Thermosensing Coordinates a Cis-regulatory Module for Transcriptional Activation of the Intracellular Virulence System in Salmonella enterica Serovar Typhimurium*

Nancy Duong†‡, Suzanne Osborne‡†, Victor H. Bustamante‡, Ana M. Tomljenovic‡, José L. Puente‡, and Brian K. Coombes†‡§

From the †Department of Biochemistry and Biomedical Sciences, McMaster University, Hamilton, Ontario L8N 3Z5, Canada, the ‡Departamento de Microbiología Molecular, Instituto de Biotecnología, Universidad Nacional Autónoma de México, Cuernavaca, Morelos 62251, Mexico, and §Public Health Agency of Canada, Laboratory for Foodborne Zoonoses, Guelph, Ontario N1G 3W4, Canada.

The expression of bacterial virulence genes is tightly controlled by the convergence of multiple extracellular signals. As a zoonotic pathogen, virulence gene regulation in Salmonella enterica serovar Typhimurium is responsive to multiple cues from the general environment as well as from multiple niches within animal and human hosts. Previous work has identified combined magnesium and phosphate limitation as an environmental cue that activates genes required for intracellular virulence. One unanswered question is how virulence genes that are expressed within the host are inhibited in non-host environments that satisfy the phosphate and magnesium limitation cues. We report here that thermosensing is the major mechanism controlling incongruous activation of the intracellular virulence phenotype. Bacteria grown at 30 °C or lower were unable to activate the intracellular type III secretion system even under strong inducing signals such as synthetic medium, contact with macrophages, and exposure to the murine gut. Thermoregulation was fully recapitulated in a Salmonella bongori strain engineered to contain the intracellular virulence genes of S. enterica sv. Typhimurium, suggesting that orthologous thermoregulators were available. Accordingly, virulence gene repression at the nonpermissive temperature required Hha and H-NS, two nucleoid-like proteins involved in virulence gene control. The use of combined environmental cues to control transcriptional “logic gates” allows for transcriptional selectivity of virulence genes that would otherwise be superfluous if activated in the non-host environment. Thus, thermosensing by Salmonella provides integrated control of host niche-specific virulence factors.

Salmonellae are Gram-negative bacteria that infect humans and animals using virulence factors that promote invasiveness and intracellular survival. Such zoonoses frequently cause gastrointestinal, but the spectrum of disease can include major extraintestinal morbidity following systemic spread. Costs associated with human salmonellosis in the United States are in the several billion dollar range (1), making this pathogen an enormous economic and public health problem.

One of the major virulence factors in Salmonella enterica is a type III secretion system (T3SS) encoded in the Salmonella pathogenicity island 2 (SPI-2). This horizontally acquired genomic island contains genes whose products activate and assemble the T3SS that is required during intracellular infection (2, 3) and that injects into host cells the effector proteins required for intracellular survival and systemic infection of animals (4–6). SPI-2 T3SS activity directs intraphagosomal bacteria to a protective compartment within host cells by disengaging these vacuoles from the classical phagosome maturation pathway that terminates at lysosome fusion and degradation of vacuolar contents. Activation of the SPI-2 T3SS is achieved in part by the genetically linked two-component regulatory system comprising a sensor kinase, SsrA, and a response regulator, SsrB (7, 8).

One barrier to bacteria benefiting from horizontal gene transfer is appropriate integration of new genes into the regulatory circuits of the host bacterium. To understand this problem, we have been studying the regulation of the SPI-2 T3SS and characterizing the positive activators (9) and repressors (10, 11) that coordinate the fine-tuned expression of intracellular virulence during infection (7, 8). Although the exact nature of SsrA-SsrB activation is not known, we and others (9, 12) have determined that this system is activated in vitro by acidification of culture medium containing micromolar concentrations of Mg\(^{2+}\) and PO\(_4\)\(^{3-}\) ions. To understand the virulence associated with S. enterica sv. Typhimurium, we are interested in defining the full range of environmental signals necessary to activate intracellular virulence in this organism. Temperature is one environmental

---

* This work was supported in part by Canadian Institutes of Health Research Operating Grant MOP-82704 (to B. K. C.), the Public Health Agency of Canada, and by Dirección General de Asuntos del Personal Académico Grant PAPIIT IN227306-3 (to J. L. P.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

† Supported by research training grants from McMaster University.

‡ Recipient of a New Investigator Award from the CIHR (MSH-83721) and a Young Investigator Award from the American Society of Microbiology. To whom correspondence should be addressed: Dept. of Biochemistry and Biomedical Sciences, McMaster University, 1200 Main St. West, Health Sciences Centre 4H17, Hamilton, Ontario L8N 3Z5, Canada. Tel.: 905-525-9140 (ext. 22159); Fax: 905-522-9033; E-mail: coombes@mcmaster.ca.

