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Detection and characterization of the *S. typhimurium* HilA protein

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Abstract

Background: Virulence genes on *Salmonella* pathogenicity island 1 (SPI1) are coordinately regulated by HilA, a member of the OmpR/ToxR family of transcription factors. Although a great deal is known about the complex regulation of *hilA* gene expression, very little is known about the HilA protein.

Results: In order to detect and localize the HilA protein in *S. typhimurium*, we raised polyclonal antiserum against purified His-tagged HilA. This allowed us to study the effect of environmental conditions on the production of HilA. We also used the antiserum to examine the fractionation properties and SDS-PAGE mobility of native HilA. Our results indicate that *S. typhimurium* initiates translation of HilA from the first AUG codon in the *hilA* open-reading frame (ORF), producing a soluble 553 amino acid (63 kDa) protein product.

Conclusion: Materials and methods are now available to study the environmental regulation of the HilA protein in *S. typhimurium*. Our results also indicate that future *in vitro* studies of the interaction between HilA and DNA should utilize soluble preparations of HilA. Previous analyses used preparations of HilA in which the protein fractionated with the membrane, greatly limiting the types of experiments that could be conducted.

Background

Salmonella pathogenicity island 1 (SPI1), a 40 kb region of the *Salmonella typhimurium* chromosome, encodes a type III secretion system that is important for virulence [1,2]. SPI1 genes that encode components of the type III secretion apparatus are directly activated by HilA [3,4]. HilA is a member of the OmpR/ToxR family of transcription regulators based on homology of its N-terminal domain to the conserved OmpR/ToxR DNA binding and transcription activation domain [5]. Members of this family typically bind degenerate direct repeats as a dimer [6,7]. Although HilA also appears to activate gene expression by binding to direct repeats, other features of HilA deviate from those of well-characterized family members [5,7,8]. Whereas OmpR and PhoB are regulated by phosphorylation of an N-terminal phosphoryl-acceptor domain, HilA lacks homology to such a domain. Although ToxR and CadC also lack a phosphoryl-acceptor domain, HilA lacks a predicted membrane spanning domain which is characteristic of these two family members.

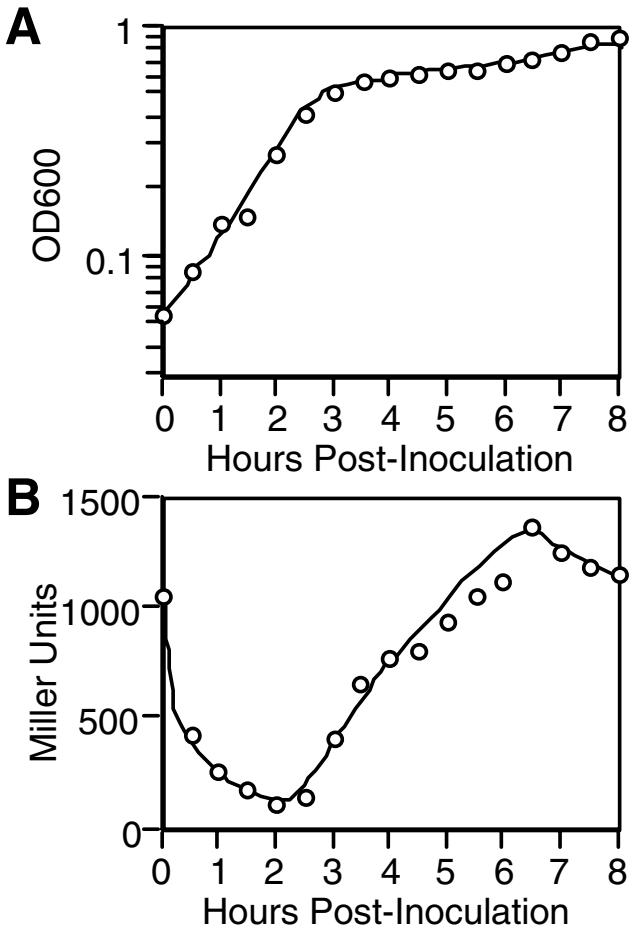

Figure 1
***S. typhimurium* grown in a high osmolarity, low aeration condition** EE658 (*hilA080::Tn5lacZY*) was grown in a high osmolarity, low aeration condition. A. Growth curve. OD600s of individual cultures plotted over an 8 hour growth period. B. *hilA-lacZ* expression. Miller Units of individual cultures plotted over an 8 hour growth period. Values represent the average from three independent cultures assayed at each time point.

