VirF-Independent Regulation of Shigella virB Transcription is Mediated by the Small RNA RyhB

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Abstract

Infection of the human host by Shigella species requires the coordinated production of specific Shigella virulence factors, a process mediated largely by the VirF/VirB regulatory cascade. VirF promotes the transcription of virB, a gene encoding the transcriptional activator of several virulence-associated genes. This study reveals that transcription of virB is also regulated by the small RNA RyhB, and importantly, that this regulation is not achieved indirectly via modulation of VirF activity. These data are the first to demonstrate that the regulation of virB transcription can be uncoupled from the master regulator VirF. It is also established that efficient RyhB-dependent regulation of transcription is facilitated by specific nucleic acid sequences within virB. This study not only reveals RyhB-dependent regulation of virB transcription as a novel point of control in the central regulatory circuit modulating Shigella virulence, but also highlights the versatility of RyhB in controlling bacterial gene expression.

Introduction

Shigellosis, a severe diarrheal disease caused by members of the Shigella genus (S. dysenteriae, S. flexneri, S. sonnei and S. boydii) remains a worldwide health concern with a conservative estimate of 165 million cases resulting in over one million deaths each year [1]. Following ingestion and transit through the gastrointestinal tract of the host, Shigella invade cells of the colonic epithelium, replicate within the cytoplasm of the infected cell and spread to neighboring epithelial cells via actin based motility [2,3]. These virulence-associated processes are mediated by the coordinated production and activity of several Shigella virulence factors.

The complex and coordinated regulation of Shigella virulence gene expression is accomplished largely via the activity of two transcriptional activators, VirF and VirB [4,5]. Activity of the VirF/VirB regulatory cascade, and thus Shigella virulence, is modulated in response to specific environmental signals such as changes in temperature, pH and osmolarity, as well as carbon source and iron availability [1,6,7,8]. In response to precise environmental conditions, specifically those encountered within the host, VirF is produced and directly activates the transcription of two virulence-associated genes, icsA and virB. IcsA facilitates intracellular spread of Shigella by mediating actin-based motility [9], while VirB directly promotes the expression of multiple virulence-associated genes [Figure 1]. The VirB regulon includes genes encoding components of the Type Three Secretion System (TTSS), icsP encoding the protease that modulates IcsA activity, and mxiE encoding a transcriptional activator of additional virulence associated genes [1,3,4,10,11]. As a central pathway controlling the coordinated expression of several virulence-associated genes, any factor that influences the production or activity of VirF or VirB consequently impacts Shigella virulence.

Expression of genes within the VirF and VirB regulons appears to be coupled: factors that impact the VirF regulon, also impact the VirB regulon. The coupled expression of genes within the VirF and VirB regulons results from the fact that both transcriptional and post-transcriptional regulation of virB is mediated by factors or environmental conditions that also modulate the expression of virF. Specifically, transcriptional regulation of virB is mediated by VirF itself, or by H-NS and IHF, proteins known to influence virF expression [5,12,13]. Post-transcriptional regulation of virB expression, via destabilization of the virB mRNA, is mediated in response to changes in environmental pH, osmolarity and temperature [14,15,16], conditions that also influence expression of virF [12,17,18,19]. No study to date has identified a regulatory factor that controls the transcription of genes within the VirB regulon, such as icsP or mxiE, without also regulating icsA, a gene that lies solely within the VirF regulon (Figure 1).

Of the environmental conditions known to modulate Shigella virulence factors via regulation of the VirF/VirB cascade, the impact of iron-availability is the least well characterized to date. Under conditions of iron-limitation, the small RNA RyhB is produced and functions to inhibit icsB expression, as evidenced by a reduction in the steady state level of icsB mRNA [6], a decrease in the production of proteins within the VirB regulon [6,20] and an inhibition of epithelial cell invasion by the bacterium [6]. While it is clear that RyhB functions to reduce the steady state level of virB mRNA and inhibit VirB activity [6], it
remains unclear whether RyhB-dependent regulation of \( \text{virB} \) mRNA is achieved through the modulation of VirF activity, whether the regulation occurs in a VirF-independent manner at the level of \( \text{virB} \) transcription, or whether the \( \text{virB} \) message is simply targeted for accelerated degradation in the presence of RyhB. A greater understanding of the RyhB-dependent regulation of \( \text{virB} \) expression is needed to improve our knowledge of the VirF/VirB cascade, which controls virulence gene expression in Shigella.

Materials and Methods

Growth Conditions

Bacterial strains and plasmids used in this study are shown in Table 1. *Escherichia coli* was cultured in Luria-Bertani (LB) broth (1% tryptone, 0.5% yeast extract and 1% NaCl) or on LB agar plates at 37°C. *Shigella dysenteriae* was cultured in LB broth or on tryptic soy broth (Becton, Dickenson and Company, Sparks, MD) agar plates containing 0.01% (wt/vol) Congo red dye (ISC Bioexpress, Kaysville, UT) at 37°C.

![Figure 1. Schematic of the VirF/VirB cascade. VirF is produced under permissive conditions and positively regulates the expression of icsA and virB. VirB in turn activates the expression of many virulence-associated genes including those encoding components of the Type Three Secretion System and associated effectors (mxi, spa and ipa) as well as IcsP, the protease specific for the actin based motility protein IcsA.](image)

Transcriptional Regulation of VirB by RyhB

Oligonucleotide Primers and Probes

The name, nucleic acid sequence and function of each oligonucleotide primer and probe used in this study are presented in Table 2.

