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Suzanne E. Osborne
McMaster University

Don Walthers
University of Illinois

Ana M. Tomljenovic
McMaster University

David T. Mulder
McMaster University

Uma Silphaduang
McMaster University

See next page for additional authors

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Osborne, Suzanne E.; Walthers, Don; Tomljenovic, Ana M.; Mulder, David T.; Silphaduang, Uma; Duong, Nancy; and Lowden, Michael J., "Pathogenic Adaptation of Intracellular Bacteria by Rewiring a Cis-Regulatory Input Function" (2009). *Open Dartmouth: Faculty Open Access Articles*. 1505.
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Authors
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Pathogenic adaptation of intracellular bacteria by rewiring a *cis*-regulatory input function

Suzanne E. Osborne[a], Don Walthers[b], Ana M. Tomljenovic[a], David T. Mulder[a], Uma Silphaduang[a], Nancy Duong[a], Michael J. Lowden[c], Mark E. Wickham[d], Ross F. Waller[e], Linda J. Kenney[b], and Brian K. Coombes[a,f,1]

[a]The Michael G. DeGroote Institute for Infectious Disease Research, Department of Biochemistry and Biomedical Sciences, McMaster University, Hamilton, ON, Canada L8N 3Z5; [b]Department of Microbiology and Immunology, University of Illinois, Chicago, IL 60612; [c]Department of Chemistry, Dartmouth College, Hanover, NH 03755; [d]Phillips Ormonde & Fitzpatrick, 367 Collins Street, Melbourne, Victoria, 3000, Australia; [e]School of Botany, University of Melbourne, Melbourne, Victoria, 3010, Australia; and [f]Public Health Agency of Canada, Laboratory for Foodborne Zoonoses, Guelph, ON, Canada N1G 3W4

Edited by Roy Curtiss III, Arizona State University, Tempe, AZ, and approved January 8, 2009 (received for review November 17, 2008)

The acquisition of DNA by horizontal gene transfer enables bacteria to adapt to previously unexploited ecological niches. Although horizontal gene transfer and mutation of protein-coding sequences are well-recognized forms of pathogen evolution, the evolutionary significance of *cis*-regulatory mutations in creating phenotypic diversity through altered transcriptional outputs is not known. We show the significance of regulatory mutation for pathogen evolution by mapping and then rewiring a *cis*-regulatory module controlling a gene required for murine typhoid. Acquisition of a binding site for the *Salmonella* pathogenicity island-2 regulator, SsrB, enabled the *srfN* gene, ancestral to the *Salmonella* genus, to play a role in pathoadaptation of *S. typhimurium* to a host animal. We identified the evolved *cis*-regulatory module and quantified the fitness gain that this regulatory output accrues for the bacterium using competitive infections of host animals. Our findings highlight a mechanism of pathogen evolution involving regulatory mutation that is selected because of the fitness advantage the new regulatory output provides the incipient clones.

bacterial pathogenesis | *cis*-regulatory mutation | evo-devo | pathoadaptation | regulatory evolution

A common source of genetic diversity among bacterial pathogens is horizontal acquisition of mobile genetic elements, which can harbor virulence genes required during various stages of host infection (1). In the *Salmonella enterica* species, two major genetic acquisitions were the *Salmonella* pathogenicity islands-1 and -2, each coding for a type III secretion system (T3SS) that translocates protein effectors into host cells during infection. The SPI-1 T3SS is common to the genus *Salmonella* and injects effectors for invasion of non-phagocytic cells. Serovars within the *S. enterica* species additionally possess SPI-2, which is absent from *S. bongori*, the other recognized species of *Salmonella*. The SPI-2 T3SS injects proteins required for intracellular survival and infection of mammals (2, 3) and represented a second phase in the evolution of *Salmonella* virulence. Of particular interest is a horizontally acquired 2-component regulatory system, SsrA-SsrB, that facilitated niche expansion from the environment into mammalian hosts because of regulation of the coinherited T3SS in SPI-2. This regulatory system includes a sensor kinase, SsrA, and response regulator, SsrB, that integrates expression of the SPI-2 T3SS with other horizontally acquired effectors by binding to *cis*-regulatory elements upstream of promoters within and outside of SPI-2 (4, 5).

Virulence is a quantitative trait resulting from the interaction between bacterial and host gene products (6). Accordingly, the intracellular virulence phenotype is regulated by multiple transcription factors that integrate signals from the bacterial cell surface to coordinate expression of niche-specific genes (7). Modularity of virulence gene-promoter architecture is evident in SPI-2, where combinatorial input from PhoP (8), OmpR (9, 10), and SlyA (4, 11) on some promoters is required to launch an integrated virulence program in the host. Combinatorial control of virulence gene regulation by modular regulatory units provides plasticity in the amplitude of transcriptional outputs depending on the environment, for example in response to a more resistant host genotype (12) or in response to metabolic demands, as shown in the Boolean gate-logic architecture of the *lac* operon in *Escherichia coli* (13).