HilA is thought to coordinate the regulation of SPI1 genes in response to environmental and regulatory factors. Previous studies have concluded that the regulation of SPI1 genes is primarily controlled by changes in *hilA* transcription [9–11]. It has been assumed that there is a direct correlation between *hilA* transcription, production of the HilA protein and HilA-activated gene expression. Here, we test this hypothesis by examining the effects of environmental conditions on HilA protein levels in *S. typhimurium*.

Results and Discussion

Induction and identification of HilA in S. typhimurium

SPI1 gene expression is induced in high osmolarity and low oxygen conditions [10]. Although standing cultures have been used to achieve these inducing conditions, such cultures require 14 hours for growth and are not convenient for following SPI1 regulatory events as they occur. We developed an alternative high osmolarity, low oxygen culture condition and examined the induction of *hilA* expression. We diluted a stationary phase EE658 (*hilA080::*Tn*5lacZY*) culture 1:100 into poorly aerated LB (see Methods). As seen in Figure 1A, such a bacterial culture achieves mid-log, late-log, and early-stationary phases during an 8 hour period. Measurement of β-galactosidase, in Miller Units, expressed from the chromosomal *hilA080::*Tn*5lacZY* fusion, shows that there is a significant level of β-galactosidase already present in the stationary phase inoculum (Fig. 1B). Interestingly, soon after inoculation, β-galactosidase levels decrease, presumably due to poor *hilA* expression during the first 2 hours post-inoculation and dilution of the pre-existing β-galactosidase by cell growth. Approximately 3 hours post-inoculation, conditions apparently become favorable for *hilA* expression as evidenced by an increase in Miller Units. Previous studies would suggest that reduced oxygen levels, caused by the increasing density and insufficient aeration of these bacterial cultures, trigger the induction of *hilA*[12–14].

In order to detect the HilA protein in *S. typhimurium*, we solubilized and purified a His-tagged version of HilA (HilA-His) in 0.5% SDS for injection into a rabbit. The resultant antiserum was used to identify and characterize the HilA protein in a *hilA*$^+$ strain. Western blots show that our antiserum recognizes two *S. typhimurium* proteins (Fig. 2). The 65.5 kDa reactive protein is present in both the *hilA*$^+$ and the *hilA*$^-$ strain, whereas the 63 kDa reactive protein is clearly *hilA*-specific. Interestingly, similar to the β-galactosidase levels expressed from the *hilA080::*Tn*5lacZY* fusion, the *hilA*-specific protein is present early after dilution of the stationary phase inoculum, disappears by 2 hours post-inoculation, and then increases 3 hours post-inoculation. These results suggest that production of the HilA protein correlates with *hilA* gene expression in these growth conditions.

Effect of a shift to aerobic growth conditions on SPI1 gene expression and HilA protein levels in S. typhimurium

Previous studies have shown that SPI1 gene expression is repressed by steady-state aerobic growth conditions [10,15]. We decided to examine SPI1 gene expression and HilA protein production in *S. typhimurium* after a shift from inducing low oxygen to repressing aerobic growth conditions. First, we grew strains CL87 and EE656 in our high osmolarity, low aeration growth conditions. CL87

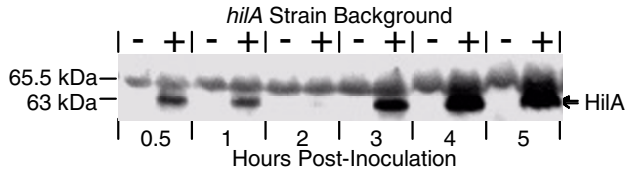

Figure 2
Detection of HilA protein in *S. typhimurium* *hilA*$^+$ strain EE656 (+) and *hilA*$^-$ strain EE668 (-) were grown in high osmolarity, low aeration conditions. Whole cell samples were harvested over a 5 hour growth period and proteins were subjected to Western analysis using polyclonal antiserum raised against HilA-His.

contains the *iagB87::lacZY* fusion, which is located immediately downstream of *hilA* and is used to monitor chromosomal *hilA* expression [11]. EE656 contains the *prgH020::*Tn*5lacZY* fusion [10]. HilA binds upstream of and directly activates the *prgH* promoter [4]. So, we used the *prgH020::*Tn*5lacZY* fusion to monitor HilA-activated SPI1 gene expression. After growing these strains for 3.5 hours to induce *hilA* expression and HilA protein production, we diluted the bacteria 1:10 into fresh media and shifted the cultures to a high aeration vs. a low aeration growth condition, for 0.5, 1.5 and 2.5 hours. Figure 3A shows that the diluted bacteria continue to grow in both conditions, with the high aeration condition promoting more rapid bacterial growth. Figure 3B shows that β-galactosidase levels decrease in CL87 and EE656 shifted to the high aeration condition, presumably due to aerobic repression of *hilA* and *prgH* transcription, and dilution of pre-existing β-galactosidase by cell growth. In contrast, bacteria shifted to the low aeration condition appear to maintain high, induced levels of *hilA* and *prgH* expression.