Site-directed Mutagenesis of \( \text{virB} \)

Specific nucleic acid modifications were introduced into \( \text{virB} \) by allelic exchange [21]. Briefly, splice overlap polymerase chain reaction (PCR) was used to amplify \( \text{virB} \) containing the desired nucleic acid changes and 500 bp of flanking sequence on either side [21]. Initial upstream and downstream products were amplified using oligonucleotides \( \text{virB}-5/\text{virB}-12 \) and \( \text{virB}-6/\text{virB}-13 \) respectively. PCR conditions: denaturation for 30 seconds at 95°C, annealing for 45 seconds at 50°C, and extension for 60 seconds at 72°C for 30 cycles in a Peltier thermal cycler (MJ Research, Watertown, MA). The final 2 kb product containing the desired nucleic acid changes, including an additional *Hae*III restriction endonuclease recognition site, was amplified with oligonucleotides \( \text{virB}-5 \) and \( \text{virB}-6 \). The resulting product was digested with *Xba*I and cloned into the *Xba*I site of pCVD442N2 to generate pERM124. The insert of pERM124 was sequenced to confirm that only the desired nucleic acid changes were incorporated. Osmotic shock was used to transform DH5α(\( \text{pir} \)) with pERM124, and the plasmid was subsequently moved by conjugation into *S. dysenteriae* O-4576S1-G. Primary integrants were selected by growth in the presence of 200 mg/ml streptomycin and 250 mg/ml carbenicillin. Primary integrants were analyzed by PCR using oligonucleotide sets \( \text{virB}-1/\text{virB}-10 \) and \( \text{virB}-4/\text{virB}-9 \), followed by the purification of each product and digestion with the *Hae*III restriction endonuclease. Once confirmed, the selected primary integrant was cultured to mid-logarithmic phase in LB supplemented with 0.1% sucrose at 30°C. The culture was then serially diluted and plated onto LB agar plates containing 5% sucrose. The plates were incubated overnight.

Table 1. Bacterial strains and plasmids.

| Strain or Plasmid | Description | Source |
|-------------------|-------------|--------|
| *Escherichia coli* strains |
| DH5α              | Wild-type  | Life Technologies |
| DH5α(pir)         |             | [39] |
| *Shigella* strains |
| O-4576S1          | Wild-type  | [40] |
| ND100 (wild-type) | Spontaneous Streptomycin resistant mutant of O-4576S1 | [6] |
| ND100\( \text{ryhB} \) | rhyB deletion in ND100 | [6] |
| ND100\( \text{ryhBaltvirB} \) | Site-specific mutation within \( \text{virB} \) of ND100\( \text{ryhB} \) | This study |
| 2457T             | Wild-type  | [41] |
| *Plasmids*        |
| pCVD442N2         | Suicide vector | [42] |
| pERM124           | Altered \( \text{virB} \) construct in pCVD442N2 | This study |
| pQE2              | Expression vector | QIAGEN |
| \( \text{ryhB} \)  |             | [6] |
| pCompryhB         | Compensatory \( \text{ryhB} \) in pQE-2 | This study |
| pRW50             | \( \text{lacZ} \) reporter plasmid | [24] |
| P\( \text{icsA-lacZ} \) | \( \text{icsA} \) promoter region transcriptionally fused to \( \text{lacZ} \) in pSRG12 | Gift from M. Goldberg,This study |
| P\( \text{icsP-lacZ} \) (previously pHJW20) | \( \text{icsP} \) promoter region transcriptionally fused to \( \text{lacZ} \) | [27] |

Table 1. Bacterial strains and plasmids.
at 37°C. Resulting colonies were screened for sensitivity to 250 μg/ml carbenicillin. Colonies sensitive to carbenicillin (8 of 120) were screened for the presence of the altered virB nucleic acid sequence by PCR using virB-4/virB-9 and virB-1/virB-10 oligonucleotide sets followed by digestion of each purified product with the HaeIII restriction endonuclease. Sequence analysis was used to verify that only the desired nucleic acid changes were present in the resulting altered virB open reading frame.

**Real-time Polymerase Chain Reaction**

RNA was isolated using RNeasy® Midi Kit (QIAGEN, Valencia, CA) per the product directions, from bacteria cultured for approximately 20 hours at 37°C on TSB agar plates supplemented with 0.1% Congo red, 250 μg/ml carbenicillin and 200 μM IPTG. Each RNA sample was then treated with 16 units of amplification grade DNaseI (Invitrogen, Carlsbad, CA), ethanol precipitated and dried. The RNA pellet was resuspended in DEPC-treated water, and the nucleic acid concentration was measured using an ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE). No more than 10 μg of total RNA was used to generate cDNA with the High Capacity cDNA Archival Kit (Applied Biosystems, Foster City, CA) per the product directions. Each cDNA sample was diluted 10 fold in water, and 5 μl was used as the template for each amplification reaction. All Taqman based reactions utilized TaqMan Universal Master Mix (Applied Biosystems, Foster City CA). Primers and FAM labeled minor groove binding probes were designed using Primer Express software (Applied Biosystems, Foster City, CA). All SYBR based reactions utilized iQ SYBR Green Supermix (Bio-Rad, Hercules, CA) and primers were designed using Beacon Designer 7 software (Premier Biosoft, Palo Alto, CA). Regardless of the chemistry, rrsA was used as the normalizer for each sample, and all values were calibrated to the value obtained from the given parental strain carrying the vector control plasmid using the ΔΔCt method [22]. All primer pairs and amplification conditions were validated by the inclusion of a standard curve on each reaction plate, from which efficiency was calculated. All reactions were run in a 7300 Real-Time PCR system (Applied Biosystems, Foster City, CA) under standard reaction conditions.

**Table 2. Primers and Probes.**

| Primers          | Sequence                                      | Use in study         |
|------------------|-----------------------------------------------|----------------------|
| virB2-for        | TCCAATCGCGTCAAGAACTTAACCT                      | Real-time primer-Taqman |
| virB2-rev        | CCCTTAATATTGAGGTAGTGAAGACTAAGAGATTCT          | Real-time primer-Taqman |
| sodB1-for        | CTGGAAMGTGCGTCAAGGCT                        | Real-time primer-Taqman |
| sodB1-rev        | CGGTTGAATTAAATGCGGAAAGAAGAAGAAGAAGAAGAAGAAGA | Real-time primer-Taqman |
| rrsA-for         | CAGATTACTAGCAATTTACTCA                       | Real-time primer-Taqman |
| rrsA-rev         | GCGCGAAAGTACACAGCCACACTCCAC                  | Real-time primer-Taqman |
| virB 3’-for      | GCGCGAAGTACACAGCCACACTCCAC                  | Real-time primer-SYBR |
| virB 3’-rev      | GAGATTCAATAGCCATTTGCACTCC                     | Real-time primer-SYBR |
| virB 5’-for      | AACAGAGAATACATTGACAGGACT                    | Real-time primer-SYBR |
| sodB1-for        | TCGGGTATCCAGCACCCAGGCA                      | Real-time primer-SYBR |
| sodB1-rev        | GCCCGAAGTACACAGCCACACTCCAC                  | Real-time primer-SYBR |
| rrsA-for         | AACGTCATAGCAAGCAACGATGTC                    | Real-time primer-SYBR |
| rrsA-rev         | TACGGGAGACGCACTTGG                         | Real-time primer-SYBR |
| MB608            | TCCAGAATTCCATTTGCACT                       | Construction of pSRG12 |
| MB607            | ACAAAAGCTGAACTTGGCTAGTATTCC                 | Construction of pSRG12 |
| virB5            | GTCTAGAGAAGAATAGTGAACCACTTCCCC               | Construction of altered virB |
| virB6            | GTCTAGAGAAGAATAGTGAACCACTTCCCC               | Construction of altered virB |
| virB12           | GCATTGTGAG TTGGGATCCGCTAAATTTCGCCAAATGAGG    | Construction of altered virB |
| virB13           | TAAGGCGCATGACACCACTCAGCATGACATGTC           | Construction of altered virB |
| sodB probe for   | CTACTGAGAAGCAGCC                        | Amplification of northern blot probe |
| sodB probe rev   | GTGCTCCAGATAGCAG                         | Amplification of northern blot probe |