Evolution of regulatory DNA, or *cis*-regulatory elements (CRE), has been invoked to explain the bulk of higher organismal diversity in the context of developmental evolution ("evo-devo") (14–16) with an expanding volume of examples. However, little is known about the evolutionary significance of *cis*-regulatory mutations that may underlie bacterial pathogenesis and adaptation to various ecological niches. In the present study, we validate this evolutionary principle in a prokaryotic system by establishing that mutation of the *cis*-regulatory input for an ancestral bacterial gene (*srfN*) contributes to pathoadaptive fitness during enteric and typhoid disease in animals. Our results demonstrate that *cis*-regulatory mutation between closely related species contributes to pathoadaptive trait gain with functional consequences for the host-pathogen interaction.

Results

Identification and Regulation of *srfN*. Given the importance in virulence of the SsrB-response regulator encoded in the SPI-2 pathogenicity island, we sought to identify other genes in the SsrB regulon for their role in pathogenesis. Analysis of SsrB coregulated genes using transcriptional arrays (for methods, see ref. 17) identified a gene whose expression under SPI-2-inducing conditions was strongly SsrB-dependent. STM0082 mRNA was reduced ≈8-fold in Δ*ssrB* cells compared to wild type, which was similar in magnitude to that of SsrB-regulated T3SS effectors *sseF* (8.6-fold) and *sspH2* (7.1-fold). STM0082 is found in pathogenic enterobacteriaceae and other γ-proteobacteria, including *S. bongori*, *S. enterica*, *Yersiniae*, and the human and coral pathogen, *Serratia marcescens*, and is part of a larger family of uncharacterized bacterial "y" genes (pfam07338) (18) (Fig. S1). The gene synteny around STM0082 is identical between *S. typhimurium* and *S. bongori*, suggesting it was ancestral to the *Salmonella* genus before the divergence of lineages giving rise to *S. bongori* and *S. enterica*, which would predate the acquisition of SsrB by the *S. enterica* lineage. We named STM0082 *srfN* (SsrB-regulated factor N) and determined that SrfN localizes to the inner bacterial membrane and is not secreted or translocated by the coexpressed type III secretion system (Fig. S2).

SsrB Controls *srfN* Directly. *S. typhimurium* and *S. bongori* both contain *srfN*, yet the expression of this gene in *S. typhimurium* is

Author contributions: S.E.O., D.W., U.S., L.J.K., and B.K.C. designed research; S.E.O., D.W., A.M.T., U.S., N.D., M.J.L., R.F.W., and B.K.C. performed research; R.F.W. contributed new reagents/analytic tools; S.E.O., D.W., D.T.M., R.F.W., L.J.K., and B.K.C. analyzed data; and S.E.O., D.W., M.E.W., R.F.W., L.J.K., and B.K.C. wrote the paper.

The authors declare no conflict of interest.

This article is a PNAS Direct Submission.

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[1]To whom correspondence should be addressed. E-mail: coombes@mcmaster.ca.

This article contains supporting information online at www.pnas.org/cgi/content/full/0811669106/DCSupplemental.