Western blots show that high levels of HilA are present in the pre-shifted cells as well as in the bacteria shifted to the low aeration condition (Fig. 3C). However, HilA levels decrease in bacteria shifted to the high aeration condition, which correlates with the reduced transcription of *hilA* and *prgH*. These results suggest that repression of *hilA* transcription by oxygen leads to a decrease in HilA protein production, which in turn reduces the expression of HilA-activated genes, such as *prgH*.

SDS-PAGE mobility of native HilA vs. engineered forms of HilA

The *hilA* ORF contains two possible AUG start codons that are separated by 22 codons. We examined native HilA in SDS-PAGE to see if it corresponds to the predicted AUG1-TAA translation product (553 amino acids, 63 kDa) or to the predicted AUG2-TAA translation product (531 amino acids, 60.4 kDa). We compared the migration of HilA produced by wild-type *S. typhimurium* with engineered deriv-

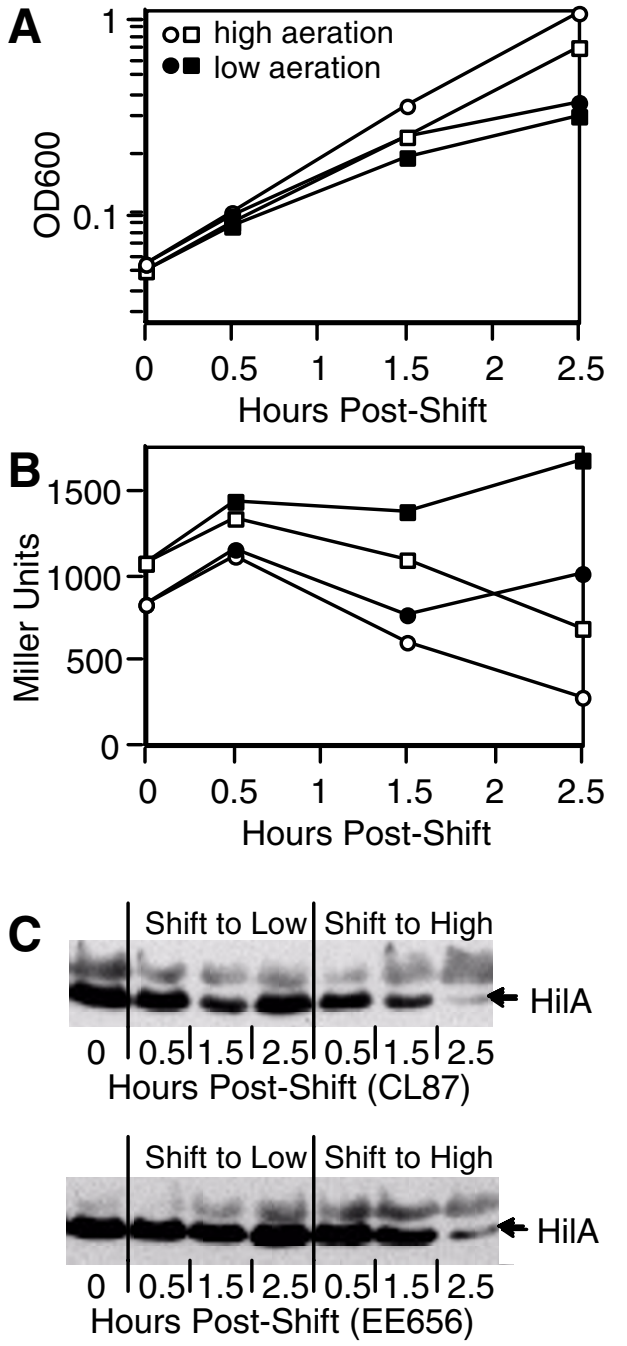

Figure 3
Effect of shifts in aeration conditions on growth, SPI1 gene expression and HilA levels CL87 (*iagB87::lacZY*) (circles) and EE656 (*prgH020::Tn5lacZY*) (squares) were grown for 3.5 hours in high osmolarity, low aeration conditions and then shifted to high aeration (open symbols) and low aeration (closed symbols) growth conditions for an additional 0.5, 1.5, 2.5 hours. A. Growth curves. OD600s of individual cultures. B. *iagB-lacZ* and *prgH-lacZ* expression. Miller Units of individual cultures. C. HilA protein levels. Western blots of protein samples from individual cultures.