§ The abbreviations used are: T3SS, type III secretion system; SPI-2, Salmonella pathogenicity island 2; LPM, low phosphate and magnesium; RIVET, recombinase-based in vivo expression technology; BAC, bacterial artificial chromosome.
Thermoregulation of Salmonella Virulence

TABLE 1

Strains or plasmids used in this study

| Strain or plasmid | Genotype or description | Source or Ref. |
|-------------------|-------------------------|---------------|
| *S. enterica* sv. Typhimurium | | |
| BKC1-1 | SL1344, wild type, Sm<sup>a</sup> | 44 |
| BKC3-25 | SL1344, p<sub>ssrB</sub>:<sup>p</sup>ssrB<sub>cat</sub>:tnpR: lacZ | 9 |
| BKC5-49 | SL1344 ΔΔdgT | 11 |
| BKC9-49 | SL1344 hha<sup>−</sup>:<sup>p</sup>Hha<sub>cat</sub>:tnpR: lacZ | 10 |
| BKC9-60 | SL1344 ΔΔdgT hha<sup>−</sup>:<sup>p</sup>Hha<sub>cat</sub>:tnpR: lacZ | 10 |
| BKC1-55 | SL1344 ssrB<sup>−</sup>:<sup>p</sup>ssrB<sub>cat</sub>:tnpR: lacZ | 45 |
| BKC1-25 | SL1344 ssrA<sup>−</sup>:<sup>p</sup>ssrA<sub>cat</sub>:tnpR: lacZ | 11 |
| BKC5-56 | SL1344 ssrB<sup>−</sup>:<sup>p</sup>ssrB<sub>cat</sub>:tnpR: lacZ | This study |
| BKC8-70 | SL1344 sseA<sup>−</sup>:<sup>p</sup>ssesA<sub>cat</sub>:tnpR: lacZ | This study |
| BKC4-79 | SL1344 sseD<sup>−</sup>:<sup>p</sup>ssesD<sub>cat</sub>:tnpR: lacZ | This study |
| BKC5-57 | SL1344 ΔΔdgT P<sup>ssrA</sup><sub>cat</sub>:<sup>p</sup>ssrA<sub>cat</sub>:tnpR: lacZ | This study |
| BKC9-73 | SL1344 hha<sup>−</sup>:<sup>p</sup>Hha<sub>cat</sub>:tnpR: lacZ | This study |
| BKC9-75 | SL1344 ΔΔdgT hha<sup>−</sup>:<sup>p</sup>Hha<sub>cat</sub>:tnpR: lacZ | This study |
| BKC5-67 | SL1344 ushA<sup>−</sup>:<sup>p</sup>ushA<sub>cat</sub>:tnpR: lacZ | 11, 18 |
| BKC8-80 | SL1344 ΔΔdgT ushA<sup>−</sup>:<sup>p</sup>ushA<sub>cat</sub>:tnpR: lacZ | 9 |
| BKC8-81 | SL1344 sseA<sup>−</sup>:<sup>p</sup>ssesA<sub>cat</sub>:tnpR: lacZ | This study |
| BKC6-11 | SL1344 p<sub>ssrA</sub>:<sup>p</sup>ssrA<sub>cat</sub>:tnpR: lacZ | This study |
| BKC6-12 | SL1344 p<sub>ssrB</sub>:<sup>p</sup>ssrB<sub>cat</sub>:tnpR: lacZ | 9 |
| BKC8-90 | SL1344 sseE<sup>−</sup>:<sup>p</sup>ssesE<sub>cat</sub>:tnpR: lacZ | This study |
| BKC8-81 | SL1344 sseA<sup>−</sup>:<sup>p</sup>ssesA<sub>cat</sub>:tnpR: lacZ | This study |
| IPTM2 | SL1344 sseA<sup>−</sup>:<sup>p</sup>3xFLAG-Kan<sup>a</sup> | |
| pMPM-T6Ω | p15A derivative containing the araBAD promoter, Te<sup>a</sup> | 46 |
| pT6-HNS-wt | pMPM-T6Ω expressing wild type hns from araBAD promoter | |
| pT6-HNS-Q92am | pMPM-T6Ω expressing N terminus dominant-negative hns from araBAD promoter. Amber mutation at position 92 | |

<sup>a</sup>V. H. Bustamante and J. L. Puente, unpublished data.

*cue sensed by bacterial pathogens (13–17); however, the role of thermoregulation of the Salmonella Typhimurium SPI-2 virulence system has not been addressed. We report here that environmental cues such as low phosphate and magnesium, interaction with host cells, and exposure to intestinal contents is not sufficient for induction of intracellular virulence genes in *S. enterica* sv. Typhimurium. A necessary environmental cue accompanying these other signals is mammalian body temperature, and we determine that the intracellular virulence phenotype is strictly thermoregulated by the SsrA-SsrB activator system and two nucleoid-like repressor proteins, Hha and H-NS (10, 17). The strict requirement of mammalian body temperature for virulence gene activation in this pathogen thus prevents premature expression of virulence genes in non-host environments that may happen to satisfy the phosphate and magnesium limitation cues but that would be incongruent with virulence-associated type III secretion. We suggest that thermosensing is an evolutionary strategy used by *Salmonella* to coordinate environmental cues at the cell surface and activate appropriate promoter elements controlling niche-specific virulence factors.