| Probes          | Sequence                                      | Use in study         |
|-----------------|-----------------------------------------------|----------------------|
| virB2           | 6FAMAGGGACTTGAAGAAGGCTMGBNFQ                 | Real-time probe-Taqman |
| sodB1           | 6FAMCCGACATTTTCTGGCMGBNFQ                   | Real-time probe-Taqman |
| rrsA            | 6FAMATCGAGTGCAGGACTGAGGGAGMGBNFQ           | Real-time probe-Taqman |
| ssvirB-sense    | TGCTTCTGACTATAGTGCTACGATGCTCTTGCTCAATGAGGCT | Northern blot |
| ssvirB-antisense| TAGCATCGCAGATACTGATTGCTCTTAATTTTCTCGTAAATGAGGAGGATGTCCT | Northern blot |

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Tissue Culture and in vivo Plaque Assays

Henle cell monolayers were cultured in 6 well polystyrene tissue culture plates (Corning Inc. Costar, Corning, NY) in Gibco Minimum Essential Medium (MEM) (Invitrogen Corp. Carlsbad, CA) supplemented with 10% fetal bovine serum (FBS), 2 mM glutamine and a 1 X final concentration of non-essential amino acids (Lonza, Switzerland). Plates were incubated at 37°C in an atmosphere of 5% CO₂.

Plaque assays were performed as published with minor modifications [23]. Briefly, bacterial strains were grown overnight at 37°C on TSB agar plates containing the appropriate antibiotic. Ten colonies were used to inoculate a 3 ml LB culture containing 250 µg/ml carbenicillin, 0.1% DOG, and 200 µM IPTG. Cultures were grown to mid-logarithmic phase at 37°C. 10⁴ bacteria, diluted in phosphate buffered saline (PBS), were used to infect the Henle cell monolayer in each tissue culture well containing 2 ml MEM supplemented with 250 µg/ml carbenicillin and 200 µM IPTG. Plates were centrifuged for 10 minutes at 600 x g in a Centra GP8 (International Equipment Company, Needham Heights, MA). Plates were incubated at 37°C for 1.5 hours, washed with 2 ml PBS, then covered with a 2 ml overlay composed of MEM supplemented with 0.3% glucose, 250 µg/ml carbenicillin, 200 µM IPTG, and 20 µg/ml gentamicin to kill any bacteria that had not invaded into a eukaryotic cell. Following incubation for 72 hours at 37°C, the plates were washed with 2 ml PBS, stained with Wright-Giemsa stain (Camco, Ft. Lauderdale, FL), washed with distilled water, and air dried.

Construction of the PicsA-lacZ Reporter Plasmid pSRG12

The PicsA-lacZ reporter plasmid pSRG12 is derived from the low-copy number, broad host range, lacZ expression vector pSR50 [24] and carries the S. flexneri icsA promoter region (397 bp upstream of the beginning of the icsA gene) on an EcoRI/Hind III restriction fragment. This places the lacZ gene directly under the control of the icsA promoter.

mRNA Stability Assay

Bacterial strains were grown on TSB agar plates containing 0.01% Congo red and 250 µg/ml carbenicillin. Following overnight incubation at 37°C, a single colony of each strain was used to inoculate a 3 ml culture of LB containing carbenicillin. Each culture was incubated overnight at 37°C in a shaking incubator. 250 µl of each overnight culture was used to inoculate a 25 ml LB culture containing carbenicillin and 0.1% DOG which was incubated at 37°C in a shaking incubator to an optical density (OD₆₀₀) of 0.7. Next, 200 µM IPTG was added to each culture followed two minutes later by the addition of 250 µg/ml rifampicin. The time of rifampicin addition was designated t = 0. At each time point, 1 ml of culture was removed and mixed with 250 µl of RNAAlater (Ambion, Austin, TX) to preserve the mRNA profile of the sample. RNA isolation and Real-time PCR was performed using SYBR technology as detailed above.

Northern Blot Analysis

Northern blots were performed using the Ambion Northern Max kit (Ambion, Austin, TX) per kit directions. No more than 4 µg of total RNA was loaded into the wells of a 0.8% agarose gel and BrightStar Biotinylated RNA Millenium Markers (Ambion, Austin, TX) were used as size standards. Blots were hybridized overnight with 10 pM of the indicated probe at 46°C or 42°C with either single stranded, biotin labeled DNA probe (IDT, Coralville, IA) or double stranded DNA probe labeled using the BrightStar Psoralen-Biotin Nonisotopic Labelling Kit (Ambion, Austin, TX), respectively. Blots were visualized using the BrightStar Biodetect Kit (Ambion, Austin, TX) per the directions.