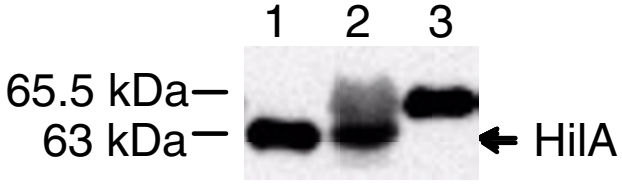

Figure 4
Mobility of native HilA and engineered forms of HilA The migration of proteins from wild-type *S. typhimurium* SL1344 grown for 3 hours in high osmolarity, low aeration conditions (lane 2) is compared to that of a 553 amino acid (63 kDa) derivative of HilA produced by pCR53 (lane 1) and to that of a 575 amino acid (65.5 kDa) HilA-Myc-His derivative produced by pCH112 (lane 3). 50-fold less of the pCH112 and pCR53 samples were run on the gel, as compared to the SL1344 sample, to compensate for the higher level of HilA produced from the arabinose-inducible plasmid constructs. Western analysis was conducted using polyclonal antiserum raised against HilA-His.

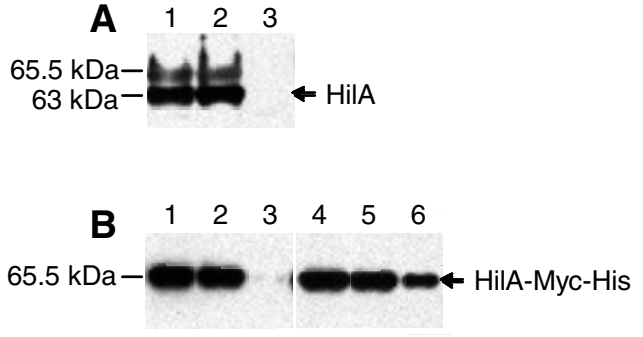

Figure 5
Subcellular fractionation of HilA proteins Proteins from cleared cell lysates (lanes 1 and 4), soluble fractions (lanes 2 and 5), and membrane pellet fractions (lanes 3 and 6) were detected by Western analysis using polyclonal antiserum raised against HilA-His. A. Native HilA expressed in *S. typhimurium* strain EE656 grown in high osmolarity, low aeration conditions for 3 hours. B. HilA-Myc-His expressed in *E. coli* DH5α/pCH112. Samples harvested one hour after induction with arabinose (lanes 1,2,3) vs. two hours after induction with arabinose (lanes 4,5,6).

atives of HilA. pCR53 was designed to express the AUG1-TAA product (63 kDa). pCH112 was designed to express a HilA derivative tagged with an extra 22 amino acids (LEQKLISEEDLNSAVDHHHHHH), which we call HilA-Myc-His (65.5 kDa). As shown in Figure 4, native HilA co-migrates with the engineered 63 kDa form of HilA. Our electrophoresis conditions can clearly distinguish the migration of HilA proteins that differ by 2.5 kDa, indicating that native HilA does not correspond to the AUG2-TAA 60.4 kDa product. Instead, our results indicate that native HilA is produced by translation of the *hilA* ORF from the first AUG codon. This conclusion is consistent with results showing that pCR53 expresses transcriptionally active HilA (data not shown). Unfortunately, we have not been able to construct a plasmid that expresses the AUG2-TAA translation product and so cannot examine this form in SDS-PAGE or in functional assays.