**EXPERIMENTAL PROCEDURES**

**Bacterial Strains and Growth Conditions**—Routine propagation of *Salmonella* was in LB broth supplemented with antibiotics as appropriate. When used, antibiotic concentrations were as follows: streptomycin, 50 μg ml<sup>−1</sup>; tetracycline, 12 μg ml<sup>−1</sup>; chloramphenicol, 10 μg ml<sup>−1</sup>; ampicillin, 100 μg ml<sup>−1</sup>. *Salmonella* mutants were isogenic derivatives of the wild type strain SL1344 (*S. enterica* serovar Typhimurium). SL1344 *p<sub>ssrB</sub>*::<sup>p</sup>ssesA<sub>cat</sub>-<sup>p</sup>ssrB<sub>cat</sub>:tnpR::<sup>p</sup>ssrB<sub>cat</sub>:tnpR: lacZ *ussA*:<sup>p</sup>ussA<sub>cat</sub>:tnpR: lacZ used was used for recombinase-based transcriptional reporter experiments and has been described elsewhere (18). *Salmonella bongori* SARC12 containing a bacterial artificial chromosome encoding the SPI-2 genomic island (pB6) was obtained from Dr. Michael Hensel (Erlangen, Germany) (19). For SsrB experiments, an allelic replacement of wild type *ssrB* with *ssrB*:3xFLAG was performed in SL1344 using lambda red recombination as described previously (20). A strain of all strains used in this study is listed in Table 1. An acidic minimal medium low in phosphate and magnesium (LPM) used for the induction of SPI-2 has been described elsewhere (9).

**Protein Expression Assays**—Overnight cultures of *Salmonella* were washed in LPM and subcultured (1:50) into LPM at 37, 30, or 25°C with shaking for 5 h (9). Whole bacterial cells were solubilized in SDS sample buffer (100 mM Tris-HCl (pH 6.8), 20% (v/v) glycerol, 4% (w/v) SDS, 0.002% (w/v) bromophenol blue, 200 mM dithiothreitol) adjusted according to the optical density of the culture. Proteins were analyzed by SDS-PAGE and immunoblotting using mouse anti-hemagglutinin antibody clone HA.11 (1:1000, Cedarlane Laboratories), mouse anti-DnaK antibody (1:5000, StressGen), rabbit anti-SseB antiserum (1:2000), and mouse anti-FLAG monoclonal antibody clone M2 (1:2000, Sigma). Secondary antibodies were conjugated to horseradish peroxidase and detected using chemiluminescence.

**Transcriptional Reporter Experiments**—Transcriptional activity of the *sseA* promoter within SPI-2 was examined using a chemiluminescent β-galactosidase assay (10) and recombinase-based in vivo expression technology reporters (18). First, *sseA* promoter activity was examined using transcriptional fusions to lacZ and a chemiluminescence assay described previously (10). Briefly, a single copy chromosomal fusion of lacZ to the *sseA* promoter (P<sub>sseA</sub>:lacZ) was constructed in wild type *Salmonella* and various mutants by homologous recombination. Reporter strains were cultured overnight in LB medium containing ampicillin and then washed in either LB or LPM medium and subcultured in fresh medium. Cultures were incubated with shaking at 225 rpm at 37 or 25°C. Relative light units of β-galactosidase activity were measured in either white microtiter plates (for experiments performed in noninducing medium) or black microtiter plates (for experiments performed in LPM) using a top-reading plate luminometer (Molecular Devices, Sunnyvale, CA). To facilitate direct comparisons...
between strains, β-galactosidase activity was normalized to the optical density of the paired culture.

H-NS Dominant-negative Assay for the Thermoregulation of SsrB-FLAG—Plasmids pMPM-T6Δ, pT6-HNS-WT, and pT6-HNS-Q92am (Table 1) were introduced into S. enterica serovar Typhimurium strain SL1344 (ssrB::3xFLAG). The resulting transformants were grown overnight in 5 ml of LB with shaking. Cultures were then washed and subcultured into 3 ml of LPM medium as described above for protein assays, in the presence or absence of 0.1% arabinose to induce dominant-negative hns expression. Total protein extracts from lysed bacterial cells were probed by Western blot using monoclonal anti-FLAG® M2 antibody (1:2000; Sigma) and anti-maltose-binding protein antibody (1:10,000; New England Biolabs).

Recombinase-based in Vivo Expression and Infection of Murine Macrophages—RAW264.7 cells were cultured using Dulbecco’s modified Eagle’s medium (HyClone, Logan, UT) supplemented with 10% fetal bovine serum. The day prior to infection, 5 × 10^5 cells were seeded into 24-well cell culture plates and incubated overnight at 37 °C in 5% CO_2_. For transcriptional reporter activity using RIVET strains (18), RAW264.7 macrophage cells were infected with opsonized stationary-phase S. enterica sv. Typhimurium RIVET strains for 30 min at 4 °C and then washed and shifted to either 37 or 25 °C. Intracellular bacteria were harvested at various time points as indicated by selective lysis of macrophages with 1% Triton X-100, 0.1% SDS. Bacteria were plated on streptomycin-containing medium and then replica stamped onto chloramphenicol medium to calculate resolution. For RIVET reporter experiments using tissue from the murine intestine, small and large intestine was harvested from C57BL/6 mice and placed into ice-cold sterile phosphate-buffered saline. Tissues were immediately homogenized, diluted, and incubated with S. enterica sv. Typhimurium RIVET reporter strains for various times at either 37 or 25 °C. Resolution was determined as described above.

