Pathogenic Adaptation of Intracellular Bacteria by Rewiring a Cis-Regulatory Input Function

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**Pathogenic adaptation of intracellular bacteria by rewiring a cis-regulatory input function**

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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.

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**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 ΔssrB 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*, *Yersinia*, and the human and coral pathogen, *Serratia marcescens*, and is part of a larger family of uncharacterized bacterial “γ” genes (pamt07338) (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...
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 expropiate 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. 1A). We used primer extension to map the location of the srfN promoter and transcription start site in S. typhimurium. RNA isolated from an hns::pO5 background (+) or an isogenic ssrB mutant (−). (C) SsrB binding to srfN promoter DNA. The purified C-terminal DNA binding domain of SsrB (SsrBc) protects from DNaseI a 20-bp site between −65 and −65 from the transcription start site. Black bar and coordinates indicate location of SsrBc binding sites 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 SsrBc binding is indicated with a solid red line and the probable −35 and −10 hexamers are shown.

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::pO5 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 −65 and −65 from the transcription start site. Black bar and coordinates indicate location of SsrBc binding sites 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 SsrBc binding is indicated with a solid red line and the probable −35 and −10 hexamers are shown.
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. 1D). Interestingly, one S. enterica serovar that showed divergence 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 S. Typhimurium chromosome, 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 tested the fitness of the mutant in competitive infections 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.
These data suggested a conservation of the ancestral transcription regions (Fig. S5). Next, we precisely swapped the start of translation and near-consensus –10 and –35 hexamer showed a transcriptional start site at position –115 bp relative to the regulatory sequence plus 680 bp of upstream DNA (to include the similar protein levels in from the this regulatory input could drive transcriptional start site for the ancestral transcriptional machinery required for expressing component, SseB, whose gene is expressed in an SsrB-dependent SPI-2. This result raised the question whether the component, SseB, whose gene is expressed in an SsrB-dependent SPI-2 into S. bongori (which evolved distinctly from S. typhimurium) was also cotransferred along with 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. 4A), 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. 4B) and in the absence and presence of SPI-2 in S. bongori (Fig. 4C). 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. 4D), which we verified by deleting phoP in S. bongori and testing expression in low-magnesium minimal medium (Fig. 4E). 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 ∆srfN with srfN expressed from the promoter evolved in S. typhimurium (PSTM) 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. 5A). In mouse experiments designed to test whether rewiring srfN to the 5′UTR evolved in S. bongori (PSSR-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. 5A), which was indistinguishable from the ∆srfN strain (0.22, 95% CI 0.08–0.63) (see Fig. 3A). 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

Promoter Swapping Experiments with S. bongori. The regulatory data for srfN indicated an evolved dependence on the horizontally acquired virulence regulator SsrB. We first verified that srfN was under SsrB control by cloning the regulatory region upstream of srfN in front of a S. typhimurium 5′UTR. These experiments showed that the S. typhimurium 5′UTR was SsrB-dependent, as indicated by similar protein levels in ssrB null bacteria and wild-type cells (Fig. 4B) and in the absence and presence of SPI-2 in S. bongori (Fig. 4C). These data suggested a conservation of the ancestral transcription factors governing control of srfN, prompting us to investigate the

competitive (Fig. 3B) 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.

**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 (PSTM) or S. bongori (PSSR). 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 (PSTM) or (B and C) the regulatory input evolved in S. bongori is shown by Western blot. Psr 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).
alignment from Fig. 1D. 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. 5B). 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. 5C). Evidence exists to suggest that SsrB 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 orthogonal 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 has been well studied in euukaryotic 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 commandeering virulence-associated transcription factors is a mechanism of regulatory evolution (34, 40) to be considered to fully understand the evolutionary potential of bacterial pathogens.


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 $10^9$ cfu of S. typhimurium 01:1 M Heps (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 loads 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: (mutant/wild type $\text{mutant/wild type}_{\text{CI}}$). Log-transformed competitive infection data were analyzed using an one-sample t test.

Genetic Analyses. Detailed references to genome sequences and genomic analyses are found in SI Text. Nucleotide alignments of the srnF 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 (LTZ nucleotides 741 to 851). 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 repeat categories and invariable sites estimated from the data. Trees were inferred using WEIGHBORG 1.0.1a (45).

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