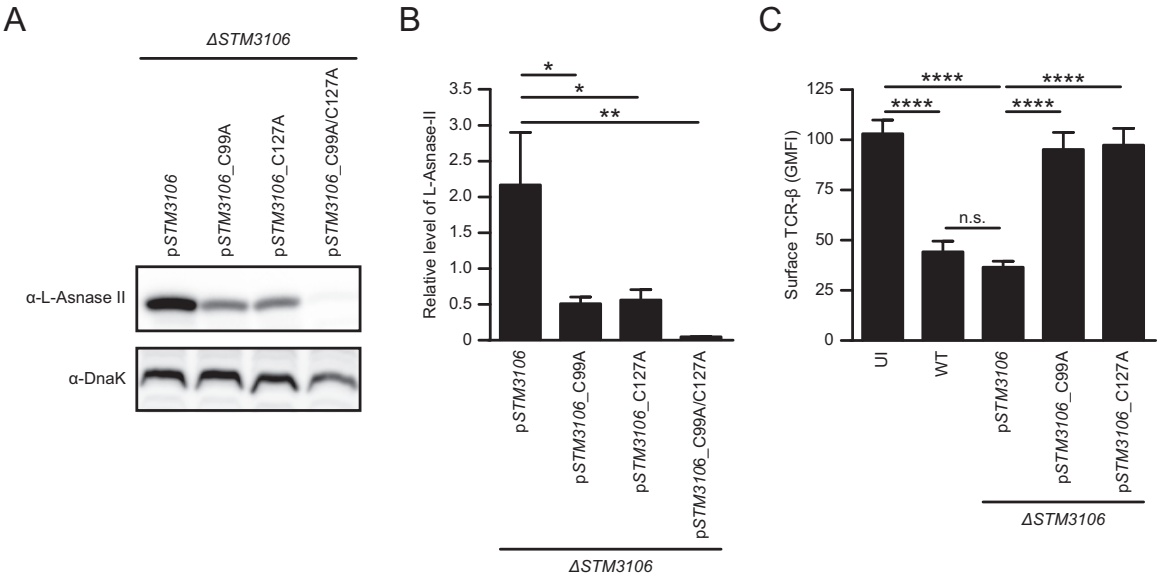

FIG 3 Cysteine residues at positions 99 and 127 are essential for L-asparaginase II stability and function. (A) Western blot analysis of whole-cell lysates of Δ*STM3106 S.* Typhimurium carrying plasmid p*STM3106* (Δ*STM3106/*p*STM3106* strain) or derivatives of p*STM3106* containing site-specific mutations in *STM3106* that result in alanine substitutions for cysteine residues at positions 99 (Δ*STM3106/*p*STM3106*_C99A strain), 127 (Δ*STM3106/*p*STM3106*_C127A strain), or both 99 and 127 (Δ*STM3106/*p*STM3106*_C99A/C127A strain). Expression of L-asparaginase II and DnaK was detected with anti-L-asparaginase II antibody (α-L-Asnase-II) and anti-DnaK antibody (α-DnaK), respectively. DnaK was used as a loading control for Western blotting. (B) Quantification of Western blot data shown in panel A. Electrophoretic densitometry was used to quantify the relative levels of L-asparaginase II normalized to DnaK. (C) Expression of surface TCR-β by anti-CD3ε/CD28-stimulated T cells left uninfected (UI) or cultured with the Δ*STM3106/*p*STM3106*, Δ*STM3106/*p*STM3106*_C99A, or Δ*STM3106/*p*STM3106*_C127A strain. Data are representative of (A) or show means with standard errors of the means obtained from (B and C) at least four independent experiments. Data were analyzed by repeated measures one-way ANOVA with Tukey's multiple comparisons posttest (B and C); *P* values of <0.05 were considered to be statistically significant. Asterisks indicate statistically significant differences for designated posttest comparisons (****, $P < 0.0001$; **, $P < 0.01$; *, $P < 0.05$; n.s. not significant). See also Fig. S1 in the supplemental material.

supernatants. We subjected aliquots of these samples to Western blot analysis. Consistent with published evidence indicating that L-asparaginase II of *E. coli* localizes to the periplasm (19), we found L-asparaginase II of *S.* Typhimurium to be present primarily in the periplasmic fraction (Fig. 4A). These results indicate that L-asparaginase II of *S.* Typhimurium is a secreted protein that localizes primarily to the periplasm, where it may function to hydrolyze nutritional asparagine.

We have shown previously that Δ*STM3106 S.* Typhimurium grows as well as wild-type *S.* Typhimurium (14). Consistent with this notion, Δ*STM3106 S.* Typhimurium grew normally in M9 minimal medium with ammonium chloride as the sole nitrogen source (Fig. 4B). However, Δ*STM3106 S.* Typhimurium grew significantly less well than wild-type *S.* Typhimurium in M9 minimal medium with asparagine as the sole nitrogen source (Fig. 4C). The presence of *STM3106* in *trans* reversed the phenotype of Δ*STM3106 S.* Typhimurium (Fig. 4C). Collectively, these results indicate that L-asparaginase II is a predominantly periplasmic protein used by *S.* Typhimurium to catabolize asparagine and assimilate nitrogen.

L-Asparaginase II and L-asparaginase I act together to provide *S.* Typhimurium the ability to assimilate nitrogen. The partial growth defect of Δ*STM3106 S.* Typhimurium in M9 minimal medium when asparagine is the sole nitrogen source (Fig. 4C) suggests that *S.* Typhimurium encodes at least one other asparagine hydrolase capable of functionally complementing L-asparaginase II. *STM1294* encodes a putative cytoplasmic asparagine hydrolase that is 94.67% identical to L-asparaginase I of *E. coli* at the amino acid level (Fig. S3A) and is syntenic. To establish the role of this putative asparagine hydrolase in nitrogen assimilation, we generated and tested an *S.* Typhimurium strain lacking *STM1294* (Δ*STM1294* strain). This strain grew normally in M9 minimal medium with ammonium chloride or asparagine as the sole nitrogen source