RESULTS

Expression of Intracellular Virulence Genes Is Thermoregulated in S. enterica sv. Typhimurium—The SPI-2 T3SS of S. enterica sv. Typhimurium is a horizontally acquired genomic island needed for virulence in the mammalian host environment (21–23). During our work with temperature-sensitive σ factor mutants of Salmonella, we took notice of a temperature dependence for expression of the SPI-2-encoded T3SS. To further understand the environmental cues controlling intracellular virulence, we cultured wild type S. enterica sv. Typhimurium. Typhimurium and mutants lacking either the SsrA sensor kinase or the SsrB response regulator in LPM synthetic medium that is a potent activator of SPI-2 gene expression (9). Bacteria were grown at 37 and 25 °C or shifted from 25–37 °C for 5 h and then bacterial lysates were probed by Western blot for the needle filament protein of the T3SS, SseB, and for DnaK as a control. Temperature was the result of reduced transcriptional activity of the SsrA-SsrB two-component regulatory system and a temperature cue. We hypothesized that lack of SseB at the nonpermissive temperature was the result of reduced transcriptional activity of the sseB promoter element (P_{sseA}) (9). To address this, we constructed a transcriptional fusion of P_{sseA} to lacZ and integrated a single copy into the S. enterica sv. Typhimurium chromosome. Reporter bacteria grown in inducing LPM medium showed high level expression of β-galactosidase at 37 °C and upon shifting from 25 to 37 °C. However, β-galactosidase reporter activity was not induced in bacteria grown at 25 °C or at any temperature when we introduced an ssrB deletion into

these strong inducing conditions. In contrast, DnaK expression was similar at both temperatures. SseB production at the permissive temperature required the SsrA sensor kinase and SsrB response regulator because SseB was absent in mutants deleted for ssrA or ssrB.

S. bongori is a Salmonella species that lacks the SPI-2 genomic island and is a commensal of cold-blooded animals. To confirm the thermoregulation of the SPI-2 T3SS, we used an S. bongori strain engineered to contain the SPI-2 genomic island, including SsrA-SsrB, on a bacterial artificial chromosome (BAC) (3, 19). S. bongori containing an empty BAC (pBelo) did not express SseB as expected because it lacks SPI-2. However, upon transfer of SPI-2 to S. bongori, temperature-dependent accumulation of SseB was recapitulated (Fig. 1B), similar to that observed in S. enterica sv. Typhimurium.

We hypothesized that lack of SseB at the nonpermissive temperature was the result of reduced transcriptional activity of the sseB promoter element (P_{sseA}) (9). To address this, we constructed a transcriptional fusion of P_{sseA} to lacZ and integrated a single copy into the S. enterica sv. Typhimurium chromosome. Reporter bacteria grown in inducing LPM medium showed high level expression of β-galactosidase at 37 °C and upon shifting from 25 to 37 °C. However, β-galactosidase reporter activity was not induced in bacteria grown at 25 °C or at any temperature when we introduced an ssrB deletion into
Thermoregulation of Salmonella Virulence

Bacterial reporter strains were constructed with the sseA promoter (P_{sseA}) or the sseE promoter (P_{sseE}) linked to lacZ to create single copy integrated transcriptional fusions. Reporter strains were cultured in LPM medium at the different temperatures indicated, and at various time points transcriptional activity was monitored using β-galactosidase activity expressed as relative light units. (***, p < 0.0001). Data are the means of quadruplicate determinations performed three times. C. mammalian body temperature is required for SPI-2 transcriptional activity following prolonged growth in LB. Wild type (wt) S. enterica sv. Typhimurium containing a P_{sseE}-lacZ reporter was cultured in LB and then split into fresh LB at either the permissive temperature (37 °C) or the nonpermissive temperature (25 °C). β-Galactosidase activity expressed as relative light units (left ordinate axis) and optical density (right ordinate axis) was measured for cultures grown at 37 °C (gray bars and open squares) and at 25 °C (black bars and open circles).

The reporter strain (Fig. 2A). To show that thermoregulation was not specific to the sseA promoter, we constructed another transcriptional fusion that reports on promoter activity from the sseE promoter (9), and we performed a time course of β-galactosidase activity in reporter strains grown at different temperatures. No transcriptional activity was detected from the sseE promoter when the reporter strain was cultured at 25 °C; however, temperature-inducible activity was detected in a similar manner to P_{sseA} when the strains were grown at 37 °C or shifted to this temperature after preincubation at 25 °C (Fig. 2B).