β-Galactosidase Assays

β-Galactosidase assays were performed as detailed previously [21]. Briefly, strains were grown overnight at 37°C on TSB with Congo red containing 250 µg/ml ampicillin and 20 µg/ml tetracycline. Single colonies were selected and cultured in 3 ml LB with appropriate antibiotics in a shaking incubator overnight at 30°C. 130 µl of each culture was used to inoculate a 3 ml subculture in LB with antibiotics and 200 µM IPTG. Each subculture was incubated in a 37°C shaking incubator and grown to an OD₆₅₀ between 0.5 and 0.6. The bacteria present in 1 ml of each culture were pelleted and resuspended in 1 ml Z buffer. 400 µl of the resuspended bacteria were then diluted 1:1 in Z buffer. Bacteria were then permeabilized by the addition of 50 µl 1% sodium dodecyl sulfate (SDS) and 100 µl chloroform and mixed by vortexing on high for 10 sec. The permeabilized bacteria were incubated at 30°C for 15 min prior to the addition of 160 µl of ortho-Nitrophenyl-β-galactopyranoside (ONPG), diluted to 4 mg/ml in Z buffer. Each tube was mixed by vortexing on high for 5 seconds and incubated again at 30°C until a color change was observed. The time required for each reaction to change color was noted. Each reaction was stopped by the addition of 400 µl of 1 M Na₂CO₃. Tubes were centrifuged at maximum speed for 2 minutes and the optical density at both 550 nM (OD₅₅₀) and 420 nM (OD₄₂₀) was measured. A reaction containing all components except the bacterial culture was used as the negative control in the experiment.

Results

RyhB does not Influence VirF Activity

Previous studies have demonstrated that the production of S. dysenteriae RyhB results in a significant decrease in the steady state level of virF mRNA [6]. These findings indicate that RyhB-dependent regulation of virF expression is not mediated indirectly via inhibition of virF transcription or by the destabilization of the virF mRNA [6], but does not rule out the possibility that RyhB-dependent regulation of virF expression is mediated indirectly via decreasing translational efficiency of the virF message, decreasing VirF protein levels or decreasing VirF activity. Since all three of these possibilities are likely to lower the activity of VirF in the cell, a necessary first step in characterizing the full impact of RyhB on the VirB/VirF regulon was to determine if RyhB influences the ability of VirF to function as a transcriptional activator.

VirF increases the activity of the icsA promoter [25,26] (Figure 1), therefore to test the impact of RyhB production on the activity of VirF, the activity of the icsA promoter was measured in the presence or absence of increased RyhB production. Wild-type S. dysenteriae and S. flexneri were transformed with PicsA-lacZ, a reporter plasmid carrying the icsA promoter fused to an otherwise promoter-less lacZ gene [27], and the ryhB expressing plasmid pysyb [6] or pQE, the empty vector control, were subsequently introduced. To ensure that RyhB production was being up-regulated from pysyb in the presence of the inducer, a PicsP-lacZ fusion plasmid [20] was used as a control since RyhB has been shown to negatively regulate this promoter via its modulation of virB [6,20].

As seen previously in S. flexneri [20], production of RyhB from the pysyb plasmid in S. dysenteriae resulted in a significant reduction in β-galactosidase activity produced from the PicsP-lacZ reporter
data indicate that RyhB production does not influence icsA expression from the ppyhB plasmid. These data indicate that RyhB production does not influence icsA promoter activity. These findings strongly suggest that RyhB does not decrease the activity of VirF, the positive regulator of icsA promoter activity.

RyhB Represses virB Transcription

The RyhB-dependent reduction of virB mRNA levels could result from lower levels of virB transcription, or from post-transcriptional regulation leading to the destabilization of the virB mRNA molecule. RyhB is known to down-regulate specific gene targets by facilitating destabilization of target mRNA molecules [28,29,30]. Furthermore, by a regulatory mechanism that is not yet fully characterized, stability of the S. sonnei virB mRNA molecule is altered in response to changes in environmental temperature, osmolarity and pH [14,15,16]. Based on these observations we next chose to determine whether or not RyhB production leads to destabilization of the virB mRNA molecule using in vivo mRNA stability assays and northern blot analyses. sodB mRNA was used as a control in each assay, as it is well established that RyhB functions to destabilize the sodB mRNA molecule [28,31].

To investigate the effect of RyhB on the stability of virB mRNA and sodB mRNA, an mRNA decay assay was performed (Figure 3A). Briefly, wild-type S. dysenteriae carrying either ppyhB or the vector control was cultured to the mid-logarithmic phase of growth and IPTG added to induce production of RyhB from ppyhB. Two minutes after the addition of IPTG, transcription was halted by the addition of rifampicin. Samples were collected, and RNA isolated at the time of rifampicin addition (t = 0) as well as 2, 3, and 10 minutes after rifampicin addition (t = 2, 3 or 10). Relative amounts of virB mRNA and sodB mRNA were quantified by Real-time PCR. Analysis of sodB mRNA levels in the presence and absence of RyhB production demonstrated that the stability of S. dysenteriae sodB mRNA, like that of E. coli sodB mRNA [31], is dramatically reduced in the presence of RyhB (Figure 3B). These findings were confirmed by northern blot analysis, demonstrating that the production of RyhB from ppyhB dramatically accelerates the rate of sodB mRNA degradation as compared to the rate of sodB mRNA degradation in the absence of increased RyhB production (Figure 3C). The obvious reduction in the amount of sodB mRNA seen at t = 0 in the strain carrying ppyhB as compared to that seen at the same time point in the strain carrying the vector control is to be expected given that RyhB causes the rapid degradation of sodB message, and that RyhB production was initiated two minutes prior to the collection of the sample (Figure 3A). These data confirm RyhB-dependent destabilization of the S. dysenteriae sodB mRNA and indicate that the in vivo assay is working as expected. Surprisingly, production of RyhB from the ppyhB plasmid had no effect on the stability of virB mRNA as determined by measuring the relative abundance of both the 3′ end (Figure 3D) and 5′ end (Figure 3E) of the message using quantitative Real-time PCR analysis. As above, these findings were confirmed by northern blot analysis (Figure 3F). Northern blot analysis of virB mRNA levels was performed using a single stranded DNA probe to eliminate the possibility that a transcript encoded anti-sense to virB was being detected (see Discussion). Together, these data clearly demonstrate that, unlike RyhB-dependent regulation of sodB mRNA, RyhB-dependent regulation of virB expression is not mediated by an increase in the rate of mRNA degradation.

Figure 2. RyhB does not influence the activity of VirF. β-galactosidase activity measured in wild-type S. dysenteriae carrying PicsP-lacZ (A), and S. dysenteriae or S. flexneri carrying PicsA-lacZ (B), in both the presence (ppyhB) and absence (pQE) of increased production of RyhB from the ppyhB plasmid. All cultures were grown in the presence of 200 μM IPTG to induce expression of ryhB from ppyhB when present. The data is the average of three independent experiments and error bars represent one standard deviation. *represents a significant difference from the activity of the strain carrying the pQE vector control (p<0.01).