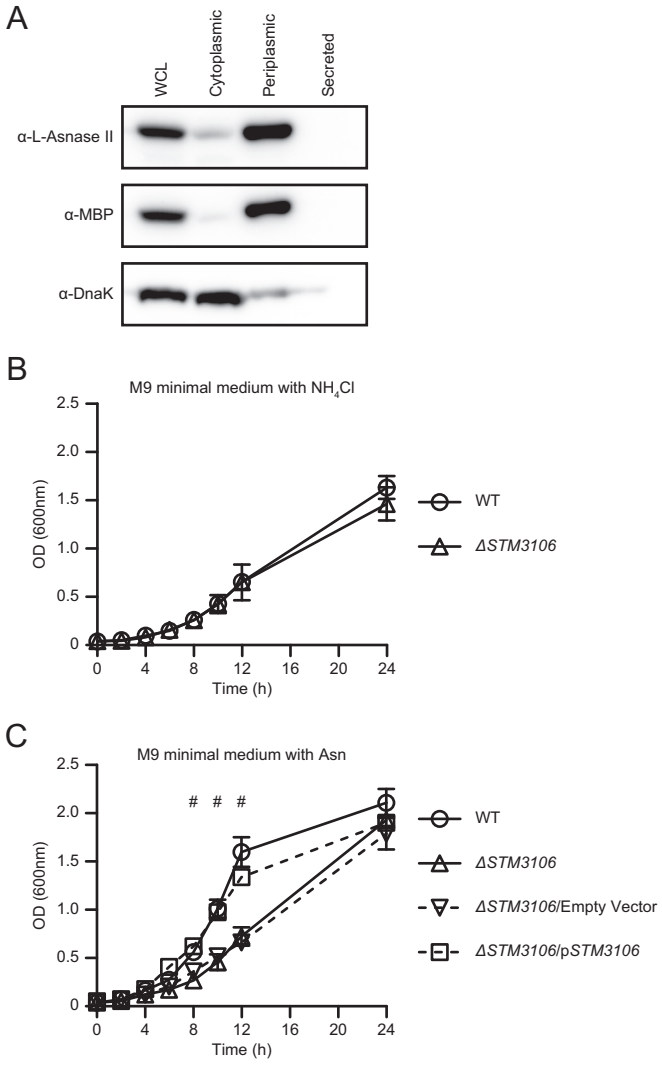

FIG 4 L-Asparaginase II localizes primarily to the periplasm and is used by *S*. Typhimurium to catabolize asparagine and assimilate nitrogen. (A) Western blot analysis of whole-cell lysates (WCL) of *S*. Typhimurium, cytoplasmic or periplasmic fractions of *S*. Typhimurium, or TCA-precipitated proteins from cell-free culture supernatants of *S*. Typhimurium (Secreted) grown in lysogeny broth. Expression of L-asparaginase II, MBP, and DnaK was detected with anti-L-asparaginase II antibody (α-L-Asnase-II), anti-MBP antibody (α-MBP), and anti-DnaK antibody (α-DnaK), respectively. MBP and DnaK were used as fractionation controls. (B) Growth of wild-type *S*. Typhimurium (WT) and Δ*STM3106 S*. Typhimurium (Δ*STM3106*) in M9 minimal medium with ammonium chloride (NH_4Cl) as the sole nitrogen source. (C) Growth of wild-type *S*. Typhimurium (WT), Δ*STM3106 S*. Typhimurium (Δ*STM3106*), Δ*STM3106 S*. Typhimurium carrying plasmid pBAD18-Cm (Δ*STM3106*/Empty Vector), and Δ*STM3106 S*. Typhimurium carrying a derivative of pBAD18-Cm bearing *STM3106* (Δ*STM3106*/p*STM3106*) in M9 minimal medium with asparagine (Asn) as the sole nitrogen source. Data are representative of (A) or show means with standard errors of the means obtained from (B and C) at least three independent experiments. Data were analyzed by repeated measures two-way ANOVA with Sidak (B) or Tukey's (C) multiple comparisons posttest; *P* values of <0.05 were considered to be statistically significant. Number (#) symbols indicate statistically significant differences for designated posttest comparisons between WT and Δ*STM3106 S*. Typhimurium at indicated time points. See also Fig. S1 in the supplemental material.

(Fig. S3B and C), suggesting that, under these conditions, *STM1294* is not required for *S*. Typhimurium to assimilate nitrogen.

To examine the possibility that *STM1294* and *STM3106* encode functionally overlapping asparagine hydrolases, we generated and tested an *S*. Typhimurium strain lacking both *STM1294* and *STM3106* (Δ*STM1294* Δ*STM3106* strain). This strain grew normally in M9 minimal medium with ammonium chloride as the sole nitrogen source (Fig. 5A) but grew poorly relative to wild-type *S*. Typhimurium when asparagine was available as the

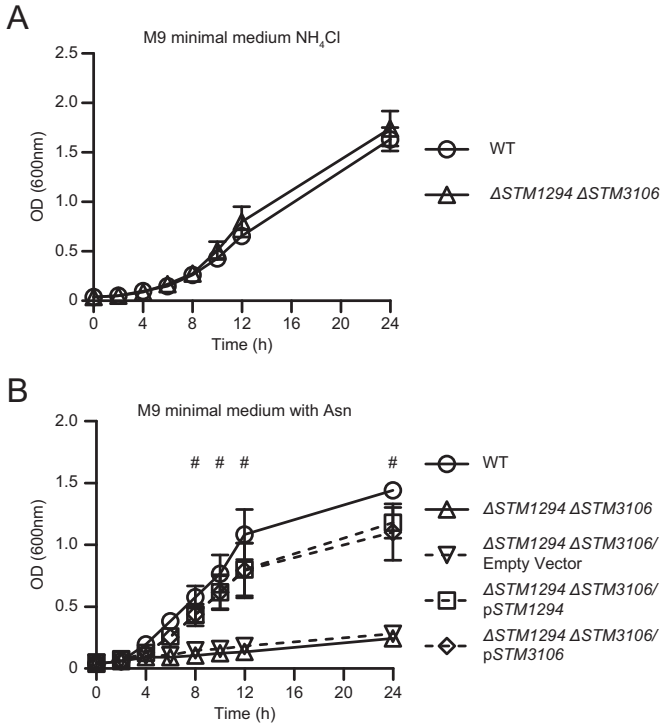

FIG 5 L-Asparaginase II and L-asparaginase I act together to provide *S.* Typhimurium the ability to assimilate nitrogen. (A) Growth of wild-type *S.* Typhimurium (WT) and Δ*STM1294* Δ*STM3106 S.* Typhimurium (Δ*STM1294* Δ*STM3106*) in M9 minimal medium with ammonium chloride (NH_4Cl) as the sole nitrogen source. (B) Growth of wild-type *S.* Typhimurium (WT), Δ*STM1294* Δ*STM3106 S.* Typhimurium (Δ*STM1294* Δ*STM3106*), Δ*STM1294* Δ*STM3106 S.* Typhimurium carrying plasmid pBAD18-Cm (Δ*STM1294* Δ*STM3106*/Empty Vector), Δ*STM1294* Δ*STM3106 S.* Typhimurium carrying a derivative of pBAD18-Cm expressing *STM1294* (Δ*STM1294* Δ*STM3106*/p*STM1294*), and Δ*STM1294* Δ*STM3106 S.* Typhimurium carrying a derivative of pBAD18-Cm expressing *STM3106* (Δ*STM1294* Δ*STM3106*/p*STM3106*) in M9 minimal medium with asparagine (Asn) as the sole nitrogen source. Data show means with standard errors of the means obtained from at least three independent experiments. Data were analyzed by repeated measures two-way ANOVA with Sidak (A) or Tukey's (B) multiple comparisons posttest; *P* values of <0.05 were considered to be statistically significant. Number (#) symbols indicate statistically significant differences for designated posttest comparisons between WT and Δ*STM1294* Δ*STM3106 S.* Typhimurium at indicated time points. See also Fig. S2 in the supplemental material.

sole nitrogen source (Fig. 5B). In fact, the Δ*STM1294* Δ*STM3106* double mutant exhibited a much stronger growth phenotype than either the Δ*STM1294* single mutant or the Δ*STM3106* single mutant (Fig. 4C and S3B and C). Finally, the presence of either *STM1294* or *STM3106* in *trans* reversed the phenotype of the Δ*STM1294* Δ*STM3106* double mutant (Fig. 5B). These results indicate that *STM1294* and *STM3106* encode functionally overlapping asparagine hydrolases that can act together to provide *S.* Typhimurium the ability to catabolize asparagine and assimilate nitrogen.

