The *Salmonella* Pathogenicity Island 2-Encoded Type III Secretion System Is Essential for the Survival of *Salmonella enterica* Serovar Typhimurium in Free-Living Amoebae

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Free-living amoebae represent a potential reservoir and predator of *Salmonella enterica*. Through the use of type III secretion system (T3SS) mutants and analysis of transcription of selected T3SS genes, we demonstrated that the *Salmonella* pathogenicity island 2 is highly induced during *S. enterica* serovar Typhimurium infection of *Acanthamoeba polyphaga* and is essential for survival within amoebae.

The importance of free-living protozoa, such as amoebae, as environmental reservoirs of food-borne pathogens is becoming increasingly recognized (1, 6, 9, 18). Such interactions may also have highly significant physiological implications, as amoebic passage of *Legionella pneumophila* enhanced bacterial virulence (3) and could resuscitate viable but nonculturable cells (20). *Salmonella enterica* serovars Typhimurium and Dublin have been shown to survive within *Acanthamoeba polyphaga* and *Acanthamoeba rhysodes* (5, 22), and induction of *fis* transcription, indicative of bacterial proliferation within contractile vacuoles, has been reported (3). The role of the *Salmonella* pathogenicity island 1 (SPI-1)-encoded type III secretion system (T3SS-1), which mediates forced bacterial uptake via subversion of actin dynamics, is unclear; however, an *S. enterica* serovar Dublin ΔhilA mutant lacking a key transcriptional activator of SPI-1 entered and survived within *A. rhysodes* at a level similar to that of the parent strain (22). A second type III secretion system encoded by SPI-2 (T3SS-2) and the PhoPQ two-component regulatory system are known to play key roles in the intracellular survival of *Salmonella* in mammalian cells, but their roles in interactions with protozoa have not been reported. In this study we investigated the roles of T3SS-1, T3SS-2, and PhoP in entry and survival of *S. enterica* serovar Typhimurium in *A. polyphaga* using defined mutant strains. Additionally, we quantified transcription of the *S. enterica* serovar Typhimurium SPI-1 gene *sipC* and the SPI-2 gene *sscC* (which encode components of the type III secretion transloc-}
described above. The sscC and sipC genes were selected as representatives of the SPI-2 and SPI-1 T3SS, respectively, as the gene products are expressed within the host cell as part of the translocon stabilizing the needle-like apparatus and we have previously demonstrated their expression within porcine and murine macrophages (14). After 1 or 4 h of incubation, flasks were centrifuged to obtain a cell pellet from which total RNA was extracted with TRI reagent and treated in solution with Turbo DNase (Ambion, Inc., Austin, TX), followed by on-column DNase treatment with RNase-free DNase. Transcription at time zero was determined in Salmonella grown in LB broth as described above. Real-time reverse transcriptase PCR (RT-PCR) was performed with the MJ Research/Bio-Rad Opticon 2 system with quantitative RT-PCR Mastermix (Eurogentec, Seraing, Belgium) using previously described conditions (14). To quantify transcription, the $2^{\Delta\Delta Ct}$ method was used for data analysis (12), and transcription was reported as $n$-fold induction normalized to the internal standard and relative to the control at time zero (14). RT-PCR data were analyzed by Student’s t test with a P value of >0.05 considered statistically significant.

Significant induction of both the SPI-1 gene sipC and the SPI-2 gene sscC was found to occur relative to yejA within amoebic cells at 1 h postinfection (Fig. 2), with an approximately sixfold increase over Salmonella grown in LB broth ($P > 0.02$). At 4 h postinfection, there was no significant change in transcription of sipC. In contrast, a dramatic increase in transcription of the SPI-2 gene sscC was found at 4 h. Transcription within amoebic cells was increased over Salmonella grown in LB broth by a mean value of 147-fold ($P = 0.001$). Previously we have shown sscC to be expressed by S. enterica serovar Typhimurium within porcine and murine macrophages at 4 h postinfection (14). A range of SPI-2 genes are also strongly induced following S. enterica serovar Typhimurium infection of J774A.1 murine macrophage-like cells (4) and epithelial cells (8).

Taken together, these data indicate that the SPI-2-encoded T3SS influences survival within amoebic cells, as is the case with macrophages. Previously we have shown that mutation of ssaU reduces the ability of a range of Salmonella serovars to survive within phagocytes and cause systemic infection (10, 11, 23). The failure of the $\Delta$phoP mutant to survive in A. polyphaga cells further supports the theory that survival within amoebae and survival within macrophages are largely analogous, as the PhoPQ two-component regulatory system, although associated with regulation of many genes, is key to activation of the SPI-2-encoded T3SS within phagocytes (7, 15). In contrast, mutation of spaS had a minor effect on Salmonella survival within amoebae, consistent with findings using a $\Delta$hilA mutant (17). Nevertheless, transcription of the SPI-1 gene sipC was induced within amoebae, in contrast to findings with S. enterica serovar Typhimurium in J774A.1 murine macrophage-like cells, where sipC transcription was downregulated 50-fold compared to growth in culture medium (4). Recently, however, SPI-1 genes were found to be induced after infection of epithelial cells, indicating that the intracellular program of bacterial gene expression is sensitive to cell type (8).

Survival within protozoa may represent an important environmental reservoir of Salmonella and confer resistance to predation in the gastrointestinal tracts of ruminants. In addition, such interactions may have exerted an evolutionary pressure leading to bacterial divergence, including variation in the lipopolysaccharide O-side chain of Salmonella and the development of virulence factors (16, 24, 25). Recent studies have indicated that Shiga toxin (Stx) of the food-borne pathogen enterohemorrhagic Escherichia coli (EHEC) aids resistance to grazing protozoa and may account for the very high stx carriage rates in EHEC isolated from ruminants (19). Furthermore, the type II and IV protein secretion systems of L. pneumophila aid survival in both waterborne amoebae and alveolar macrophages during human infection (17, 21). Taken together with
our finding that <i>S. enterica</i> serovar Typhimurium SPI-2 is induced and required for survival in <i>A. polyphaga</i>, such data suggest that traits we primarily consider virulence factors for bacterial pathogenesis in animals and humans may have originally evolved to play other roles in microbial ecology.

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