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Unlike that observed for sodB mRNA, virB mRNA levels were not dramatically different in the S. dysenteriae carrying the vector control as compared to the strain carrying the ppyhB plasmid at the
point of transcriptional inhibition (t = 0, Figure 3C and Figure 3F). This finding indicates that virB mRNA levels are not altered in the short time between the induction of RyhB production and the inhibition of transcription, further supporting the conclusion that RyhB does not function to destabilize virB mRNA.

RyhB-dependent reduction in the steady state level of virB mRNA, as seen in previous studies [6], together with the finding that this reduction is not mediated by destabilization of the transcript are consistent with RyhB regulating virB mRNA levels at the level of transcription.

**RyhB-dependent Regulation of virB Transcription is Facilitated by Nucleic acid Sequences within the virB Open Reading Frame**

Each mechanism of direct RyhB-dependent regulation characterized to date has been shown to be mediated, at least in part, by nucleic acid complementarity between RyhB and the target

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Figure 3. RyhB does not alter the stability of the virB mRNA molecule. Schematic of the procedure used to induce RyhB production, inhibit transcription, and collect RNA samples for Real-time PCR and northern blot based investigations into the impact of RyhB on the stability of virB and sodB mRNA molecules (A). The relative amount of sodB mRNA (B) and virB mRNA (D and E) was quantified by Real-time PCR in the presence (light lines) and absence (dark lines) of increased RyhB production. At each time point the amount of mRNA present is expressed relative to the level of mRNA at the time of transcriptional inhibition by the addition of rifampicin (t = 0) and is normalized to the amount of rrsA measured in the sample. The data is an average of three independent experiments and error bars represent one standard deviation. The same set of RNA samples was used to measure levels of both sodB and virB mRNA. Northern blot analysis was used to detect full-length sodB and virB mRNA in each RNA sample collected (C and F, respectively). The negative control lane in each northern blot contains RNA isolated from wild-type S. dysenteriae carrying the pQE vector grown at 30°C, as virB expression is inhibited at this temperature. Each northern blot shown is representative of analysis performed with three biological replicates.

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mRNA molecule [28,30,32,33]. The nucleic acid sequence of \textit{virB} mRNA was examined for complementarity to RyhB, however no such complementarity was found. Instead, nucleic acid complementarity was observed between RyhB and the template DNA strand within the \textit{virB} gene. Specifically, within the 930 nucleotide long \textit{virB} coding sequence, twelve of eighteen nucleotides between base numbers 403 and 420 on the template DNA strand share perfect nucleic acid complementarity to nucleic acid sequences within RyhB (Figure 4A). Furthermore, five of the seven nucleotides between base numbers 414 and 420 of the \textit{virB} gene share nucleic acid complementarity to five identically spaced nucleotides within the region of RyhB previously shown to mediate repression of \textit{virB} expression [6] (Figure 4A).

The role of nucleic acid complementarity between RyhB and the DNA sequence of the template strand within the \textit{virB} gene in mediating RyhB-dependent regulation of \textit{virB} transcription was investigated by site-directed mutagenesis of \textit{virB}. The nucleic acid mutations introduced into the \textit{virB} gene, designated Altered \textit{virB}, conserve the amino acid sequence of the encoded protein, but reduced the nucleic acid complementarity between \textit{virB} and RyhB to six of the original twelve bases (Figure 4B). The impact of the site-directed mutagenesis on RyhB-mediated repression of \textit{virB} expression was investigated using quantitative Real-time PCR. The relative amount of either wild-type or Altered \textit{virB} was measured in the presence (\textit{pyr} \textit{hB}) or absence (vector) of increased production of RyhB. It is important to note that prior to normalization the level of Altered \textit{virB} message was not significantly different than that of wild-type \textit{virB} in each strain carry the vector control (data not shown). As seen previously [6] production of RyhB from the \textit{pyr} \textit{hB} plasmid resulted in a significant decrease in the steady state levels of wild-type \textit{virB} mRNA, however production of RyhB had no significant effect on the steady state level of Altered \textit{virB} mRNA (Figure 5). These data indicate that the identified nucleic acid sequence within the \textit{virB} gene directly or indirectly facilitate RyhB-dependent regulation of \textit{virB} expression.

The Altered \textit{virB} Mutant Displays Virulence-associated Phenotypes in \textit{S. dysenteriae}, but the Phenotypes are not Affected by the Production of RyhB

\textit{Shigella} virulence is positively correlated with the ability of the bacterium to bind Congo red from an agar medium [34] and this phenotype has been used previously to determine the effects of RyhB on production of VirB-dependent gene products [6]. Therefore we next tested the ability of RyhB to suppress Congo red binding by wild-type \textit{S. dysenteriae} or an Altered \textit{virB} mutant. Each strain carrying the pQE vector control, displayed a positive Congo red phenotype, strongly suggesting that the mutations introduced into the Altered \textit{virB} mutant do not disrupt function of the encoded protein (Figure 6A). Strikingly, unlike Congo red binding by wild-type \textit{S. dysenteriae}, which was dramatically reduced

![Figure 4. Nucleic acid complementarity exists between RyhB and the template DNA strand within virB. (A) A comparison of nucleic acid sequences within the wild-type \textit{virB} gene to that within RyhB. The double stranded DNA sequence between base numbers 403 and 420 of 930 total bases in the wild-type \textit{virB} gene is shown. The encoded amino acids are indicated using the single letter code. “+” indicates the location of nucleic acid sequence complementarity between the template strand DNA in the \textit{virB} open reading frame and RyhB. (B) A comparison of nucleic acid sequences within the Altered \textit{virB} gene to that within RyhB. The double stranded DNA sequence between base numbers 403 and 420 of 930 total bases in the Altered \textit{virB} gene is shown. The encoded amino acids are indicated using the single letter code. Underlined sequences denote those that were mutated to construct Altered \textit{virB}. “+” indicates the location of nucleic acid sequence complementarity between the template strand DNA in the Altered \textit{virB} open reading frame and RyhB.](https://www.plosone.org/content/10.1371/journal.pone.0038592.g004)