Asparagine catabolism contributes to *S.* Typhimurium virulence. *S.* Typhimurium is a facultative intracellular pathogen that thrives inside phagocytes, where access to nutrients is limited. To determine if *S.* Typhimurium, like *Mycobacterium tuberculosis* (20) and *Francisella tularensis* (21), catabolizes asparagine to survive and replicate inside phagocytes, we infected bone marrow-derived macrophages cultured from 129X1/SvJ mice with wild-type *S.* Typhimurium, the Δ*STM1294* single mutant, the Δ*STM3106* single mutant, or the Δ*STM1294* Δ*STM3106* double mutant. After 18 h of infection, we recovered intracellular bacteria and found reduced numbers of the Δ*STM1294* mutant and the Δ*STM1294* Δ*STM3106* mutant relative to the level of the wild-type *S.* Typhimurium, while the Δ*STM3106* mutant followed the same trend (Fig. 6A and S4A). These results suggest that *S.* Typhimurium uses asparagine catabolism while surviving and replicating inside macrophages.

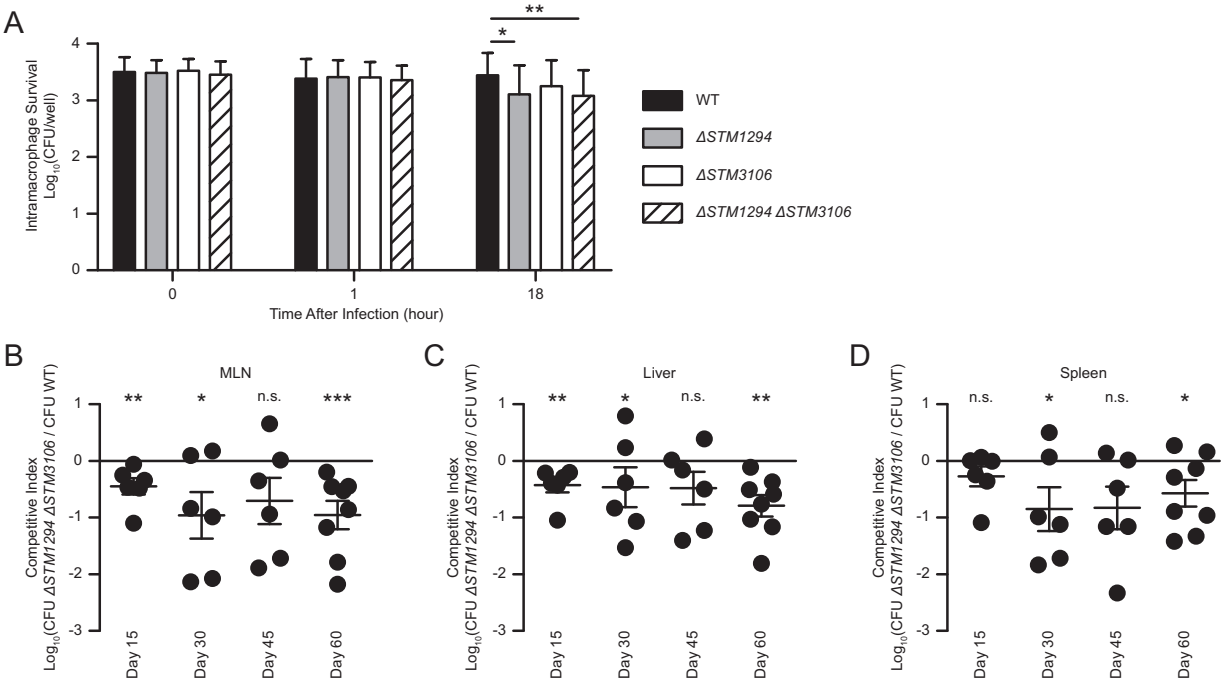

FIG 6 Asparagine catabolism contributes to *S.* Typhimurium virulence. (A) Survival and growth of wild-type *S.* Typhimurium (WT), Δ*STM1294 S.* Typhimurium (Δ*STM1294*), Δ*STM3106 S.* Typhimurium (Δ*STM3106*) or Δ*STM1294* Δ*STM3106 S.* Typhimurium (Δ*STM1294* Δ*STM3106*) inside bone marrow-derived macrophages cultured from 129X1/SvJ mice. (B to D) Competitive indices of the Δ*STM1294* Δ*STM3106* strain versus wild-type *S.* Typhimurium recovered from mesenteric lymph nodes (MLN; B), livers (C) and spleens (D) of 129X1/SvJ mice ($n = 6$ to 8 per group per time point) at various times after intragastric inoculation with 1×10^8 CFU of a 1:1 mixture of differentially marked bacterial strains. Data were analyzed by repeated measures two-way ANOVA with Tukey's multiple comparisons posttest (A), repeated-measures one-way ANOVA with Dunnett's multiple comparisons posttest (B to D; data were compared to the expected 1:1 output ratio of the Δ*STM1294* Δ*STM3106* strain versus WT *S.* Typhimurium). *P* values of <0.05 were considered to be statistically significant. Asterisks indicate statistically significant differences for designated posttest comparisons (***, $P < 0.001$; **, $P < 0.01$; *, $P < 0.01$; n.s. not significant). See also Fig. S3 in the supplemental material.

While *S.* Typhimurium must access nutrients to survive and replicate inside mammalian hosts, the exact nutrients exploited by *S.* Typhimurium during infection remain largely unknown. To establish the role of asparagine catabolism in *S.* Typhimurium virulence, we inoculated 129X1/SvJ mice intragastrically with 1×10^8 CFU of a 1:1 mixture of the wild-type strain and the Δ*STM1294* Δ*STM3106* double mutant. At various times after inoculation, we performed organ burden assays (Fig. S4B to D) and found that the wild-type strain had outcompeted the mutant for colonization of mesenteric lymph nodes (Fig. 6B), liver (Fig. 6C), and spleen (Fig. 6D). Collectively, these results establish that asparagine catabolism promotes survival and replication of *S.* Typhimurium inside macrophages and contributes to *S.* Typhimurium virulence.

DISCUSSION

We report that asparagine catabolism contributes to *S.* Typhimurium virulence. Specifically, we determined that *STM3997*, which encodes a homolog of DsbA of *E. coli*, is required to maintain the steady-state level of L-asparaginase II and that cysteine residues at positions 99 and 127 are essential for L-asparaginase II stability and function (Fig. 1 to 3). In addition, we determined that L-asparaginase II localizes primarily to the periplasm and, together with L-asparaginase I, enables *S.* Typhimurium to catabolize asparagine (Fig. 4 and 5). Importantly, we determined that, in a murine model of infection, *S.* Typhimurium lacking both L-asparaginase I and II genes competes poorly with the wild-type strain for colonization of target tissues (Fig. 6). Collectively, these results provide new insights into the competition for nutrients at the host-pathogen interface.