Previous studies showed that S. enterica sv. Typhimurium grown in medium that is noninducing for SPI-2 activity (LB or LPM, pH 7.0) could activate gene expression within SPI-2, but this activity was detectable only after prolonged growth, and the mechanism behind this was thought to be simply transition into stationary phase (9, 10). To test whether this SPI-2 activity was also thermoregulated, we grew the wild type sseA::pP_{sseA}-lacZ reporter strain overnight in LB at 25 °C, split the culture into fresh LB, and shifted it to either 37 °C or kept it at 25 °C until stationary phase was reached for both cultures. β-Galactosidase activity and culture optical density was monitored in the cells over time. As expected (10), sseA promoter activity increased in bacteria growing at 37 °C in LB as the culture density increased (Fig. 2C). This activity was strictly thermoregulated because cultures grown at 25 °C, although ultimately reaching similar densities to those at 37 °C, repressed SPI-2 transcriptional activity throughout the course of the experiment (Fig. 2C). Together, these data establish mammalian body temperature as a major environmental cue driving expression of intracellular virulence factors that are critical for S. enterica sv. Typhimurium pathogenesis.

Thermoregulation of the Horizontally Acquired Response Regulator, SsrB—Thermoregulation of some bacterial genes is controlled by the nucleoid-associated transcriptional repressor, H-NS. Because activation of the intracellular virulence regulon in S. enterica sv. Typhimurium requires the response regulator, SsrB, we designed experiments using an SL1344 ssrB::3xFLAG strain containing a mutant allele of hns to test whether thermoregulation of SPI-2 genes takes place at the level of SsrB expression. Deletion of hns has pleiotropic effects leading to toxicity in S. enterica sv. Typhimurium unless additional compensatory mutations are introduced (24). We overcame this by choosing a dominant-negative strategy to examine the role of H-NS on SPI-2 gene expression. hns^{Q92am} is a dominant-negative hns mutant that gives rise to a C-terminal truncation at position 92 due to introduction of an amber mutation (25). We constructed an hns^{Q92am} allele in pMPM-T6Ω for arabinose-inducible expression of H-NS^{Met1–Ala91} from the pBAD promoter and transferred the construct into SL1344 expressing SsrB-FLAG from the chromosome. At 37 °C, SsrB-FLAG was robustly expressed in a wild type strain containing the empty plasmid, pMPM-T6Ω, but was almost undetectable from the same strain grown at 25 °C, thus establishing thermoregulation of the response regulator (Fig. 3A). We then performed expression experiments in the presence of arabinose to induce expression of either wild type H-NS (pT6-HNS-wt) or the H-NS^{Met1–Ala91} dominant-negative mutant (pT6-HNS-Q92am). Under these conditions, thermoregulation of SsrB-FLAG was maintained in the presence of wild type H-NS, but the strain expressing the H-NS^{Q92am} dominant-negative mutant exhibited a temperature-blind phenotype and accumulated SsrB-FLAG at 25 °C (Fig. 3A). Omitting arabinose from these cultures such that the dominant-negative H-NS was not expressed restored
Thermoregulation of Salmonella Virulence Genes—We speculated that the effect of H-NS on thermoregulation of SsrB would influence genes downstream of this response regulator. To test this we examined production of SseB, which lies downstream of the SsrB activation cascade, following expression of H-NSQ92am. SseB levels from whole cell lysates were examined by immunoblot following arabinose induction of H-NSQ92am at 25 and 37 °C. In accord with the regulation data for SsrB, SseB levels increased when H-NSQ92am was overexpressed at 37 °C and decreased when wild type H-NS was overexpressed (Fig. 3B). In addition, overexpression of H-NSQ92am caused a temperature-blind phenotype leading to SseB accumulation at the nonpermissive temperature of 25 °C, which could be corrected if the dominant-negative construct was not induced with arabinose (Fig. 3B).

Several other small regulatory proteins are members of the nucleoid-like family of transcriptional regulators that includes H-NS (26, 27). To understand further the molecular mechanism of thermoregulation, we turned our attention to two small nucleoid-like proteins, YdgT and Hha, which we have shown previously to repress transcription of SPI-2 genes (10, 11). We installed the P_{ssea}-lacZ reporter into single ydgT and hha deletion mutants and also into a ydgT hha double mutant and performed β-galactosidase activity assays on these strains during growth in LPM medium at the permissive (37 °C) and nonpermissive temperature (25 °C). At 37 °C, both ydgT and hha mutants showed increased β-galactosidase activity from the transcriptional reporter compared with wild type as shown previously (10, 11). At the nonpermissive temperature (25 °C), deletion of hha, but not ydgT, restored β-galactosidase activity to the wild type level at 37 °C. Consistent with this “temperature-blind” phenotype (Fig. 4A), this phenotype was similar for the hha ydgT double mutant as well. The difference in β-galactosidase activity at 37 and 25 °C in the hha mutant was not significantly different indicating that Hha is essential for thermoregulation of SPI-2 gene expression.