![Figure 5. Alteration of nucleic acid sequences within \textit{virB} reduces the efficiency of RyhB-mediated repression. Real-time PCR analysis of the amount of wild-type \textit{virB} mRNA and Altered \textit{virB} mRNA in the absence (vector) and presence (\textit{pyr} \textit{hB}) of \textit{pyr} \textit{hB} expressed from an inducible plasmid promoter. The amount of mRNA is reported relative to the level of each in the strain carrying the vector control and is normalized to the amount of \textit{rrsA} mRNA present in each sample. Each set of data is an average of three independent experiments. * denotes the existence of a significant difference between the indicated data points (p=0.05). doi:10.1371/journal.pone.0038592.g005](https://www.plosone.org/content/10.1371/journal.pone.0038592.g005)
following production of RyhB from the \textit{pryhB} plasmid, Congo red binding by wild-type and Altered \textit{virB} in \textit{S. dysenteriae} in the presence (\textit{pryhB}) and absence (\textit{pQE2}) of increased production of RyhB. All strains were cultured in the presence of 200 μM IPTG to induce expression of \textit{ryhB} from \textit{p PryhB}. B. Quantification of plaques formed by \textit{S. dysenteriae} expressing wild-type (dark bar) or Altered \textit{virB} (light bar) in the presence of \textit{ryhB} expressed from the inducible plasmid promoter of \textit{p PryhB} and that measured in each strain carrying the vector control. All strains were cultured in the presence of 200 μM IPTG to induce expression of \textit{ryhB} from \textit{p PryhB}. Data represents the average of four independent experiments. *represents a significant difference from the number of plaques formed by the strain carrying the \textit{pQE} vector control (\(p < 0.01\)).

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Transcriptional Regulation of VirB by RyhB

The ability of \textit{Shigella} to form plaques within a Henle cell monolayer is a quantifiable virulence-associated phenotype that is dependent on the activity of VirB [23]. As a means to evaluate the ability of RyhB to inhibit VirB activity, the impact of RyhB production on plaque formation by wild-type \textit{S. dysenteriae} and the Altered \textit{virB} mutant was measured. As demonstrated by the number of plaques formed by wild-type \textit{S. dysenteriae} carrying the vector control (149+/− 25) and the Altered \textit{virB} mutant carrying the vector control (111+/− 27), the nucleic acid changes introduced into the mutant did not significantly affect the ability of this strain to form plaques as compared to wild-type \textit{S. dysenteriae} (\(p = 0.149\)) (Figure 6B). Consistent with previous data [6], production of RyhB from the \textit{p PryhB} plasmid significantly repressed plaque formation by wild-type \textit{S. dysenteriae} as compared to the strain carrying the \textit{pQE} vector control (\(p = 0.002\)) (Figure 6B). In contrast, in the Altered \textit{virB} mutant, increased production of RyhB had no significant effect on plaque formation, as compared to the strain carrying the \textit{pQE} vector control (\(p = 0.188\)).
Together, these data clearly demonstrate that alteration of nucleic acid sequences within \( \text{virB} \) that reduce the level of nucleic acid complementarity between RyhB and the DNA sequence of the template strand within \( \text{virB} \) decrease the ability of RyhB to inhibit each VirB-dependent virulence-associated phenotype investigated.

**Discussion**

The VirF/VirB regulatory cascade plays a central role in *Shigella* virulence, coordinately regulating the production of several virulence factors in response to environmental conditions encountered by the pathogen during the course of a natural infection. The hierarchical regulation of the VirF and VirB regulons [1] and the observation that factors affecting the post-transcriptional regulation of \( \text{virB} \) mRNA also affect \( \text{virF} \) expression [1,14,15,16] has suggested that the expression of the VirF and VirB regulons is linked. This study reveals that RyhB modulates \( \text{virB} \) transcription and that this regulation is independent of VirF, providing the first experimental evidence of differential regulation of genes within the VirF and VirB regulons.

So far, three mechanisms of RyhB-dependent regulation have been characterized in *E. coli*. These mechanisms are exemplified by the regulation of \( \text{sodB} \), the \( \text{isc} \) operon (not to be confused with the *Shigella* \( \text{ics} \) genes, also discussed in this work) and \( \text{shiA} \) [28,29,30,31,32,33]. RyhB controls the expression of *E. coli* \( \text{sodB} \), encoding superoxide dismutase, by binding to and ultimately destabilizing the \( \text{sodB} \) mRNA molecule [28,29,30]. Additionally, RyhB can facilitate differential gene expression from polycistronic mRNA molecules by altering the stability of a portion of the message, as has been demonstrated for the \( \text{isc} \) operon [33]. Finally, RyhB is predicted to promote the translational efficiency of *E. coli* \( \text{shiA} \) by binding to the 5’ untranslated region of the message and altering the structure such that an inhibitory element is eliminated [32]. Although the molecular mechanisms vary, common to each identified mechanism of RyhB-dependent regulation is the fact that regulation occurs following transcription and is dependent upon nucleic acid complementarity between RyhB and the target mRNA molecule.

As demonstrated in this study, several features of RyhB-mediated regulation of \( \text{virB} \) differentiate it from RyhB-dependent regulation of previously characterized gene targets. Unlike other targets of RyhB-mediated regulation that are chromosomally located, \( \text{virB} \) is encoded on a large virulence plasmid [4]. The regulation of a horizontally acquired, genus specific, virulence factor, by a conserved, chromosomally located, regulatory RNA, provides further evidence of the versatility of RyhB in controlling bacterial gene expression. The lack of nucleic acid complementarity between RyhB and the \( \text{virB} \) mRNA, as well as the presence of nucleic acid complementarity within the \( \text{virB} \) gene distinguishes \( \text{virB} \) regulation from that of previously characterized RyhB targets.

The presence of nucleic acid complementarity between RyhB and template strand DNA within \( \text{virB} \) raises the possibility that RyhB is complementary to an mRNA or sRNA encoded anti-sense to \( \text{virB} \). If present, the anti-sense transcript may play an intermediary role in facilitating RyhB-dependent regulation of \( \text{virB} \) transcription. In this study, the presence of such a transcript was investigated by northern blot using a single-stranded DNA probe with nucleic acid identity to \( \text{virB} \). This analysis failed to detect a transcript anti-sense to \( \text{virB} \) in the region of the message with sequence similarity to RyhB (data not shown). These findings strongly suggest that RyhB affects \( \text{virB} \) mRNA levels via its complementarity with the template strand of \( \text{virB} \).