We previously showed that L-asparaginase II produced by *S.* Typhimurium inhibits T cell responses and mediates virulence (14) and that asparagine deprivation such as that mediated by L-asparaginase II of *S.* Typhimurium causes inhibition of activation-induced

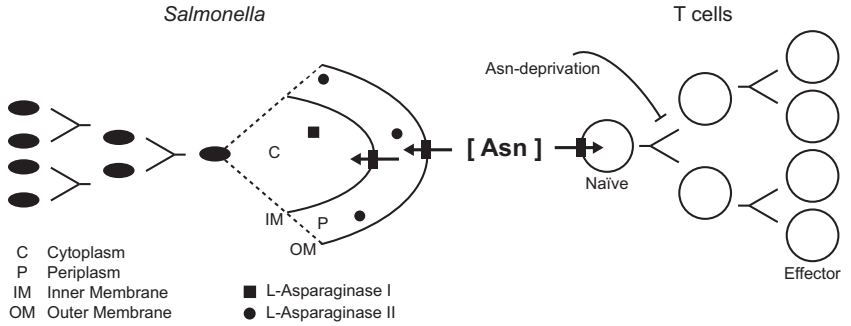

FIG 7 Model showing the competition for nutritional asparagine between *S*. Typhimurium and T cells. Our published findings (14, 15) and results described in the present study support a model in which *S*. Typhimurium competes with the host for nutritional asparagine, causing the local depletion of a key resource required for T cell activation. See the text for details.

T cell metabolic reprogramming (15). These previous findings and the findings described in this study are consistent with the notion that nutrient availability, including amino acid availability, is a key determinant that affects the outcome of infection. We propose that *S*. Typhimurium competes with the host for nutritional asparagine, causing the local depletion of a key resource required for the induction of a protective T cell response (Fig. 7).

Pathogens require biosynthetic precursors to survive and replicate inside the mammalian host. To access these precursors, pathogens must express appropriate transporters and enzymes in key metabolic pathways that permit precursor use or biosynthesis.

Asparagine catabolism appears to be a key metabolic pathway that promotes *S*. Typhimurium virulence. Carbon and nitrogen are essential components of many biomolecules, and we suggest that during infection *S*. Typhimurium accesses both by catabolizing asparagine (Fig. 7). The mechanism(s) *S*. Typhimurium uses to import nutritional asparagine during infection is not well characterized. Recent studies have shown that asparagine biosynthesis is also important for *S*. Typhimurium virulence. *S*. Typhimurium bacteria lacking both asparagine biosynthesis genes *asnA* (*STM3877*) and *asnB* (*STM0680*) are attenuated for survival and replication inside host cells (22), and are unable to compete with wild-type *S*. Typhimurium for colonization of murine spleen (23). Furthermore, it has been reported that *S*. Typhimurium exploits fructose-asparagine for growth in the inflamed intestine (24). Understanding the different mechanisms used by *S*. Typhimurium to metabolize (i.e., catabolize and synthesize) asparagine during infection will require further study.

Asparagine catabolism is important for the virulence of a number of significant human pathogens, including *S*. Typhimurium (14; also the present study), *Mycobacterium tuberculosis* (20), *Helicobacter pylori* (25), *Campylobacter jejuni* (26), and *Francisella tularensis* (21). A number of these pathogens can cause chronic or persistent infections, and immunity to these pathogens is dependent, at least in part, on T cells (8, 9, 27–31), which require nutritional asparagine for activation (14, 15). Thus, pathogens competing with the host for nutrients may have evolved so as to exploit asparagine catabolism as a means to limit T cell responses (Fig. 7).

Bacterial L-asparaginases have been a cornerstone of acute lymphoblastic leukemia treatment since the late 1970s. These enzymes are used for remission induction and intensification treatment in all pediatric regimens as well as in the majority of adult treatment protocols and act through a mechanism that likely involves asparagine deprivation (32). Our published findings (14, 15) and the results described in this study indicate that pathogens competing with the host for nutrients may similarly use asparagine deprivation to cause inhibition of protective T cell responses. Therefore, the targeting of asparagine metabolism (e.g., bacterial asparagine catabolism) holds great potential for the development of new strategies to fight infectious diseases.

MATERIALS AND METHODS

Ethics statement. All procedures that used mice were approved by the Institutional Animal Care and Use Committee at Stony Brook University and were conducted in accordance with the recommendations outlined in the *Guide for the Care and Use of Laboratory Animals* adopted by the National Institutes of Health (33). Euthanasia of mice was performed by inhalation of carbon dioxide, a method that is consistent with the recommendations of the Panel on Euthanasia of the American Veterinary Medical Association.

Bacterial strains, strain construction, and culture conditions. With the use of standard microbiological techniques, bacteria were grown aerobically at 37°C in lysogeny broth or on lysogeny agar except where otherwise noted. Kanamycin (60 μg/ml) was added to the culture medium to select for various mutant strains of *S.* Typhimurium in the 14028 or IR715 strain background (see below). Chloramphenicol (30 μg/ml) and L-arabinose (0.075%, wt/vol; 5 mM) were added to the culture medium to maintain plasmids *in vivo* and induce expression of plasmid-encoded L-asparaginase II, respectively. In the context of mouse infections, nalidixic acid (50 μg/ml) or kanamycin was added to the culture medium to select for wild-type or mutant *S.* Typhimurium in the IR715 strain background (see below).

S. Typhimurium strain 14028 (American Type Culture Collection, Manassas, VA, USA) was used as the wild-type strain. For mouse infections, *S.* Typhimurium strain IR715 (34), a spontaneous nalidixic acid-resistant derivative of *S.* Typhimurium strain 14028, was used as the wild-type strain. The library of targeted, multigene deletion mutants of *S.* Typhimurium strain 14028 has been published previously (16). An isogenic derivative of *S.* Typhimurium strain 14028 lacking *STM3106* (Δ*STM3106* strain), Δ*STM3106 S.* Typhimurium carrying the bacterial gene expression vector pBAD18-Cm (American Type Culture Collection) (Δ*STM3106*/pBAD strain; here referred to as the Δ*STM3106*/empty vector strain), and Δ*STM3106 S.* Typhimurium carrying a derivative of pBAD18-Cm bearing *STM3106* (Δ*STM3106*/pBAD-*STM3106* strain; here referred to as the Δ*STM3106*/p*STM3106* strain) have also been published previously (14).

Isogenic derivatives of *S.* Typhimurium strain 14028 lacking *STM1294* (Δ*STM1294* strain) or *STM3997* (Δ*STM3997* strain) were generated by use of the lambda red recombinase method (35). Briefly, the kanamycin resistance gene carried by plasmid pCLF4 (36) was amplified by use of PCR with primers ΔSTM1294-F and ΔSTM1294-R to delete *STM1294* or primers ΔSTM3997-F and ΔSTM3997-R to delete *STM3997* (see Table S1 in the supplemental material).