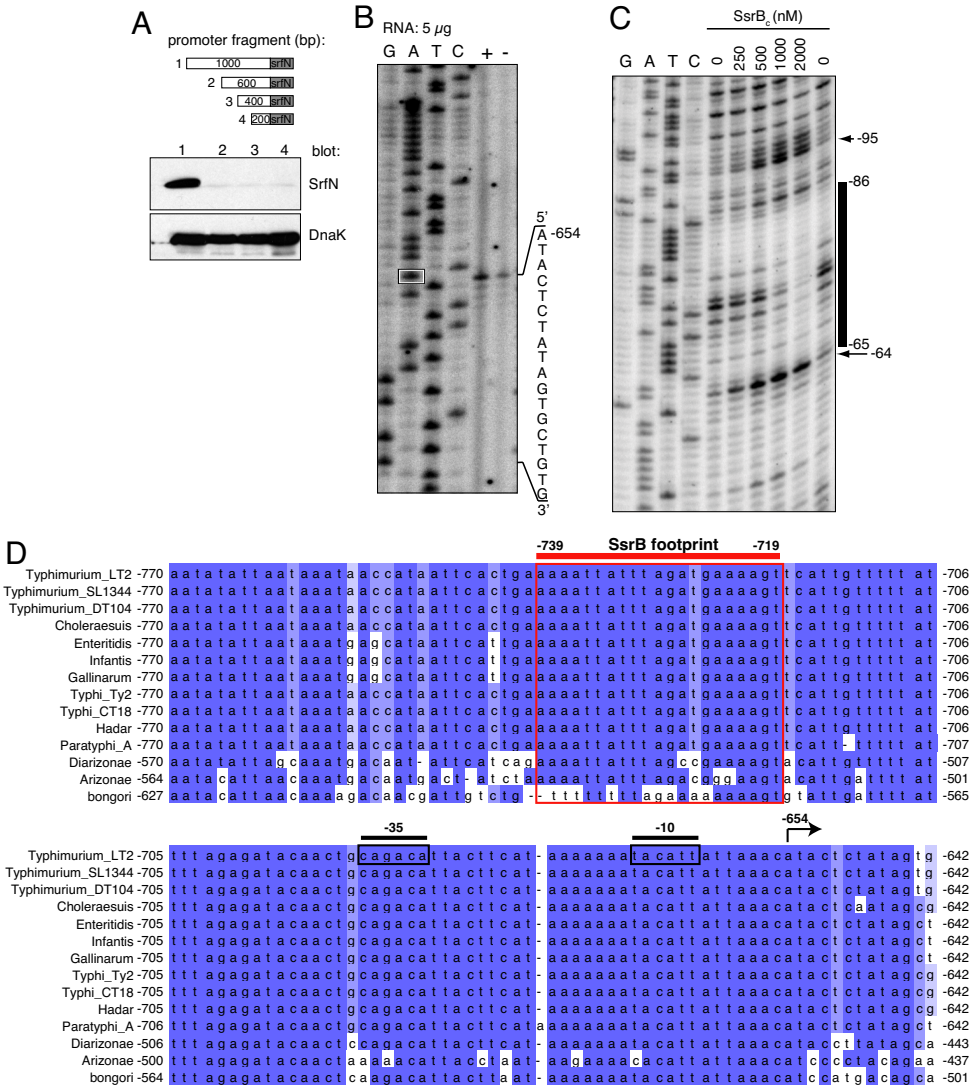

Fig. 1. Mapping the *cis*-regulatory input for *srfN*. (*A*) Production of SrfN requires regulatory DNA between −600 and −1,000 base pairs from the translation start site. Different lengths of DNA upstream of *srfN* were tested for their ability to promote *srfN* expression. Data are Western blots of SrfN and DnaK from whole-cell lysates following growth of cells in inducing medium. (*B*) Primer extension reveals an SsrB-specific transcription start site −654 nt from the *srfN* translation start site. Boxed adenine indicates the start of the transcript. From left to right, the lanes in each panel contain G, A, T, or C sequencing ladders and reactions performed on RNA isolated from an *hns*/*rpoS* background (+) or an isogenic *ssrB* mutant (–). (*C*) SsrBc binds to *srfN* promoter DNA. The purified C-terminal DNA binding domain of SsrB (SsrBc) protects from DNaseI a 20-bp site between −86 and −65 from the transcription start site. Black bar and coordinates indicate location of SsrBc binding site relative to transcriptional start site. Arrows indicate DNaseI hypersensitive sites. (*D*) Alignment of the *srfN cis*-regulatory region from *S. enterica* serovars and *S. bongori.* Numbers to the left and right of the alignment correspond to base-pair distances from the *srfN* translational start site from the respective species. The region of SsrB binding is indicated with a solid red line and the probable −35 and −10 hexamers are shown.

driven by SsrB. The absence in *S. bongori* of SPI-2, and thus the SsrA-SsrB 2-component regulatory system, suggested adaptive evolution of the *srfN cis*-regulatory element in *S. typhimurium,* allowing SsrB to expropriate control of *srfN* during intracellular infection. Deletion analysis of the *srfN cis*-regulatory region identified a putative CRE between 600 and 1,000 base pairs upstream of the translational start site that was absolutely required for *srfN* expression (Fig. 1*A*). We used primer extension to map the location of the *srfN* promoter and transcription start site in *S. typhimurium*. RNA isolated from an *hns* mutant (5) and an isogenic *ssrB* mutant revealed a single *srfN*-specific transcript ending 654 nucleotides upstream of the translation start site (Fig. 1*B*). *srfN* mRNA was significantly reduced in the absence of SsrB, consistent with protein levels. To determine whether gene expression by SsrB was a consequence of direct binding to the *srfN* regulatory region, we used purified SsrBc in footprinting protection experiments (4). SsrBc protected ≈20 base pairs of DNA flanked by multiple DNase I hypersensitive sites between 86 and 65 base pairs upstream of the transcription start site (Fig. 1*C*), consistent with a direct role of SsrB in transcriptional activation of *srfN*. The location of protection validates the long 5′ untranslated leader sequence identified by primer extension and the extent of protection is consistent with binding by a single SsrB dimer (46), and is similar to that of another response regulator family member, NarL (19). We further verified SsrB binding to this DNA region in vivo using ChIP followed by PCR (Fig. 2*A*) and microarray analysis (ChIP-on-chip), with a strain carrying a chromosomal *ssrB*-FLAG allele. Thirty-two ChIP-chip probes located near *srfN*, verified that SsrB was loaded onto intergenic chromosomal DNA at the precise location mapped by SsrB footprinting, but not at sites flanking this region (Fig. 2*B*), or at another chromosomal location (>5,000 bp in length) not regulated by SsrB (Fig. 2*C*). Sequence alignment of the *srfN* regulatory