**Intracellular Environment (low iron)**

![Diagram of RyhB-dependent repression of virB transcription]

Figure 7. Working model of RyhB-dependent repression of \( \text{virB} \) transcription under iron poor conditions encountered within the intracellular environment. Under iron poor conditions, mimicking those likely encountered within the eukaryotic cell, Fur does not inhibit RyhB production and the RyhB functions to, directly or indirectly, inhibit \( \text{virB} \) transcription. Under such conditions RyhB-dependent VirF-independent regulation of \( \text{virB} \) expression facilitates the differential modulation of TTSS activity and actin based motility.

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While disruption of the nucleic acid complementarity between RyhB and the template strand of icsA [6], Figure 4), reduces the efficiency of both RyhB-dependent regulation of icsA expression (Figure 5) and RyhB-dependent modulation of virulence phenotypes (Figure 6), the precise role of these sequences in facilitating regulation remains unknown.

Finally, in contrast to previously characterized mechanisms of RyhB-dependent gene regulation, we have demonstrated that RyhB-dependent regulation of icsA expression is mediated, directly or indirectly, at the level of icsA transcription. Aside from RyhB and VirF, all previously identified factors shown to regulate icsA transcription also influence virF expression. Thus, if RyhB-dependent regulation of icsA transcription is indirect, it is mediated via the regulation of an as-yet unidentified transcriptional regulator. Future in vitro assays will explore the molecular mechanism underlying RyhB-dependent regulation of icsA transcription.

In *Shigella* species, the RyhB-dependent regulation of VirB activity may allow the VirB regulon (genes encoding the TTSS) to be regulated, without affecting the expression of genes in the VirF regulon (*icsA*, which is required for actin-based motility). Independent regulation of the VirF and VirB regulons may be advantageous to the bacterium at specific times during an infection within the human host. Specifically, once within the intracellular environment *Shigella* must repress production of the TTSS in order to prevent premature lysis of the eukaryotic cell, while allowing the production of IcsA in order to facilitate intracellular spread. As transcription of *ryhB* is under control of the iron-responsive regulator Fur [6,28,35], and gene expression analysis has demonstrated that several Fur-regulated genes are expressed within the intracellular environment of epithelial cells [36,37,38], it is reasonable to expect that RyhB will be produced when *S. dysenteriae* is within this intracellular environment, as discussed in Africa et al [20]. Under these conditions, RyhB may function to inhibit *virF* transcription, thus reducing production of TTSS while simultaneously allowing VirF-activated transcription of *icsA* to proceed (Figure 7).

In conclusion, this study demonstrates that *virF* transcription can be uncoupled from the activity of VirF by RyhB, revealing a unique mechanism by which genes within the VirF and VirB regulons can be differentially regulated. These findings not only expand the current understanding of the regulatory circuit controlling *Shigella* virulence gene expression, but also highlight the versatility of RyhB in regulating bacterial gene expression.

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### Author Contributions

Conceived and designed the experiments: WHB HJW SMP ERM. Performed the experiments: WHB NE ERM. Analyzed the data: WHB NE HJW SMP ERM. Contributed reagents/materials/analysis tools: HJW SMP ERM. Wrote the paper: WHB HJW SMP ERM.

### References

1. Jennison AV, Verma NK (2004) *Shigella flexneri* infection: pathogenesis and vaccine development. FEBS Microbiol Rev 28: 43–58.
2. Clerc P, Baudry B, Sansonetti PJ (1988) Molecular mechanisms of entry, intracellular multiplication and killing of host cells by *shigella*. Curr Top Microbiol Immunol 130: 3–13.
3. Dorman CJ, Porter ME (1998) The *Shigella* virulence gene regulatory cascade: a paradigm of bacterial gene control mechanisms. Mol Microbiol 29: 677–684.
4. Adler B, Sasakawa C, Tobe T, Makino S, Komatsu K, et al. (1989) A dual transcriptional activation system for the 230 kb plasmid genes coding for virulence-associated antigens of *Shigella flexneri*. Mol Microbiol 3: 627–635.
5. Tobe T, Yoshikawa M, Mizuno T, Sasakawa C (1993) Transcriptional control of the invasion regulatory gene *virB* of *Shigella flexneri*: activation by VirF and repression by H-NS. J Bacteriol 175: 6142–6149.
6. Murphy ER, Payne SM (2007) RyhB, an Iron-Responsive Small RNA Molecule Regulates *Shigella dysenteriae* Virulence. Infect Immun 75: 3470–3477.
7. Gore AL, Payne SM (2010) *cis* and *trans* influence *Shigella flexneri* pathogenesis. Infect And Immunity 78: 4674–4682.
8. Tohe T, Nagai S, Okada N, Adler B, Yoshikawa M, et al. (1991) Temperature-regulated expression of invasion genes in *Shigella flexneri* is controlled through the transcriptional activation of the *virB* gene on the large plasmid. Molecular Microbiology 5: 887–893.
9. Goldberg MB, Theriot JA, Sansonetti PJ (1994) Regulation of surface presentation of IcsA, a Shigella protein essential to intracellular movement and spread, is growth phase dependent. Infection And Immunity 62: 5664–5668.
10. Wing HJ, Yan AW, Goldman SR, Goldberg MB (2004) Regulation of IcsA, the outer membrane protease of the *Shigella* actin tail assembly protein IcsA, by virulence plasmid regulators VirF and VirB. Journal Of Bacteriology 186: 699–705.
11. Le Gall T, Mavris M, Marinno MC, Bernardini ML, Denaruf E, et al. (2005) Analysis of virulence plasmid gene expression defines three classes of effectors in the type III secretion system. *Shigella flexneri* Microbiology [Reading, England] 151: 951–962.
12. Proseda G, Fridiani PA, Di Lorenzo M, Falconi M, Micheli G, et al. (1998) A role for H-NS in the regulation of the *sci* gene of *Shigella* and enteroinvasive *E. coli*. Research In Microbiology 149: 15–25.
13. Porter ME, Dorman CJ (1997) Positive regulation of *Shigella flexneri* virulence genes by integration host factor. J Bacteriol 179: 6537–6550.
14. Mitobe J, Arakawa E, Watanabe H (2005) A sensor of the two-component system CspE affects expression of the type III secretion system through posttranscriptional processing of InvE. Journal Of Bacteriology 187: 107–113.
15. Mitobe J, Morita-Ishihara T, Ishihama A, Watanabe H (2008) Involvement of RNA-binding protein Hfq in the post-transcriptional regulation of *sciE* gene expression in *Shigella sonnei*. The Journal Of Biological Chemistry 233: 5738–5747.
16. Mitobe J, Morita-Ishihara T, Ishihama A, Watanabe H (2009) Involvement of RNA-binding protein Hfq in the osmotic-response regulation of *sciE* gene expression in *Shigella sonnei*. BMC Microbiology 9: 110–110.
17. Nakayama S, Watanabe H (1995) Involvement of *cspE*, a sensor of a two-component regulatory system, in the pH-dependent regulation of expression of *Shigella sonnei* *sciF* gene. J Bacteriol 177: 5062–5069.
18. Falconi M, Colonna B, Proseda G, Micheli G, Guaderriz CO (1998) Thermoregulation of *Shigella* and *Escherichia coli* EEFC pathogenicity. A temperature-dependent structural transition of DNA regulates accessibility of *sciF* promoter to transcriptional repressor H-NS. The EMBO Journal 17: 7033–7043.
19. Falconi M, Proseda G, Giorgianni M, Beghetto E, Colonna B (2001) Involvement of FIS in the H-NS-mediated regulation of *sciF* gene of *Shigella* and enteroinvasive *E. coli*. Molecular Microbiology 42: 439–452.
20. Africa LAA, Murphy ER, Egan NR, Wigley AF, Wing HJ (2011) The Iron-Responsive Fur/RyhB Regulatory Cascade Modulates the *Shigella* Outer Membrane Protease IcsP. Infection And Immunity 79: 4543–4549.
21. Sambrook J, Russell DW (2001) *Molecular Cloning: A Laboratory Manual*. 3rd ed. Cold Spring Harbor: Cold Spring Harbor Laboratory Press.
22. Livak KJ, Schmittgen TD (2001) Analysis of Relative Gene Expression Data Using Real-Time Quantitative PCR and the 2-ΔΔCT Method. Methods 25: 402–408.
23. Osa KE, Wingfield ME, Formal SB (1983) Plaque formation by virulent *Shigella flexneri*. Infect Immun 46: 124–129.
24. Lodovici F, Faur J, Busby S, Gimauro P, Kamini NR (1992) Bread host range plasmids carrying the *Escherichia coli* lactose and galactose operons. FEBS Microbiology Letters 74: 271–276.
25. Porter ME, Dorman CJ (1997) Differential regulation of the plasmid-encoded genes in the *Shigella flexneri* virulence regulon. Mol Gen Genet 256: 93–103.
26. Sakai T, Sasakawa C, Yoshikawa M (1988) Expression of four virulence antigens of *Shigella sonnei* is controlled through the promoter to transcriptional repressor H-NS of *S. sonnei*. Microbiology (Reading, England) 134 (Pt 2): 201–207.
27. Castellanos MI, Harrison DJ, Smith JM, Labahn SK, Levy KM, et al. (2009) VirB alleviates H-NS repression of the *sciF* promoter in *Shigella* from sites more than one kilobase upstream of the transcription start site. Journal Of Bacteriology 191: 4047–4050.
28. Masse E, Gottesman S (2002) A small RNA regulates the expression of genes involved in iron metabolism in *Escherichia coli*. Proc Natl Acad Sci U S A 99: 4620–4625.
29. Afonyushkin T, Vecerek B, Moll I, Blasi U, Kaberdin VR (2005) Both RNase E and RNase III control the stability of sodB mRNA upon translational inhibition by the small regulatory RNA RyhB. Nucleic Acids Res 33: 1678–1689.