To generate an *S.* Typhimurium strain lacking both *STM1294* and *STM3106*, plasmid pCP20 (37), which encodes the FLP recombinase, was electroporated into Δ*STM3106 S.* Typhimurium. This strain was generated by use of the aforementioned lambda red recombinase method and is resistant to kanamycin. Following FLP recombinase-mediated excision of the kanamycin resistance gene, the resulting strain was cured of plasmid pCP20, yielding a strain with an unmarked deletion of *STM3106*. Next, the *STM1294* deletion mutation was moved from Δ*STM1294 S.* Typhimurium into the unmarked Δ*STM3106 S.* Typhimurium strain by bacteriophage P22-mediated transduction, yielding an *S.* Typhimurium strain lacking both *STM1294* and *STM3106* (Δ*STM1294* Δ*STM3106* strain). A similar approach was used to delete *STM1294* in *S.* Typhimurium strain IR715 lacking *STM3106* (14).

The bacterial gene expression vector pBAD18-Cm was used to generate isogenic derivatives of Δ*STM3993–8*, Δ*STM1294*, and Δ*STM1294* Δ*STM3106 S. Typhimurium* strains carrying plasmids bearing the *STM1294*, *STM3993*, *STM3994*, *STM3995*, *STM3996*, *STM3997*, or *STM3998* gene. The open reading frames corresponding to these genes were amplified from the chromosome of *S.* Typhimurium strain 14028 by PCR with gene-specific primer pairs (Table S1). Restriction sites for endonucleases NheI and XbaI were incorporated into the primer sequences to facilitate cloning of the PCR products into pBAD18-Cm immediately downstream of the arabinose-inducible *araC* promoter. The resulting constructs (or empty vector) were verified by DNA sequencing and introduced into the Δ*STM3993–8*, Δ*STM1294*, or Δ*STM1294* Δ*STM3106 S.* Typhimurium strain by electroporation. Transformants were selected for on lysogeny agar supplemented with chloramphenicol. Isolated single colonies were picked, and the presence of the plasmids was confirmed. The resulting strains grew normally in lysogeny broth (supplemented with chloramphenicol) following induction with L-arabinose (data not shown).

Site-directed mutagenesis was used to introduce site-specific mutations into *STM3106*, resulting in an alanine substitution for cysteine at position 99 or 127. Site-directed mutagenesis was performed on plasmid pBAD-*STM3106* (p*STM3106*) by use of Q5 DNA polymerase (New England BioLabs, Ipswich, MA, USA) and primer pair STM3106.C99A-F and STM3106.C99A-R (for position 99) or primer pair STM3106.C127A-F and STM3106.C127A-R (for position 127). After PCR, the parental DNA was digested by use of DpnI endonuclease (Table S1). The resulting constructs, p*STM3106*_C99A and p*STM3106*_C127A, were verified by DNA sequencing and introduced into Δ*STM3106 S.* Typhimurium by electroporation. Transformants were selected for on lysogeny agar supplemented with chloramphenicol. Isolated single colonies were picked, and the presence of the plasmids was confirmed. The resulting strains grew normally in lysogeny broth (supplemented with chloramphenicol) following induction with L-arabinose (data not shown). To introduce two site-specific mutations into *STM3106*, resulting in alanine substitutions for cysteine residues at positions 99 and 127, site-directed mutagenesis was performed on plasmid p*STM3106*_C127A by use of the aforementioned PCR-based approach and primers STM3106.C99A-F and STM3106.C99A-R.

Bacterial growth curve assays. Overnight cultures were washed twice with sterile water and diluted (1:200) into fresh lysogeny broth. Chloramphenicol and L-arabinose were added to the culture medium to maintain plasmids *in vivo* and to induce expression of plasmid-encoded L-asparaginase II, respectively. Bacteria were grown aerobically at 37°C in a rotary shaker incubator (250 rpm). Bacterial growth was monitored over time by measuring the change in absorbance of the cultures at an optical density of 600 nm (OD_{600}). M9 minimal medium was used where indicated to perform bacterial growth assays under

nutrient-limiting conditions. M9 minimal medium was prepared with glycerol (0.2%) as the primary carbon source and ammonium chloride (18.7 mM) or asparagine (12 mM) as the sole nitrogen source.

Bacterial transcript analysis. Overnight cultures were diluted (1:200) into 3 ml of fresh lysogeny broth. Bacteria were grown aerobically at 37°C until the OD_{600} reached 0.5, when L-arabinose (5 mM) was added to the cultures to induce expression of plasmid-encoded DsbA. After 1 h of induction, RNA was isolated by use of an RNeasy Protect bacteria minikit (Qiagen, Valencia, CA, USA) and TRI reagent (Molecular Research Center, Cincinnati, OH, USA). A total of 100 ng of RNA was converted into cDNA by use of a Verso cDNA synthesis kit (Thermo Fisher Scientific, Grand Island, NY, USA). Quantitative PCR was performed by use of an ABI 7300 real-time PCR system (Thermo Fisher Scientific), PerfeCTa SYBR green FastMix ROX (QuantaBio, Beverly, MA, USA), and the primer pair qSTM3106-F and qSTM3106-R (for *STM3106*) or the pair qSTM3835-F and qSTM3835-R (for *STM3835*). The $2^{-\Delta\Delta CT}$ (where C_T is threshold cycle) method was used to analyze the data and determine relative expression of *STM3106* (*ansB*) normalized to that of *STM3835* (*gyrB*).

T cell enrichment and T cell assay. Splenocytes harvested from naive C57BL/6J mice (6- to 8-week-old females; The Jackson Laboratory, Bar Harbor, ME, USA) were used as a source of T cells. Following the lysis of red blood cells in ACK lysing buffer (0.15 M NH_4Cl, 10 mM $KHCO_3$, and 0.1 mM Na_2EDTA, pH 7.2 to 7.4), CD90.2 MicroBeads and magnetic cell separation technology (Miltenyi Biotec, San Diego, CA, USA) were used to enrich for $CD90.2^+$ cells. CD90.2 is a pan-T cell marker expressed by both $CD4^+$ and $CD8^+$ T cells. Enriched populations of T cells were suspended in RP-10 medium (RPMI 1640 medium [Gibco, Grand Island, NY, USA] supplemented with 10% fetal bovine serum, 0.2 M glutamine, 0.1 M HEPES, and 50 μM 2-mercaptoethanol) and used in T cell assays.

To measure the effect of *S.* Typhimurium on TCR-β surface expression, blastogenesis, and IL-2 secretion, enriched populations of T cells suspended in RP-10 medium supplemented with 1 μg/ml anti-CD28 monoclonal antibody (MAb) (clone E18; BioLegend, San Diego, CA, USA) were seeded at 1×10^5 cells per well into flat-bottom 96-well tissue culture plates coated with 5 μg/ml anti-CD3ε MAb (clone 145-2C11; BioLegend). The T cells were then cultured in the absence or presence of *S.* Typhimurium at a multiplicity of infection of ~60. L-Arabinose was added to the cultures for continued induction of plasmid-encoded L-asparaginase II expression by L-arabinose-pretreated *S.* Typhimurium. After 2 h of incubation at 37°C in 5% CO_2, the T cells were pelleted by centrifugation and resuspended in RP-10 medium supplemented with gentamicin (50 μg/ml) and with penicillin and streptomycin (2%) to kill all remaining bacteria. After an additional 20 h of incubation at 37°C in 5% CO_2, the T cells were harvested, stained, and analyzed by flow cytometry. Additionally, the culture supernatants were collected, and IL-2 concentrations were determined by IL-2 enzyme-linked immunosorbent assay (ELISA).