Sub-cellular fractionation of native HilA vs. HilA-Myc-His

Previous studies have shown that HilA-Myc-His, expressed from an arabinose-inducible promoter, binds to DNA containing the HilA box and activates gene expression [3]. Interestingly, HilA-Myc-His was found to pellet with bacterial membrane fractions [3]. The regulatory properties of many bacterial transcription factors are determined by their ability to associate with membranes [16–19]. We examined whether native HilA associates with the membrane by separating *S. typhimurium* cells into soluble and membrane fractions. We found native HilA in the soluble fraction (Fig. 5A). To investigate why HilA-Myc-His pelleted with the membrane in previous experiments, we induced the expression of HilA-Myc-His in *E. coli* by addition of arabinose as was done previously. We examined the subcellular fractions of cells harvested at one hour and 2 hours post-induction. Interestingly, we found that HilA-Myc-His is soluble at the one hour time period, but appears in both the soluble and membrane pellet fractions at the 2 hour time period (Fig. 5B). Previously, cell extracts in which HilA-Myc-His was only found in the membrane fraction had been prepared from cells induced with arabinose for 4–5 hours. These results indicate that native HilA and the engineered HilA-Myc-His protein are inherently soluble. The fractionation of HilA-Myc-His with membranes may be an artifact of overproduction.

Conclusions

The activity of many OmpR/ToxR family members is modulated by environmental conditions [8]. HilA has a unique C-terminal domain, which could mediate its modulation by environmental or regulatory factors. Our current results support the idea that the regulation of SPI1 gene expression by oxygen is primarily mediated by regulating *hilA* transcription. However, it is possible that other regulatory signals control SPI1 gene expression by affecting HilA post-translationally. By developing materials and methods to detect HilA protein in *S. typhimurium*, we can now investigate whether HilA activity is modulated. We may find conditions in which HilA protein levels are high, but expression of HilA-activated SPI1 genes is extremely low, suggesting that HilA activity is down-modulated by these conditions. Alternatively, we may find a condition which down-modulates HilA activity by triggering its deg-

radation. *S. typhimurium* encounters many different intracellular and extracellular environments during infection. The complex regulation of SPI1 virulence genes may be important to activate SPI1 gene expression during infection, but may also be important to down-regulate SPI1 gene expression when production of the type III secretion system would induce a bacteriocidal host response [20,21]. Our current work on the HilA protein is an important first step towards future studies aimed at discovering non-transcriptional mechanisms that regulate expression of SPI1 virulence genes.

Methods

S. typhimurium strains and growth conditions

S. typhimurium strains used are all derivatives of SL1344. EE658 (*hilA080::*Tn*5lacZY*), CL87 (*iagB87::lacZY*), EE656 (*prgH020::*Tn*5lacZY*) and EE668 (*hilA339::kan prgH020::*Tn*5lacZY*) are described in [10,11,22]. LM558 (*hilA339::kan ΔaraBAD22 invF12-5::*Tn*5lacZY*) carrying pCR53 and pCH112 were used to express engineered versions of HilA from an arabinose-inducible promoter. The *hilA* ORF contains two possible AUG start codons that are separated by 22 codons. pCR53 expresses a 553 amino acid (63 kDa) derivative of HilA, which starts at the first AUG codon and ends at the natural stop codon of the chromosomal *hilA* ORF. pCH112 expresses a 575 amino acid (65.5 kDa) derivative of HilA, which starts at the first AUG codon and ends with an extra 22 amino acid C-terminal Myc-His-tag [3]. *S. typhimurium* cultures were grown in 16 × 150 mm tubes at 37°C in Luria-Bertani (LB) medium composed of 0.5% Bacto-yeast extract, 1% Bactotryptone, and 1% NaCl.

To induce *hilA* expression and the production of HilA protein, *S. typhimurium* were grown under high osmolarity, low aeration conditions. Bacteria were first grown to saturation in 3 ml LB, by rolling tubes at an angle overnight. The overnight culture was then diluted 1:100 in fresh LB and 10 ml aliquots were shaken at 150 rpm in upright tubes. Aliquots were harvested at various times post-inoculation for analysis. To analyze SPI1 gene expression and HilA protein levels in*S. typhimurium* shifted from inducing conditions to high aeration vs. low aeration conditions, bacteria were grown in 10 ml aliquots as described above. At 3.5 hours post-inoculation, the induced culture was diluted 1:10 in fresh LB. The shift to high aeration conditions was achieved by rolling tubes containing 2 ml aliquots at an angle. The shift to low aeration conditions was achieved by shaking 10 ml aliquots in upright tubes at 150 rpm. β-Galactosidase assays were performed and Miller Units were calculated as described [23].

To compare native HilA to engineered HilA derivatives, SL1344 or LM558, carrying pCR53 or pCH112, were grown as described above in high osmolarity, low aeration conditions, except that the media for the LM558 strains was supplemented with 0.2% arabinose and 200 μg/ml ampicillin. Cells were harvested by centrifugation after 4 hours and boiled in 1 × SDS loading buffer.