Because previous data indicated that prolonged growth at 37 °C induced SPI-2 gene expression under classically noninducing conditions (LB; Fig. 2B), we wanted to test whether hha was also involved in temperature-dependent induction of SPI-2 gene expression under these conditions. As expected, transcriptional activity of the sseA promoter was low in wild type Salmonella grown in LB at all temperatures (Fig. 4B) and SPI-2 activity increased in the hha mutant (10). At 25 °C, β-galacto-
Thermoregulation of Salmonella Virulence

A contact with the host cell surface is not sufficient for virulence gene activation. Macrophages were infected with a RIVET reporter strain that measures transcriptional activation of the sseA promoter as described under “Experimental Procedures.” Cells were infected at 4 °C to allow for bacterial attachment, followed by a temperature shift to either 25 °C (closed squares) or 37 °C (open circles). Data shown are the mean percent resolution of a resolvable genetic reporter cassette (with S.E.). *(p < 0.05) between 37 and 25 °C temperatures. B, intracellular bacteria enumerated as part of data collection for A, shown as the mean ± S.E. C, thermoregulation of virulence gene expression following host activation cues improves bacterial fitness in the intracellular environment of macrophages. *Salmonella was primed at either 25 or 25 °C and infected into macrophages at 37 °C. Survival of *Salmonella during the first 2 h of intracellular infection is shown as the number of intracellular colony-forming units (CFU) recovered at 5 min and 2 h post-infection. Data shown are the mean number of colony-forming units from triplicate determinations from two separate experiments. *(p < 0.01). D, interaction with the enteric environment is not sufficient for virulence gene activation. Activation of a resolvable genetic reporter cassette was measured following incubation of *Salmonella with murine small and large intestinal contents. Data represent cassette resolution immediately prior to incubation with intestinal contents. Data are the mean percent resolution with standard errors. *(p < 0.05).

The intracellular virulence phenotype of *Salmonella sv. Typhimurium is thermoregulated. A, contact with the host cell surface is not sufficient for virulence gene activation. Macrophages were infected with a RIVET reporter strain that measures transcriptional activation of the sseA promoter as described under “Experimental Procedures.” Cells were infected at 4 °C to allow for bacterial attachment, followed by a temperature shift to either 25 °C (closed squares) or 37 °C (open circles). Data shown are the mean percent resolution of a resolvable genetic reporter cassette (with S.E.). *(p < 0.05) between 37 and 25 °C temperatures. B, intracellular bacteria enumerated as part of data collection for A, shown as the mean ± S.E. C, thermoregulation of virulence gene expression following host activation cues improves bacterial fitness in the intracellular environment of macrophages. *Salmonella was primed at either 25 or 25 °C and infected into macrophages at 37 °C. Survival of *Salmonella during the first 2 h of intracellular infection is shown as the number of intracellular colony-forming units (CFU) recovered at 5 min and 2 h post-infection. Data shown are the mean number of colony-forming units from triplicate determinations from two separate experiments. *(p < 0.01). D, interaction with the enteric environment is not sufficient for virulence gene activation. Activation of a resolvable genetic reporter cassette was measured following incubation of *Salmonella with murine small and large intestinal contents. Data represent cassette resolution immediately prior to incubation with intestinal contents. Data are the mean percent resolution with standard errors. *(p < 0.05).

The Intracellular Virulence Phenotype of *Salmonella sv. Typhimurium Is Thermoregulated—Intracellular virulence of *Salmonella sv. Typhimurium requires the T3SS encoded in the SPI-2 pathogenicity island. We hypothesized that the temperature cue was required for activating the virulence program to cause pathogenesis in a host niche. To test this, we infected mouse macrophages with *Salmonella sv. Typhimurium at 4 °C to allow for bacterial attachment to the cell surface without phagocytosis. Upon shifting the infected cells to either 25 or 37 °C, transcriptional activity of the SPI-2 sseA promoter was monitored over time using recombinase-based in vivo expression technology (RIVET). In these experiments, there was a rapid induction of sseA promoter activity within 5 min following the temperature shift to 37 °C that was not observed if the infected cells were instead shifted to 25 °C (Fig. 5A). Resolution of the transcriptional reporter remained at background levels throughout the experiment when host cells remained at 25 °C indicating that host cell cues alone are insufficient in activating the intracellular virulence program and instead must be accompanied by the permissive temperature cue for proper activation.