Cell staining and analysis by flow cytometry. All antibodies and reagents described in this section were purchased from BioLegend. Cells were stained in the presence of Fc block (clone 93) with allophycocyanin-conjugated anti-mouse CD90.2 MAb (clone 30-H12), phycoerythrin-conjugated anti-mouse CD25 MAb (clone PC61), and fluorescein isothiocyanate-conjugated anti-mouse TCR-β MAb (clone H57-597). Data were acquired on a FACSCalibur with CellQuest Pro software (BD Biosciences, Franklin Lakes, NJ, USA) and analyzed with FlowJo, version X, software (Tree Star, Ashland, OR, USA).

Live cells expressing CD90.2 and CD25 were identified as anti-CD3ε- and anti-CD28-stimulated T cells. Size and granularity of live cells were analyzed by use of forward and side scatter. Blastogenesis was calculated by determining the percentage of live cells that, in response to treatment with anti-CD3ε and anti-CD28 MAb, had transformed from small lymphocytes into larger, more granular cells resembling blast cells.

IL-2 ELISA. IL-2 concentrations in collected T cell supernatants were determined by use of a mouse IL-2 ELISA MAX Deluxe kit (BioLegend).

Bacterial fractionation. Overnight cultures were diluted (1:200) into 55 ml of fresh lysogeny broth and grown aerobically at 37°C until the OD_{600} reached ~0.5, after which the cultures were placed on ice to stop bacterial growth. Next, the cultures (50 ml) were subjected to centrifugation (10,000 $\times$ *g* for 10 min at 4°C). To obtain secreted protein fractions, proteins in the resulting supernatants were precipitated with trichloroacetic acid (TCA) and suspended in 1 ml of 1$\times$ Laemmli sample buffer. To obtain cytoplasmic and periplasmic fractions, the bacterial pellets were washed in 20 mM Tris (pH 8), suspended in 1 ml of TSE buffer (200 mM Tris, pH 8, 500 mM sucrose, 1 mM EDTA) (38), and incubated for 1 h on ice, after which the samples were subjected to centrifugation (10,000 $\times$ *g* for 20 min at 4°C). The supernatants were collected, yielding periplasmic fractions, and the pellets were resuspended in 1 ml of TSE buffer. The resuspended pellets were sonicated while on ice water by use of a Microson Ultrasonic Cell Disruptor XL (4 cycles of 15 s on and 15 s off; power, 5 [Misonix, Farmingdale, NY, USA]) and subjected to centrifugation (10,000 $\times$ *g* for 10 min at 4°C). The supernatants were collected, yielding cytoplasmic fractions. Laemmli sample buffer (6$\times$) was added to the cytoplasmic and periplasmic fractions at a final concentration of 1$\times$. Aliquots (5 ml) of the bacterial cultures were subjected to centrifugation, and the resulting pellets were suspended in 0.1 ml of 1$\times$ Laemmli sample buffer to ultimately yield whole-cell lysates (see below). All samples containing Laemmli sample buffer were then heated to 95°C for 30 min and subjected to PAGE and Western blot analysis.

PAGE and Western blot analysis. Aliquots of whole-cell lysates, cytoplasmic and periplasmic fractions, and samples containing proteins precipitated from cell-free culture supernatants mixed with Laemmli/SDS sample buffer were boiled for 15 min, subjected to centrifugation to pellet debris, and loaded onto 16% Tris-tricine polyacrylamide gels with discontinuous buffering systems (39). Following gel electrophoresis, proteins were transferred onto polyvinylidene difluoride membranes (Bio-Rad, Hercules, CA, USA) by use of a Trans-Blot SD Semi-Dry Transfer Cell system (Bio-Rad).

Expression of L-asparaginase II was detected with polyclonal antibody (1:10,000) from rabbits immunized with recombinant L-asparaginase II of *E. coli* (catalog number 21013; Abcam, Cambridge, MA, USA). Expression of the chaperone protein DnaK was detected with MAb (1:30,000) from mice immunized with recombinant DnaK of *E. coli* (clone 8E2/2; Abcam). Expression of maltose-binding protein (MBP) was detected with MAb (1:2,000) from rats immunized with recombinant MBP of *E. coli* (clone YM-2; BioLegend, San Diego, CA, USA).

For detection, horseradish peroxidase-conjugated goat anti-rabbit IgG secondary antibodies (catalog number 7074; Cell Signaling Technology, Danvers, MA, USA), goat anti-mouse IgG secondary antibodies (170-5047; Bio-Rad), or goat anti-rat IgG secondary antibodies (112-035-003; Jackson ImmunoResearch Laboratories, West Grove, PA, USA) were used (1:10,000) in combination with ECL Western blotting detection reagent (GE Healthcare Life Sciences, Piscataway, NJ, USA). An ImageQuant LAS 500 imaging system (GE Healthcare Life Sciences) was used for chemiluminescence detection and gel documentation. Electrophoretic densitometry (Image Studio Lite; Li-Cor, Lincoln, NE, USA) was used to quantify the relative levels of L-asparaginase II normalized to DnaK.

Macrophage culture and macrophage infection assay. Bone marrow-derived macrophages were cultured from 129X1/SvJ mice, as described previously (40). Briefly, bone marrow cells isolated from femurs of 129X1/SvJ mice were suspended in medium (Dulbecco's modified Eagle's medium [DMEM] with 4.5 g/liter glucose [Gibco] supplemented with 20% heat-inactivated fetal bovine serum [FBS; Atlanta Biologicals, Lawrenceville, GA, USA], 30% L-cell conditioned medium, 2 mM glutamine, and 1 mM sodium pyruvate) and seeded into 100-mm-diameter petri dishes at 4×10^6 cells per plate. After 5 days of incubation at 37°C in 5% CO_2, macrophages were collected and used for infection assays.

Macrophage infection assays were performed by use of a standard gentamicin protection assay. Briefly, bone marrow-derived macrophages were suspended in medium (same as above, except that concentrations of FBS and L-cell conditioned medium were reduced to 10% and 15%, respectively) and seeded at 1.5×10^6 cells per ml per well into a 24-well tissue culture plate. After overnight incubation at 37°C in 5% CO_2, the medium was replaced with 0.5 ml of fresh medium, and the macrophages were infected with bacteria at a multiplicity of infection of 1. Upon addition of bacteria, the plate was centrifuged for 5 min at 1,000 rpm to facilitate bacterial contact with the macrophage monolayer. After 20 min of incubation at 37°C in 5% CO_2, the wells were washed three times with 1 ml of phosphate-buffered saline (PBS) to remove non-cell-associated bacteria, after which fresh medium supplemented with gentamicin (25 μg/ml) was added to kill all extracellular bacteria. This was referred to as the 0-h time point. After 1 and 18 h of incubation at 37°C in 5% CO_2, the wells were washed three times with 1 ml of PBS to remove the gentamicin-containing medium, and the macrophages were lysed by use of 0.5 ml of Triton X-100 (0.1%) to release intracellular bacteria. Viable bacteria were enumerated by plating for CFU onto lysogeny agar. To determine the number of cell-associated bacteria at the 0-h time point, macrophages were lysed immediately following the initial washes with PBS, after which viable bacteria were enumerated by plating for CFU onto lysogeny agar.