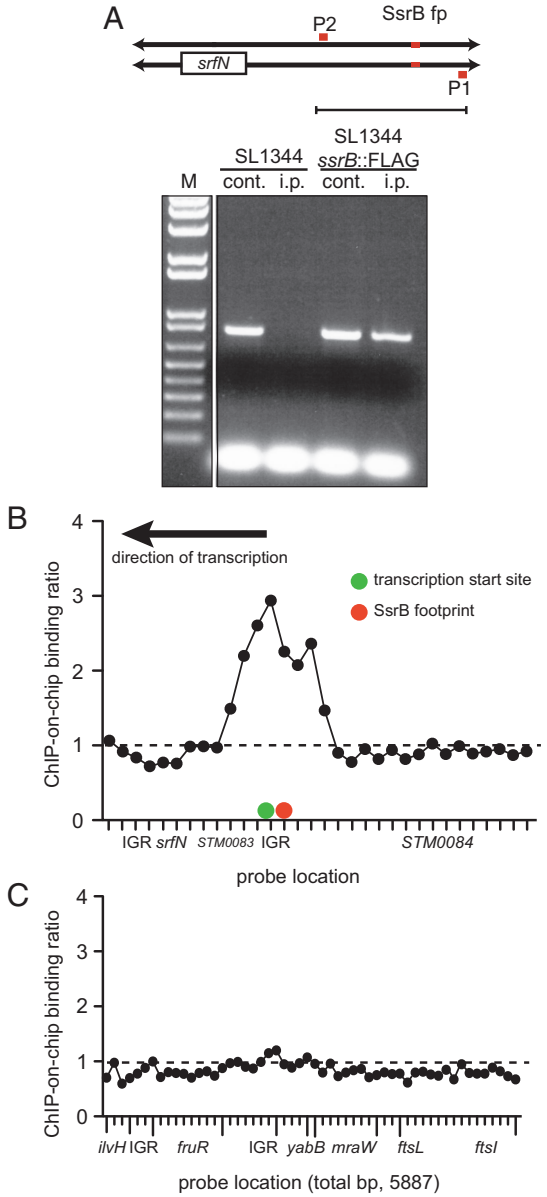

Fig. 2. SsrB binds to the *srfN cis*-regulatory element in vivo. (*A*) Location of ChIP-PCR primers and experimentally mapped SsrB binding site (*red*) with respect to *srfN* ORF. Bacterial cells were grown in SsrB-inducing LPM medium and sonicated DNA fragments bound to SsrB-FLAG in vivo were isolated by ChIP (i.p.), along with similarly processed DNA fragments from wild-type cells containing untagged-SsrB. The ability of SsrB-FLAG to selectively bind to the *srfN cis* regulatory element was determined by PCR, along with samples of pre-IP DNA (cont.). (*B*) ChIP-on-chip analysis of the genomic region surrounding *srfN*. SsrB binding to 32 syntenic probes located near *srfN* plotted against probe location. Each data point is a unique probe with averaged data from 3 biological replicates. Direction of transcription is shown. The location of the SsrB footprint (*red circle*) and the transcription start site (*green circle*) with respect to probe location is shown. IGR, intergenic region. (*C*) SsrB binding profile for a chromosomal region not regulated by SsrB. The genomic region (5,887 bp) is represented by 54 unique probes. Each data point represents averaged data from 3 biological replicates.

region from serovars of *S. enterica* and *S. bongori* for which sequence data were available revealed a DNA signature unique to 11 of 13 serovars of *S. enterica* that mapped to the chromosomal location protected by SsrB. This region was divergent in *S. bongori*, with only 11 out of 21 bases conserved with the *S. enterica* species (Fig. 1*D*). Interestingly, one *S. enterica* serovar that showed diver-

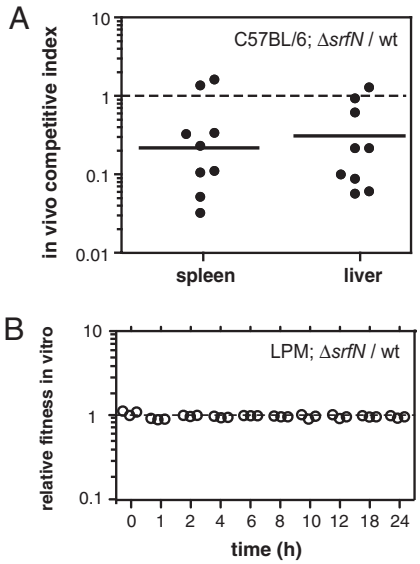

Fig. 3. SrfN increases in-host fitness. (*A*) Δ*srfN* and wild-type cells were competed in mixed infections of mice for 3 days. Competitive indices (CI) for the mutant were determined in the spleen and liver following oral infection. Each data point represents 1 animal and horizontal bars indicate geometric means. Data are from 2 experiments performed independently. $P = 0.038$ (spleen), 0.006 (liver). (*B*) Relative in vitro fitness of Δ*srfN* was assessed by growth competitions with wild-type cells in LPM. Relative fitness of the mutant was determined and plotted over time, where each data point represents an independent competitive culture.

gence in the SsrB footprint region (Diarizonae) belongs to the IIIb subspecies that are symbionts of reptiles but rarely associated with human infection. Although subspecies IIIb contains SsrB, it forms a distinct phylogenetic branch that diverged from the class I subspecies (responsible for 99% of mammalian infections) between 20 and 25 Myr ago (20). We performed analyses of relative genetic distances of the *srfN cis*-regulatory region, the entire upstream DNA sequence, and the *srfN* ORF between *S. enterica* serovars and *S. bongori*. In neighbor joining phylogenies of all regions (Fig. S3), *S. enterica* subspecies I (Enterica) forms a distinct clade from subspecies IIIb (Diarizonae) and *S. bongori*, as is also seen for housekeeping genes and invasion genes (21). The region adapted to SsrB regulation (from the SsrB footprint to the start of transcription) shows less overall divergence within subspecies class I compared to the rest of the 5′UTR, or even the *srfN* ORF (see Fig. S3), consistent with selection imposed on this regulatory region. These data identified a conserved CRE involved in regulatory evolution of *srfN* in *Salmonella* subspecies infecting warm-blooded mammals.