To determine whether thermoregulation of intracellular virulence in *Salmonella sv. Typhimurium has functional consequences for the bacterial population during infection, bacteria were primed by growth at either 37 or 25 °C, opsonized to promote complement receptor-mediated phagocytosis, and then infected into macrophages at 37 °C. The level of bacterial uptake by macrophages 5 min after infection was similar for bacteria primed at either temperature. However, bacteria primed at 37 °C were recovered in significantly greater numbers at 2 h after infection than those primed at the nonpermissive temperature (Fig. 5C). These data suggest that initial thermosensing in the host environment, in addition to other host-derived cues, primes bacteria for intracellular survival by activation of the SPI-2 T3SS, a phenotype we have shown is important in vivo (11). Previous work has established that the SPI-2 T3SS is expressed in the lumen of the gut, prior to bacterial penetration of the intestinal mucosa (18). To verify that host cues alone were not sufficient to overcome the requirement for temperature induction, we incubated the RIVET reporter strain in homogenized murine gut tissue at 37 or 25 °C and measured sseA promoter activity by resolution of the chloramphenicol reporter after 30 min. Under these conditions, promoter activity at 25 °C remained at base-line levels, whereas promoter activity at 37 °C increased significantly (Fig. 5D), indicating that mammalian body temperature sensing is an essential signal that must accompany additive host cues to activate the intracellular virulence program.
DISCUSSION

Virulence factor expression in bacterial pathogens relies on collections of interconnected regulators to compute a transcriptional output based on a given set of inputs. Inputs are defined by the environmental context in which the organism finds itself. In the case of zoonotic pathogens with non-host lifestyles, the use of environmental cues to control the so-called transcriptional logic gates (28–30) of virulence genes allows for niche-specific transcription programs that would otherwise be superfluous if activated in the environment. In *S. enterica* sv. Typhimurium, one cue to activate the expression of intracellular virulence genes encoded in the SPI-2 pathogenicity island is the concerted presence of low magnesium, low phosphate, and mild acidification of the external medium (9, 31). However, we speculated that other environmental signals must also operate in this activation pathway because ion limitation cues and acidification would also be encountered in non-host environments that are incongruous with virulence gene expression. Therefore, none of the genes identified here as being thermoregulated are known to be required for bacterial growth in the environment, but rather they are involved exclusively in the pathogenic nature of the organism in mammalian hosts. We have identified mammalian body temperature as a necessary cue that activates intracellular virulence gene expression in *S. enterica* sv. Typhimurium, and we have determined that the nucleoid-like proteins H-NS and Hha are required for virulence gene silencing at nonpermissive temperatures below 30 °C.

Thermoregulation of the SPI-2 virulence system of *S. enterica* sv. Typhimurium was fully recapitulated in *S. bongori*, which lacks SPI-2, suggesting that the thermoregulators involved in this mechanism were common to both species. A comparison of the *S. enterica* sv. Typhimurium and *S. bongori* genome sequence revealed orthologues encoding the repressors *hha* (94% sequence identity), *ydgT* (91% sequence identity), and *hns* (97% sequence identity); however, *tlpA*, a gene whose protein product was described as a temperature-dependent regulator in *S. enterica* sv. Typhimurium (32) is absent in *S. bongori*. Accordingly, Hha and H-NS were shown to fully control thermoregulation of the intracellular virulence system in *S. enterica* sv. Typhimurium under various conditions. Even in the absence of temperature-dependent repressors, the response regulator SsrB remained essential for SPI-2 gene activation (10), unlike in an *Escherichia coli* system where inactivation of repressors renders the positive transcriptional activator dispensable for full gene expression (15). Nevertheless, it remains possible that in addition to its role as an activator, SsrB also antagonizes the repression mediated by nucleoid-like proteins such as H-NS (33), as H-NS has recently been shown to repress genes acquired by horizontal gene transfer because of their base skew (commonly lower GC content) from the recipient genome (24, 34).

Much work has been done on the mechanism of transcriptional repression by H-NS and the nature of repressive H-NS complexes. *In vivo*, H-NS forms dimers, tetramers, and higher order complexes (35). Assembly of high order repressive complexes is inhibited by truncation mutants that oligomerize with full-length H-NS but lack DNA binding activity (36), which we took advantage of in the design of our inducible dominant-negative H-NS experiments. These data clearly showed a temperature-blind phenotype for the thermoregulation of SsrB and downstream SPI-2 targets when the DNA binding activity of H-NS was attenuated with H-NS<sup>Q22am</sup>. It has been postulated that environmental changes (such as temperature) might affect the oligomerization state of H-NS and be used as points of transcriptional regulatory control (35). Genetic and biophysical studies have shown that reducing temperature below 25 °C leads to a reduction in the amount of tetrameric H-NS (37, 38). Because tetrameric H-NS is thought to be the active form of the protein (35), the thermal switch operation of H-NS at environmental temperatures may involve other proteins *in vivo*. We focused attention on proteins Hha and YdgT, which we have shown to be repressors for virulence gene expression in *Salmonella* (10, 11). Hha and YdgT form heteromeric complexes with H-NS (39, 40) and therefore were good candidates to participate in the thermal regulation of intracellular virulence genes described here. We found that Hha but not YdgT was a key thermoregulator in *Salmonella*. Deletion of *hha* in an *hns<sup>+</sup>* background produced a temperature-blind phenotype leading to transcriptional activation of intracellular virulence gene promoters at 25 °C that was similar to that activity for wild type cells at 37 °C. The involvement of Hha in thermal regulation of gene expression has been described previously only for hemolysis expression in *E. coli* (41), and thus our data support the idea that Hha, along with H-NS, participates in the broader thermoregulation of enterobacterial virulence factors involved in animal infections. Because the interaction between Hha and H-NS is restricted to the family Enterobacteriaceae (42), these data imply an evolutionary significance to the H-NS-Hha interaction in this family of pathogens that permits colonization of a specific host niche. A microarray study of temperature-controlled genes in *Salmonella* identified other virulence genes involved in bacterial invasion as H-NS-dependent (43). Although this previous study did not identify the intracellular virulence program described here as temperature-dependent, this is likely because of the culture conditions used, which in the microarray study were optimized for expression of invasion-related genes and not the SPI-2 virulence system required for intracellular infection. Together, these data support the logic-gate design (28–30) of virulence gene control, where the integration of multiple environmental cues activates a transcriptional output to control niche-specific gene programs, such as during different stages of infection. In the case of *Salmonella*, activation of genes required for intracellular survival and evasion of host immunity require integrated extracellular signals, including acidity, micromolar concentrations of magnesium and phosphate, and mammalian body temperature as additive inputs leading to the downstream activation of the requisite gene program. Understanding how environmental cues act on transcriptional regulatory nodes illuminates key aspects of evolution in microbial pathogens.