Murine infections. Naive, 8- to 10-week-old female 129X1/SvJ mice (The Jackson Laboratory) were inoculated intragastrically with 1×10^8 CFU of a 1:1 mixture of differentially marked wild-type and Δ*STM1294* Δ*STM3106 S.* Typhimurium (in the IR715 strain background) suspended in 0.1 ml of phosphate-buffered saline. To improve the consistency of intragastric infections, food (but not water) was removed 6 to 8 h prior to inoculation. Immediately following inoculation, food was provided *ad libitum*. Tenfold serial dilutions of the inoculum were plated to confirm the inoculum titer and input ratio. At various times after inoculation, mice were euthanized, and target tissues (i.e., mesenteric lymph nodes, liver, and spleen) were harvested and processed to determine bacterial loads. *S.* Typhimurium bacteria were enumerated by serial dilution and plating. Competitive indices were calculated by dividing the number of Δ*STM1294* Δ*STM3106 S.* Typhimurium (nalidixic acid and kanamycin resistant) bacteria by the number of wild-type *S.* Typhimurium (nalidixic acid resistant) bacteria normalized to the input ratio.

Statistical analysis. Statistical analysis was performed with Prism, version 6 (GraphPad Software, La Jolla, CA, USA). Data were analyzed by one-way analysis of variance (ANOVA) with Dunnett's multiple comparisons posttest, repeated-measures one-way ANOVA with Dunnett's or Tukey's multiple comparisons posttest, or repeated measures two-way ANOVA with Sidak or Tukey's multiple comparisons posttest. A P value of <0.05 was considered to be statistically significant (see figure legends for detailed significance levels).

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at https://doi.org/10.1128/IAI.00740-16.

TEXT S1, PDF file, 0.7 MB.