HilA protein purification and production of polyclonal antisera

E. coli strain VV445 (BL21/pET15b-*hilA*) produces a C-terminal His-tagged derivative of HilA, HilA-His, that was purified and used for antibody production. pET15b-*hilA* was generated by cloning a DNA fragment containing 98% of the predicted *hilA* ORF, from the second AUG to the *Bam*HI site, into pET15b (Novagen, Inc.). 100 mL of VV445 culture was grown in LB to OD_{600} 0.4–0.6 at 37°C, induced by the addition of 0.5 mM IPTG, and cultured for an additional 1.5 hours at 30°C. Cells were harvested by centrifugation at 5000 × g and resuspended in 10 mL 50 mM Tris-HCl pH 8, 2 mM EDTA, 0.1 mg/ml lysozyme, 0.1% TritonX-100. After a 20 minute incubation, the cells were lysed by sonication and centrifuged at 12000 rpm in an SS34 rotor for 20 minutes. The membrane fraction contained HilA-His and was resuspended in 4 mL of 1 × binding buffer (1 × BB: 20 mM Tris-HCl pH 7.9, 500 mM NaCl, 0.5% SDS, 10% glycerol). After incubation for 10 minutes at 30°C, the solution was passed through a 0.45 μm low protein binding disk filter and loaded onto a 2 mL bed volume of His-bind resin at room temperature. The resin was washed extensively with 1 × BB and then with 1 × BB + 20 mM imidazole. Protein was eluted with 1 × BB + 1 M imidazole and analyzed by SDS-PAGE and Coomassie staining. A 2 mg/mL solution of the purified HilA-His protein was used to inoculate a rabbit (Cocalico Biologicals, Inc.). The initial injection contained 100 μg HilA-His and Freund's complete adjuvant, and boosters contained 50 μg HilA-His and Freund's incomplete adjuvant.

Subcellular fractionation and protein analysis

To examine the subcellular localization of native HilA in *S. typhimurium*, strain EE656 was grown in inducing, high osmolarity, low aeration conditions as described above. The *S. typhimurium* cells were harvested by centrifugation for analysis at 3 hours post-inoculation. To examine the subcellular localization of HilA-Myc-His in *E. coli*, an overnight culture of DH5α/pCH112 was diluted 1:1000 in fresh LB, shaken in a flask for 2.5 hours, and then supplemented with 0.2% arabinose. The *E. coli* cells were harvested by centrifugation 1 hour and 2 hours after the addition of arabinose. Bacterial cells were lysed by sonication in lysis buffer (20 mM HEPES pH 7.0, 1 mM DTT, 1 mM EDTA, 20% glycerol, 100 mM NaCl). Unbroken cells were removed by low-speed centrifugations and the cleared cell lysates were ultracentrifuged in a TLA100.3 rotor for 2 hours at 45 K rpm 4°C to separate the membrane and soluble fractions.

Samples were boiled for 10 minutes in 1 × SDS loading buffer and were electrophoresed on 5% stacking/7.5% resolving SDS-PAGE gels at 25 mA for 5.5–6 hours until a 45 kDa pre-stained protein standard was running off the bottom of the gel. This protocol was necessary to clearly separate the *hilA*-specific 63 kDa protein from the 65.5 kDa reactive protein that was present in both *hilA*$^-$ and *hilA*$^+$ strains. Proteins were transferred from the gel to a PVDF membrane in a semi-dry blotter at 150 mA for 2 hours. HilA was detected following Western analysis using a 1:5000 dilution of polyclonal antiserum in TBST (20 mM Tris-HCl pH 7.5, 150 mM NaCl, 0.1% Tween 20) containing 1% BSA, followed by secondary detection using a goat anti-rabbit horseradish peroxidase conjugate diluted 1:10,000 in TBST. Whole cell and subcellular samples were normalized relative to bacterial number as estimated from OD600 measurements of the original cultures. Normalization was verified by running samples on SDS-PAGE and examining protein levels by Coomassie staining.

Authors' contributions

CRR prepared a draft of this manuscript, constructed pCR53, and analyzed SPI1 gene expression, HilA levels and HilA localization in *S. typhimurium* grown in different conditions. LMS purified the His-tagged HilA and produced the polyclonal antiserum. CAL prepared the submitted manuscript and conducted some of the experiments shown.

All authors read and approved the final manuscript.

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