SrfN Is a Fitness Factor During Systemic Typhoid. The identification of a gene in *S. typhimurium* appropriated by a horizontally acquired response regulator required for infection of mammals led us to hypothesize that SrfN contributes to within-host fitness. To test this, we constructed a nonpolar, in-frame deletion of *srfN* in *S. typhimurium* and tested the fitness of the mutant in competitive infections with the isogenic parent strain. Mice were infected orally and competitive indices (17) were calculated for the cecum, liver, and spleen 3 days after infection of C57BL/6 mice. After a single round of infection, the geometric mean competitive index for the Δ*srfN* strain in the liver and spleen was 0.227 [95% confidence interval (CI) 0.081–0.639] and 0.296 (95% CI 0.091–0.562), respectively (Fig. 3*A*), showing that bacteria lacking SrfN were significantly out-competed by wild-type bacteria during systemic infection. Mutant cells were also out-competed by wild-type cells in the cecum during enteric stages of colonization with a similar magnitude of attenuation (data not shown). The *srfN* mutant was not defective for

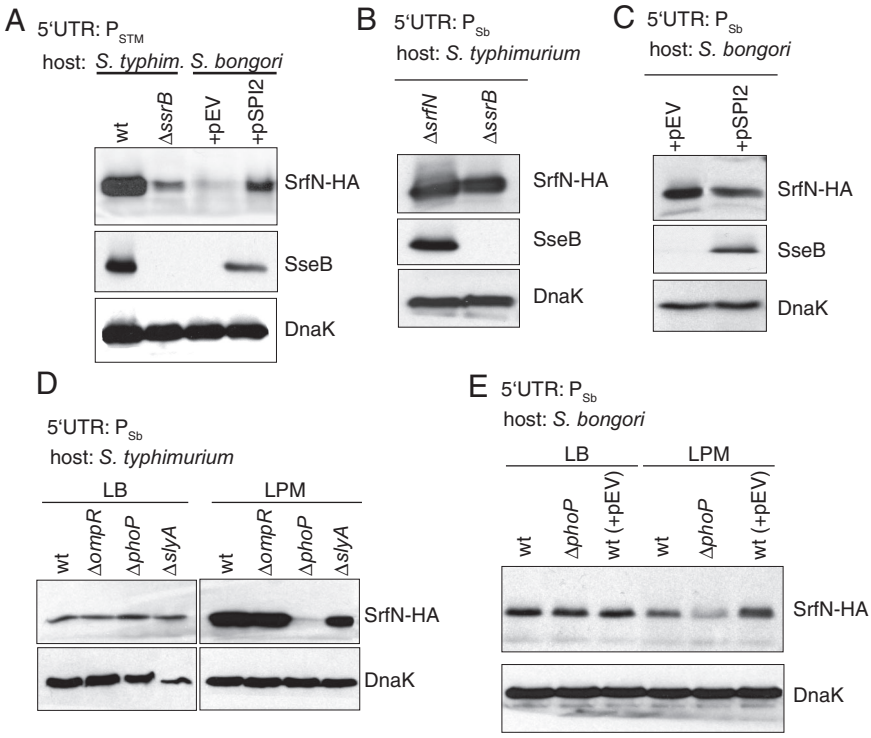

Fig. 4. The *cis*-regulatory input for *srfN* requires SsrB. Promoter swap constructs were made that express *srfN* from the *cis*-regulatory input evolved in *S. typhimurium* (P_{STM}) or *S. bongori* (P_{Sb}). Constructs were transformed into *S. typhimurium* and *S. bongori* along with a bacterial artificial chromosome (BAC) containing the SPI-2 pathogenicity island (pSPI2) or an empty BAC (pEV). The ability of *S. typhimurium* and *S. bongori* to express *srfN* from (*A*) the *S. enterica* promoter (P_{STM}) or (*B* and *C*) the regulatory input evolved in *S. bongori* is shown by Western blot. P_{Sb} is recognized as a promoter in *S. typhimurium* leading to *srfN* expression, but in an SsrB-independent manner similar to that seen in *S. bongori*. Lysates were probed using antibodies against HA to detect expression of the SrfN fusion protein, and antibodies to SseB (SsrB-dependent) and DnaK (SsrB-independent) as controls. (*D* and *E*) The regulatory input driving *srfN* expression in *S. bongori* requires the PhoP response regulator. Transcription factor mutants in *S. typhimurium* and *S. bongori* were tested for their ability to recognize the *srfN* promoter from *S. bongori*, shown in immunoblots for SrfN-HA, following growth in rich (LB) or minimal medium (LPM).

competitive (Fig. 3*B*) or noncompetitive growth (Fig. S4) in vitro in minimal or rich media, indicating that the phenotype of the *srfN* mutant was specific to within animal hosts.