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REFERENCES

1. Majowicz SE, Musto J, Scallan E, Angulo FJ, Kirk M, O'Brien SJ, Jones TF, Fazil A, Hoekstra RM, International Collaboration on Enteric Disease "Burden of Illness" Studies. 2010. The global burden of nontyphoidal *Salmonella* gastroenteritis. Clin Infect Dis 50:882–889. https://doi.org/10.1086/650733.
2. Crump JA, Mintz ED. 2010. Global trends in typhoid and paratyphoid fever. Clin Infect Dis 50:241–246. https://doi.org/10.1086/649541.
3. Gordon MA. 2011. Invasive nontyphoidal *Salmonella* disease: epidemiology, pathogenesis and diagnosis. Curr Opin Infect Dis 24:484–489. https://doi.org/10.1097/QCO.0b013e32834a9980.
4. Gilchrist JJ, MacLennan CA, Hill AVS. 2015. Genetic susceptibility to invasive *Salmonella* disease. Nat Rev Immunol 15:452–463. https://doi.org/10.1038/nri3858.
5. Kirk MD, Pires SM, Black RE, Caipo M, Crump JA, Devleesschauwer B, Dopfer D, Fazil A, Fischer-Walker CL, Hald T, Hall AJ, Keddy KH, Lake RJ, Lanata CF, Torgerson PR, Havelaar AH, Angulo FJ. 2015. World Health Organization estimates of the global and regional disease burden of 22 foodborne bacterial, protozoal, and viral diseases, 2010: a data synthesis. PLoS Med 12:e1001940. https://doi.org/10.1371/journal.pmed.1001940.
6. Pegues DA, Miller SI. 2015. *Salmonella* species, p 2559–2568. *In* Bennett JE, Dolin R, Blaser MJ (ed), Mandell, Douglas, and Bennett's principles and practice of infectious diseases, 8th ed. Elsevier, Philadelphia, PA.
7. Keestra-Gounder AM, Tsolis RM, Baumler AJ. 2015. Now you see me, now you don't: the interaction of *Salmonella* with innate immune receptors. Nat Rev Microbiol 13:206–216. https://doi.org/10.1038/nrmicro3428.
8. Dougan G, John V, Palmer S, Mastroeni P. 2011. Immunity to salmonellosis. Immunol Rev 240:196–210. https://doi.org/10.1111/j.1600-065X.2010.00999.x.
9. McSorley SJ. 2014. Immunity to intestinal pathogens: lessons learned from *Salmonella*. Immunol Rev 260:168–182. https://doi.org/10.1111/imr.12184.
10. Tsolis RM, Xavier MN, Santos RL, Baumler AJ. 2011. How to become a top model: impact of animal experimentation on human *Salmonella* disease research. Infect Immun 79:1806–1814. https://doi.org/10.1128/IAI.01369-10.
11. Bedoui S, Kupz A, Wijburg OL, Walduck AK, Rescigno M, Strugnell RA. 2010. Different bacterial pathogens, different strategies, yet the aim is the same: evasion of intestinal dendritic cell recognition. J Immunol 184:2237–2242. https://doi.org/10.4049/jimmunol.0902871.
12. Bueno SM, Riquelme SA, Riedel CA, Kalergis AM. 2012. Mechanisms used by virulent *Salmonella* to impair dendritic cell function and evade adaptive immunity. Immunology 137:28–36. https://doi.org/10.1111/j.1365-2567.2012.03614.x.
13. Eisenstein TK. 2001. Implications of *Salmonella*-induced nitric oxide (NO) for host defense and vaccines: NO, an antimicrobial, antitumor, immunosuppressive and immunoregulatory molecule. Microbes Infect 3:1223–1231. https://doi.org/10.1016/S1286-4579(01)01482-4.
14. Kullas AL, McClelland M, Yang HJ, Tam JW, Torres A, Porwollik S, Mena P, McPhee JB, Bogomolnaya L, Andrews-Polymenis H, van der Velden AWM. 2012. L-Asparaginase II produced by *Salmonella* Typhimurium inhibits T cell responses and mediates virulence. Cell Host Microbe 12:791–798. https://doi.org/10.1016/j.chom.2012.10.018.
15. Torres A, Luke JD, Kullas AL, Kapilashrami K, Botbol Y, Koller A, Tonge PJ, Chen EI, Macian F, van der Velden AWM. 2016. Asparagine deprivation mediated by *Salmonella* asparaginase causes suppression of activation-induced T cell metabolic reprogramming. J Leukoc Biol 99:387–398. https://doi.org/10.1189/jlb.4A0615-252R.
16. Porwollik S, Santiviago CA, Cheng P, Long F, Desai P, Fredlund J, Srikumar S, Silva CA, Chu WP, Chen X, Canals R, Reynolds MM, Bogomolnaya L, Shields C, Cui P, Guo JB, Zheng Y, Endicott-Yazdani T, Yang HJ, Maple A, Ragoza Y, Blondel CJ, Valenzuela C, Andrews-Polymenis H, McClelland M. 2014. Defined single-gene and multi-gene deletion mutant collections in *Salmonella enterica* sv Typhimurium. PLoS One 9:e99820. https://doi.org/10.1371/journal.pone.0099820.
17. Hatahet F, Boyd D, Beckwith J. 2014. Disulfide bond formation in prokaryotes: history, diversity and design. Biochim Biophys Acta 1844:1402–1414. https://doi.org/10.1016/j.bbapap.2014.02.014.
18. Swain AL, Jaskolski M, Housset D, Rao JK, Wlodawer A. 1993. Crystal structure of *Escherichia coli* L-asparaginase, an enzyme used in cancer therapy. Proc Natl Acad Sci U S A 90:1474–1478. https://doi.org/10.1073/pnas.90.4.1474.
19. Cedar H, Schwartz JH. 1967. Localization of the two-L-asparaginases in anaerobically grown *Escherichia coli*. J Biol Chem 242:3753–3755.
20. Gouzy A, Larrouy-Maumus G, Bottai D, Levillain F, Dumas A, Wallach JB, Caire-Brandli I, de Chastellier C, Wu TD, Poincloux R, Brosch R, Guerquin-Kern JL, Schnappinger D, de Carvalho LPS, Poquet Y, Neyrolles O. 2014. *Mycobacterium tuberculosis* exploits asparagine to assimilate nitrogen and resist acid stress during infection. PLoS Pathog 10:e1003928. https://doi.org/10.1371/journal.ppat.1003928.
21. Gesbert G, Ramond E, Rigard M, Frapy E, Dupuis M, Dubail I, Barel M, Henry T, Meibom K, Charbit A. 2014. Asparagine assimilation is critical for intracellular replication and dissemination of *Francisella*. Cell Microbiol 16:434–449. https://doi.org/10.1111/cmi.12227.
22. Popp J, Noster J, Busch K, Kehl A, zur Hellen G, Hensel M. 2015. Role of host cell-derived amino acids in nutrition of intracellular *Salmonella enterica*. Infect Immun 83:4466–4475. https://doi.org/10.1128/IAI.00624-15.
23. Jelsbak L, Hartman H, Schroll C, Rosenkrantz JT, Lemire S, Wallrodt I, Thomsen LE, Poolman M, Kilstrup M, Jensen PR, Olsen JE. 2014. Identification of metabolic pathways essential for fitness of *Salmonella* Typhimurium in vivo. PLoS One 9:e101869. https://doi.org/10.1371/journal.pone.0101869.
24. Ali MM, Newsom DL, Gonzalez JF, Sabag-Daigle A, Stahl C, Steidley B, Dubena J, Dyszel JL, Smith JN, Dieye Y, Arsenescu R, Boyaka PN, Krakowka S, Romeo T, Behrman EJ, White P, Ahmer BMM. 2014. Fructose-asparagine is a primary nutrient during growth of *Salmonella* in the inflamed intestine. PLoS Pathog 10:e1004209. https://doi.org/10.1371/journal.ppat.1004209.
25. Shibayama K, Takeuchi H, Wachino J, Mori S, Arakawa Y. 2011. Biochemical and pathophysiological characterization of *Helicobacter pylori* asparaginase. Microbiol Immunol 55:408–417. https://doi.org/10.1111/j.1348-0421.2011.00333.x.
26. Hofreuter D, Novik V, Galan JE. 2008. Metabolic diversity in *Campylobacter jejuni* enhances specific tissue colonization. Cell Host Microbe 4:425–433. https://doi.org/10.1016/j.chom.2008.10.002.
27. Philips JA, Ernst JD. 2012. Tuberculosis pathogenesis and immunity. Annu Rev Pathol 7:353–384. https://doi.org/10.1146/annurev-pathol-011811-132458.
28. Jasenosky LD, Scriba TJ, Hanekom WA, Goldfeld AE. 2015. T cells and adaptive immunity to *Mycobacterium tuberculosis* in humans. Immunol Rev 264:74–87. https://doi.org/10.1111/imr.12274.
29. Velin D, Straubinger K, Gerhard M. 2016. Inflammation, immunity, and vaccines for *Helicobacter pylori* infection. Helicobacter 21:26–29. https://doi.org/10.1111/hel.12336.
30. Janssen R, Krogfelt KA, Cawthraw SA, van Pelt W, Wagenaar JA, Owen RJ. 2008. Host-pathogen interactions in *Campylobacter* infections: the host perspective. Clin Microbiol Rev 21:505–518. https://doi.org/10.1128/CMR.00055-07.
31. Cowley SC, Elkins KL. 2011. Immunity to *Francisella*. Front Microbiol 2:26. https://doi.org/10.3389/fmicb.2011.00026.
32. Shrivastava A, Khan AA, Khurshid M, Kalam MA, Jain SK, Singhal PK. 2016. Recent developments in L-asparaginase discovery and its potential as anticancer agent. Crit Rev Oncol Hematol 100:1–10. https://doi.org/10.1016/j.critrevonc.2015.01.002.
33. National Research Council. 2011. Guide for the care and use of laboratory animals, 8th ed. National Academies Press, Washington, DC.
34. Stojiljkovic I, Baumler AJ, Heffron F. 1995. Ethanolamine utilization in *Salmonella* Typhimurium: nucleotide sequence, protein expression, and mutational analysis of the *cchA cchB eutE eutJ eutG eutH* gene cluster. J Bacteriol 177:1357–1366. https://doi.org/10.1128/jb.177.5.1357-1366.1995.
35. Datsenko KA, Wanner BL. 2000. One-step inactivation of chromosomal genes in *Escherichia coli* K-12 using PCR products. Proc Natl Acad Sci U S A 97:6640–6645. https://doi.org/10.1073/pnas.120163297.

36. Santiviago CA, Reynolds MM, Porwollik S, Choi SH, Long F, Andrews-Polymenis HL, McClelland M. 2009. Analysis of pools of targeted *Salmonella* deletion mutants identifies novel genes affecting fitness during competitive infection in mice. PLoS Pathog 5:e1000477. https://doi.org/10.1371/journal.ppat.1000477.
37. Cherepanov PP, Wackernagel W. 1995. Gene disruption in *Escherichia coli*: Tc^R and Km^R cassettes with the option of FLP-catalyzed excision of the antibiotic-resistance determinant. Gene 158:9–14. https://doi.org/10.1016/0378-1119(95)00193-A.
38. Quan S, Hiniker A, Collet J-F, Bardwell JCA. 2013. Isolation of bacteria envelope proteins, p 359–366. *In* Delcour HA (ed), Bacterial cell surfaces: methods and protocols. Humana Press, Totowa, NJ.
39. Schagger H. 2006. Tricine-SDS-PAGE. Nat Protoc 1:16–22. https://doi.org/10.1038/nprot.2006.4.
40. Pujol C, Bliska JB. 2003. The ability to replicate in macrophages is conserved between *Yersinia pestis* and *Yersinia pseudotuberculosis*. Infect Immun 71:5892–5899. https://doi.org/10.1128/IAI.71.10.5892-5899.2003.