30. Vecerek B, Moll I, Afonyushkin T, Kaberdin V, Blasi U (2005) Interaction of the RNA chaperone Hfq with mRNAs: direct and indirect roles of Hfq in iron metabolism of Escherichia coli. Mol Microbiol 50: 897–909.

31. Masse E, Escorcia FE, Gottesman S (2003) Coupled degradation of a small regulatory RNA and its mRNA targets in Escherichia coli. Genes Dev 17: 2374–2383.

32. Prevost K, Salvail H, Desnoyers G, Jacques J-Fo, Planeuf E, et al. (2007) The small RNA RyhB activates the translation of shiA mRNA encoding a permease of shikimate, a compound involved in siderophore synthesis. Molecular Microbiology 66: 1260–1273.

33. Desnoyers G, Morissette A, Prevost K, Masse E (2009) Small RNA-induced differential degradation of the polycistronic mRNA iscRSUA. The EMBO Journal 28: 1551–1561.

34. Payne SM, Finkelstein RA (1977) Detection and differentiation of iron-responsive avirulent mutants on Congo red agar. Infect Immun 18: 94–98.

35. Oglesby AG, Murphy ER, Iyer VR, Payne SM (2005) Fur regulates acid resistance in Shigella flexneri via RyhB and ydeP. Mol Microbiol 58: 1354–1367.

36. Payne SM, Wyckoff EE, Murphy ER, Oglesby AG, Boulette ML, et al. (2006) Iron and pathogenesis of Shigella: iron acquisition in the intracellular environment. Biometals: An International Journal On The Role Of Metal Ions In Biology, Biochemistry, And Medicine 19: 173–180.

37. Lucchini S, Liu H, Jin Q, Hinton JC, Yu J (2005) Transcriptional adaptation of Shigella flexneri during infection of macrophages and epithelial cells: insights into the strategies of a cytotoxic bacterial pathogen. Infect Immun 73: 80–102.

38. Runyen-Janecky LJ, Payne SM (2002) Identification of chromosomal Shigella flexneri genes induced by the eukaryotic intracellular environment. Infect Immun 70: 4379–4388.

39. Miller VI, Mekalanos JJ (1988) A novel suicide vector and its use in construction of insertion mutations: osmoregulation of outer membrane proteins and virulence determinants in Vibrio cholerae requires toxR. Journal Of Bacteriology 170: 2575–2583.

40. Milt M, Payne SM (1997) Identification of shuA, the gene encoding the heme receptor of Shigella dysenteriae, and analysis of invasion and intracellular multiplication of a shuA mutant. Infect Immun 65: 5358–5363.

41. Formal SB, Dammin GJ, Labrec EH, Schneider H (1936) Experimental Shigella infections: characteristics of a fatal infection produced in guinea pigs. Journal Of Bacteriology 75: 604–610.

42. Wyckoff EE, Mey AR, Leimbach A, Fisher CF, Payne SM (2006) Characterization of ferric and ferrous iron transport systems in Vibrio cholerae. Journal Of Bacteriology 188: 6515–6523.