Promoter Swapping Experiments with *S. bongori*. The regulatory data for *srfN* indicated an evolved dependence on the horizontally acquired virulence regulator SsrB. We first verifed that *srfN* was under SsrB control by cloning the regulatory region upstream of *srfN* in front of a *srfN-HA* allele and inducing expression in wild-type *Salmonella* and Δ*ssrB* cells. SrfN-HA protein was abundant in wild-type cells but highly reduced in the *ssrB* mutant (Fig. 4*A*). Next, we cloned *srfN* and its 5′UTR from *S. typhimurium* into a low-copy plasmid and transformed this construct into *S. bongori* (which evolved distinctly from *S. typhimurium* in the absence of SsrB). We asked whether *S. bongori* could recognize this *typhimurium*-adapted promoter for gene expression, which it could not. Although SrfN-HA was expressed in *S. typhimurium*, the protein was barely detectable in wild-type *S. bongori* (see Fig. 4*A*), unless the pathogenicity island containing SsrAB was also cotransferred on a bacterial artificial chromosome (pSPI2). Introducing SsrAB/SPI-2 into *S. bongori* resulted in an immediate accumulation of SrfN protein (see Fig. 4*A*), as well as the type III secretion-needle component, SseB, whose gene is expressed in an SsrB-dependent manner in SPI-2. This result raised the question whether the ancestral transcriptional machinery required for expressing *srfN* was present in *S. typhimurium*. To examine this, we first mapped the transcriptional start site for the *srfN* orthologue in *S. bongori*, which showed a transcriptional start site at position –115 bp relative to the start of translation and near-consensus –10 and –35 hexamer regions (Fig. S5). Next, we precisely swapped the *S. bongori* 5′UTR regulatory sequence plus 680 bp of upstream DNA (to include the divergent regulatory region) into *S. typhimurium* and asked whether this regulatory input could drive *srfN* expression. Expression of *srfN* from the *S. bongori* 5′UTR was SsrB-independent, as indicated by similar protein levels in *ssrB* null bacteria and wild-type cells (Fig. 4*B*) and in the absence and presence of SPI-2 in *S. bongori* (Fig. 4*C*). These data suggested a conservation of the ancestral transcription factors governing control of *srfN*, prompting us to investigate the regulation of the *srfN* orthologue in *S. bongori*. We deleted transcriptional regulators in *S. typhimurium* that are common to both *S. typhimurium* and *S. bongori* and tested whether these strains could express *srfN* from the *S. bongori* 5′UTR. These experiments revealed the requirement for PhoP input on the *S. bongori* CRE (Fig. 4*D*), which we verified by deleting *phoP* in *S. bongori* and testing expression in low-magnesium minimal medium (Fig. 4*E*). That the *srfN* CRE from *S. typhimurium* is not well recognized in PhoP-containing *S. bongori* cells in the absence of SsrB suggests a contemporaneous loss of direct input from the ancestral PhoP response regulator. However, a direct or indirect role for PhoP cannot be ruled out, because *ssrB* is epistatic to *phoP* in *S. typhimurium* (8). Together with the phylogenetic analysis, these data suggest that the 5′UTR controlling *srfN* in *S. typhimurium* evolved under selection because of the beneficial fitness this new regulatory input afforded incipient clones within the host.

Pathogenic Adaptation via *cis*-Regulatory Mutation. To test the evolutionary significance of *cis*-regulatory mutation-driving bacterial pathogenesis, we complemented the *S. typhimurium srfN* mutant with a wild-type copy of *srfN* driven by the promoter evolved in either *S. bongori* or *S. typhimurium* and competed the complemented strains against wild-type *S. typhimurium* cells in mixed infections of mice. Complementation of Δ*srfN* with *srfN* expressed from the promoter evolved in *S. typhimurium* (P_{St}-5′UTR) restored in-host fitness of the mutant to wild-type levels, as evidenced by a competitive index of 0.98 (95% CI 0.55–1.79) (Fig. 5*A*). In mouse experiments designed to test whether rewiring *srfN* to the 5′UTR evolved in *S. bongori* (P_{Sb}-5′UTR) could restore in-host fitness to this strain, we determined that it could not. These cells were still out-competed by wild-type cells in mixed infections in animals with a relative fitness of 0.19 (95% CI 0.10–0.26) (see Fig. 5*A*), which was indistinguishable from the Δ*srfN* strain (0.22, 95% CI 0.08–0.63) (see Fig. 3*A*). This was despite the fact that *srfN* was expressed in a PhoP-dependent manner in this strain. Finally, to verify the precise requirement of the mapped SsrB CRE for in vivo fitness, we deleted the chromosomal SsrB CRE identified by footprinting and replaced it with the analogous region from *S. bongori* defined in the