Acknowledgments—We thank Dr. Michael Hensel for providing the SPI-2 BAC used in this work and members of the Coombes laboratory for helpful reviews of the manuscript.
Thermoregulation of Salmonella Virulence

REFERENCES

1. World Health Organization (2005) Drug-resistant Salmonella, Report 139, pp. 1–4, Geneva, Switzerland

2. Hensel, M., Shea, J. E., Waterman, S. R., Mundy, R., Nikolaus, T., Banks, G., Vasquez-Torres, A., Gleeson, C., Fang, F. C., and Holden, D. W. (1998) Mol. Microbiol. 30, 163–174

3. Coombes, B. K., Lowden, M. J., Bishop, J. L., Wickham, M. E., Brown, N. F., Duong, N., Osborne, S., Gal-Mor, O., and Finlay, B. B. (2007) Infect. Immun. 75, 574–580

4. Coombes, B. K., Brown, N. F., Valdez, Y., Brumell, J. H., and Finlay, B. B. (2005) Science 310, 49804–49815

5. Hensel, M., Shea, J. E., Waterman, S. R., Mundy, R., Nikolaus, T., Banks, G., Vasquez-Torres, A., Gleeson, C., Fang, F. C., and Holden, D. W. (1998) Mol. Microbiol. 30, 163–174

6. Shea, J. E., Hensel, M., Gleeson, C., and Holden, D. W. (1995) Proc. Natl. Acad. Sci. U. S. A. 93, 7062–7065

7. Garmendia, J., Beuzon, C. R., Ruiz-Albert, J., and Holden, D. W. (2003) Mol. Microbiol. 47, 163–174

8. Worley, M. J., Ching, K. H., and Heffron, F. (2000) J. Biol. Chem. 275, 458–465

9. Coombes, B. K., Lowden, M. J., Bishop, J. L., Wickham, M. E., Brown, N. F., and Finlay, B. B. (2005) Proc. Natl. Acad. Sci. U. S. A. 102, 14740–14745

10. Deiwick, J., and Hensel, M. (1999) Electrophoresis 20, 813–817

11. Coombes, B. K., Brown, N. F., Valdez, Y., Brumell, J. H., and Finlay, B. B. (2005) Proc. Natl. Acad. Sci. U. S. A. 102, 14740–14745

12. Deiwick, J., Nikolaus, T., Erdogan, S., and Hensel, M. (1999) Mol. Microbiol. 30, 1–6

13. Hurme, R., and Hinton, J. C. (2006) Mol. Microbiol. 61, 941–953

14. Hermsen, R., Tans, S., and ten Wolde, P. R. (2006) Plos Comput. Biol. 2, e164

15. Deiwick, J., Nikolaus, T., Erdogan, S., and Hensel, M. (1999) Mol. Microbiol. 31, 1759–1773

16. Hurme, R., Berndt, K. D., Normark, S. J., and Rhen, M. (1997) Cell 90, 55–64

17. Navarre, W. W., Porwollik, S., Wang, Y., McCelland, M., Rosen, H., Libby, S. J., and Fang, F. C. (2006) Science 313, 236–238

18. Ueguchi, C., Suzuki, T., Yoshida, T., Tanaka, K., and Mizuno, T. (1996) J. Mol. Biol. 263, 149–162

19. Morgan, E., Campbell, J. D., Rowe, S. C., Bispham, J., Stevens, M. P., Bowen, A. J., Barrow, P. A., Maskell, D. J., and Wallis, T. S. (2004) Mol. Microbiol. 54, 994–1010

20. Coombes, B. K., Coburn, B. A., Potter, A. A., Gomis, S., Mirakhur, K., Li, Y., and Finlay, B. B. (2005) Infect. Immun. 73, 7161–7169

21. Coombes, B. K., Coburn, B. A., Potter, A. A., Gomis, S., Mirakhur, K., Li, Y., and Finlay, B. B. (2005) Infect. Immun. 73, 7161–7169