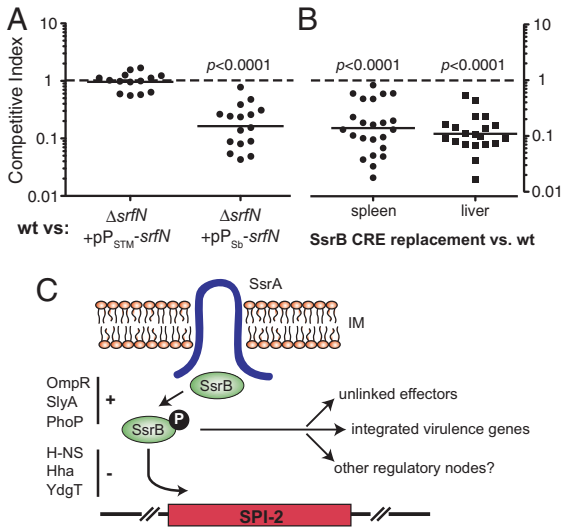

Fig. 5. The in-host fitness benefit associated with SrfN is regulatory. Effects on in-host fitness were tested following, (*A*) trans complementation of Δ*srfN* with *srfN* driven by either the *S. typhimurium* 5′UTR or *S. bongori* 5′UTR, or (*B*) in competitive infection experiments in vivo against a strain where the chromosomal SsrB CRE was replaced with the analogous region from *S. bongori* (SsrB CRE replacement). Competitive indices were determined 3 days after infection in the spleen and liver. Data points represent individual animals from at least 3 experiments and horizontal lines are geometric means. (*C*) Simplified regulatory diagram showing that phosphorylated SsrB acts as a transcription factor on genes within SPI-2, along with input from additional positive (OmpR, SlyA, PhoP) and negative (H-NS, Hha, YdgT) regulators. Activated SsrB also influences phenotypic variation by acting on unlinked regulatory DNA driving expression of T3SS effector genes, unlinked integrated virulence genes (such as *srfN*), and possibly other regulatory nodes.

alignment from Fig. 1*D*. This strain (SsrB CRE replacement) was out-competed by wild-type cells in mouse infections similar to that seen with the rewired trans-complementation constructs (CI_{spleen} 0.16, 95% CI 0.09–0.23; CI_{liver} 0.13, 95%CI 0.08–0.17) (Fig. 5*B*). Thus, a single adaptive *cis*-regulatory mutation enhances within-host fitness, emphasizing a unique source of regulatory evolution in bacterial pathogenesis.

Discussion

Genotypic variation between *S. enterica* and *S. bongori* includes the presence in *S. enterica* of a large, horizontally acquired pathogenicity island, SPI-2. This genetic island provided the incumbent species with the machinery to access a new ecological niche in warm-blooded hosts and to replicate within immune cells. The acquisition of SPI-2 also introduced a new two-component regulatory system to the *Salmonella* genome, SsrA-SsrB, which ostensibly contributed to immediate and gradual phenotypic variation among SPI-2-containing subspecies (Fig. 5*C*). Evidence exists to suggest that SPI-2 was acquired by *S. enterica* following the branching of *S. enterica* and *S. bongori*. Arguments against the presence of SPI-2 in the last common ancestor and eventual loss in *S. bongori* include the inability to find any remnants of SPI-2 in extant *S. bongori* strains. However, in the absence of a third distinct species on which to adjudicate, maximum parsimony arguments could account for either scenario.

Other forces, apart from unique gene content, contribute to phenotypic variation among closely related species, such as stochastic differences in gene regulation (22) and evolution of enhancer sequences (23) and of orthologous regulators (24). In bacterial pathogens, the regulatory interactions orchestrating virulence and fitness genes collectively shape the ecology of the host-pathogen interaction. The requirement of *srfN* for intrahost fitness highlights the selective advantage achieved by fine-tuning gene expression by regulatory evolution and suggests that SrfN may have evolved unique functions in *S. enterica* for use within the animal host. That *srfN* (STM0082) was found to be required for long-term chronic infection of mice supports this view (25). Although the exact function of SrfN is not yet known, we have excluded function in motility, resistance to reactive oxygen or serum, and in chemical-genetic interactions with 60 known-bioactive molecules. The unusually long 5′UTR that is generated by regulatory input from SsrB may be maintained because of the presence of a small predicted ORF on the opposite DNA strand between the mapped SsrB CRE and *srfN*. In addition, the length of the 5′UTR may imply additional functionality of this region, such as posttranscriptional regulation, although this has yet to be studied experimentally.

The evolution of bacterial pathogenesis has been considered in two complementary forms. "Quantum leaps" (26), with the acquisition of genomic islands, provide genes *en masse* that could contribute immediately to niche expansion and to overcoming host restriction. The acquisition of the multigene-pathogenicity islands SPI-1 and SPI-2 represents this model of evolution, providing the incumbent clones with unique cellular machinery to interface with their host environment. Another mechanism of evolution involves gene loss or gene modification to remove (or alter) genes whose products influence fitness in a given environment (27, 28). The loss of the lysine decarboxylase gene, *cadA*, from multiple strains of *Shigella* and enteroinvasive *E. coli* is an example of this type of structural mutation that prevents host cadaverine-mediated attenuation of virulence following decarboxylation of lysine (29). Yet structural mutations can give rise to antagonistic pleiotropy and functional trade-offs as organisms move from one niche to another, where selective pressures are different and the lost or modified gene product may be needed to greater or lesser extents. Instead, evolution by mutation of *cis*-regulatory input functions with concomitant changes in gene expression is well suited to quantitative trait loci involved in pathogenic adaptation. This type of adaptive divergence is well studied in eukaryotic evolutionary biology, where it has been shown to reduce pleiotropy, minimize functional trade-offs, and resolve adaptive conflicts (30, 31). Although the relative contributions of *cis*-regulatory mutations versus structural mutations driving quantitative traits have been debated (32, 33), empirical evidence supports the view that both regulatory (30, 32, 34–36) and structural mutations (37, 38) can shape adaptive evolution in higher organisms. Our work highlights how evolution of gene regulation via *cis*-regulatory mutation influences pathogenic adaptation with functional consequences for the host-pathogen interaction. Especially for zoonotic pathogens that are commensal, mutualist or pathogenic associates depending on the host, regulatory evolution offers a mechanism to resolve adaptive conflicts that arise from having alleles under negative selection in one environment but positive selection in others.

Further studies to identify variation between closely related bacterial species in the *cis*-regulatory region of homologous genes will provide clues to the selective forces driving pathogen evolution in the host environment, a topic for which very little empirical data exists (39). Understanding how horizontally acquired transcription factors influence promoter evolution and plasticity of regulatory circuits controlling bacterial virulence and fitness is in the formative stage, and the work herein exemplifies a seminal case. Our efforts here reveal a connection between virulence gene regulators and coregulation of ancillary factors critical for pathoadaptation. Rewiring regulatory DNA to commandeer virulence-associated transcription factors is a mechanism of regulatory evolution (34, 40) to be considered to fully understand the evolutionary potential of bacterial pathogens.

Materials and Methods

Bacterial Strains and Growth Conditions. *Salmonella enterica* serovar Typhimurium (abbreviated *S. typhimurium* here) was strain SL1344 and mutants were isogenic derivatives. *S. bongori* (serovar 66:z) was purchased from the *Salmonella* Genetic Stock Centre, and *S. bongori* SARC12 containing a bacterial artificial chromosome encoding the SPI-2 genomic island has been described elsewhere (41, 42). An acidic minimal medium low in phosphate and magnesium (LPM) used for the induction of SPI-2 has been described previously (43). SL1344 *ushA*::*cat* was used for competitive infections of animals and has been described elsewhere (17).

Cloning and Mutant Construction. Detailed information on cloning and mutant construction is found in *SI Text*.

Type III Secretion Assays. Assays for type III secretion were conducted as described previously (43), with details in *SI Text*.

Promoter Mapping and Footprinting. The *srfN* transcription start site was identified by primer extension performed as described previously (5) using the *srfN*-specific primer CTG TTA CTG ATA GTG TTT CTT TCG. The *srfN* regulatory region was amplified by PCR with primers CGC CGG ATA ACA TAC CGC CT and GAA TGA AGC AAC CGT TGC C and cloned into pCR2.1 (Invitrogen). The resulting plasmid, pDW106, was used as a template for generation of DNA sequencing ladders. To map the SsrB input module on the *srfN* promoter, DNase I protection footprinting was performed as described previously using the purified DNA binding domain of SsrB protein (SsrBc) (5). Primers CTG TTA CTG ATA GTG TTT CTT TCG and CAT TTA TTA CTG TAC GAG GAA GC, and plasmid pDW106 were used to generate footprinting templates and DNA sequencing ladders. Footprinting reactions on similarly constructed templates from sequence more proximal to the *srfN* coding sequence did not reveal SsrBc binding sites.

Competitive Infection of Animals. Animal protocols were approved by the Animal Ethics Committee, McMaster University. Female C57BL/6 mice (Charles River) were used throughout and infected orally with $\approx 10^6$ cfu of *S. typhimurium* in 0.1 M Hepes (pH 8.0), 0.9% NaCl. For competitive infections, mice were infected orally with a mixed inoculum containing wild-type *S. typhimurium* and an unmarked mutant strain under investigation. At 72 h after infection, bacterial load in the cecum, spleen, and liver were enumerated from organ homogenates. Detailed methods for enumeration of bacteria in vivo are found in *SI Text*. The competitive index (CI) was calculated on log-transformed cfu as: $(\text{mutant/wild type})_{\text{output}}/(\text{mutant/wild type})_{\text{input}}$. Log-transformed competitive infection data were analyzed using a one-sample *t* test.

Genetic Analyses. Detailed references to genome sequences and genomic analyses are found in *SI Text*. Nucleotide alignments of the *srfN* coding sequence and the upstream noncoding sequence were generated with ClustalX. With the exclusion of indels, phylogenetic distance analyses were performed on the coding sequence (291 nucleotides), upstream 770 nucleotides, and the 92-nucleotide *cis*-regulatory region (LT2 nucleotides −741 to −651). Distances were calculated by TREE-PUZZLE 5.2 (44) using the Hasegawa model of substitution and a uniform rate of heterogeneity. Bootstraps were calculated using puzzleboot (shell script by A. Roger and M. Holder) with 8 rate categories and invariable sites estimated from the data. Trees were inferred using WEIGHBOR 1.0.1a (45).

ACKNOWLEDGMENTS. This work was funded by grants from the Canadian Institutes of Health Research (MOP-82704) and the Public Health Agency of Canada (to B.K.C.), and by Grant GM-58746 from the National Institutes of Health and MCB-0613014 from the National Science Foundation (to L.J.K.). B.K.C. is the recipient of a New Investigator Award from the Canadian Institutes of Health Research, a Young Investigator Award from the American Society of Microbiology, and an Early Researcher Award from the Ontario Ministry of Research and Innovation. S.E.O. is the recipient of an Ontario Graduate Scholarship.

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