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Identification of HilD-Regulated Genes in *Salmonella enterica* Serovar Typhimurium

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Salmonella enterica **serovar Typhimurium (*S.* Typhimurium) pathogenicity island 1 (SPI-1) encodes a type III secretion system required for invasion of host gut epithelial cells. Expression of SPI-1 virulence genes is controlled by a complex hierarchy of transcription factors encoded within and outside SPI-1. The master regulator of SPI-1, HilA, is itself regulated by three homologous transcription factors, HilD, HilC, and RtsA. HilD activates transcription of *hilA* and other target genes in response to environmental conditions associated with the intestinal microenvironment of the host. We have mapped the binding of HilD across the *S.* Typhimurium genome using chromatin immunoprecipitation-sequencing (ChIP-seq). Thus, we have identified 17 regions bound by HilD, including 11 novel targets. The majority of HilD targets are located outside SPI-1. We demonstrate transcription activation of 8 genes by HilD; four of these genes have not been previously described as being regulated by HilD, including *lpxR*, which encodes a lipid A deacylase important for immune evasion. We also show that HilD-activated genes are frequently activated by HilC and RtsA, indicating extensive overlap of the HilD, HilC, and RtsA regulons.**

Salmonella enterica subsp. *enterica* serovar Typhimurium (*S.* Typhimurium) is a Gram-negative enteric pathogen that is a leading cause of bacterial food-borne illness worldwide. Capable of infecting a broad range of animal species, *S.* Typhimurium infection in humans typically manifests as a self-limiting gastroenteritis but in approximately 5% of cases progresses to a severe, potentially fatal bacteremia (1). In addition to its relevance as a pathogen, *S.* Typhimurium serves as an important model for host-pathogen interactions during infection (2). Successful *S.* Typhimurium pathogenesis is the consequence of complex temporal regulation of sets of virulence genes, each specific to a particular microenvironment encountered within the host.

An essential early step in the progression of *S.* Typhimurium infection is bacterial invasion of host intestinal epithelial cells. Invasion is facilitated by genes in a 40-kb region of horizontally acquired DNA known as *Salmonella* pathogenicity island 1 (SPI-1), one of several pathogenicity islands on the *Salmonella* chromosome (3). SPI-1 encodes a type III secretion system (T3SS) that is required for invasion of host cells and intestinal disease (4). The SPI-1 T3SS is a multiprotein, needle-like complex that delivers effector proteins to the host cell cytoplasm; these effectors directly induce rearrangements of the actin cytoskeleton, leading to membrane ruffling and uptake of invading *Salmonella* by these otherwise nonphagocytic cells (5).

Appropriate expression of the SPI-1 T3SS is controlled by a combination of regulatory proteins encoded both inside and outside the pathogenicity island. The OmpR/ToxR family transcription factor HilA is considered to be the master regulator of SPI-1, as it is capable of activating expression of all SPI-1 genes required for the assembly of a functional T3SS (6). However, HilA activation is dependent upon a coupled, positive-feedback loop comprising the AraC/XylS family transcription factors HilD, HilC, and RtsA, which drive HilA expression above the SPI-1 activation threshold (7, 8). HilD, HilC, and RtsA are capable of self-activating expression in addition to activating expression of one another and of *hilA*. Of these three regulators, HilD is considered to be dominant, as deletion of *hilD* nearly abolishes *hilA* expression (9–11), whereas deletion of either *hilC* or *rtsA* does not. Additionally, HilD serves as the integration point of various activating environmental signals, including low oxygen, iron limitation, and osmolarity, which together indicate the intestinal microenvironment of the host appropriate for invasion gene expression (12, 13).

HilD has mainly been studied in the context of *hilA* regulation (7) and regulation of other known virulence genes (14). Previously described HilD regulatory targets are *hilD* itself, *hilA*, *hilC*, *rtsA*, *invF*, and downstream genes (the *inv-spa* and *sic-sip* operons), *invR*, *dsbA*, *slrP*, *ssrAB* (6), and genes in SPI-4 (15). Most of these genes were identified as being regulated by HilD by using targeted experiments. Next-generation sequencing methods, such as chromatin immunoprecipitation-sequencing (ChIP-seq), can be used to probe regulatory effects in a much more comprehensive and unbiased manner. ChIP with microarray technology (ChIP-chip) and ChIP-seq enable quantitative, genome-wide mapping of both the position and magnitude of transcription factor binding sites (16). Even for well-studied transcription factors, ChIP-chip and ChIP-seq experiments have repeatedly revealed novel binding sites, including those previously considered to be noncanonical in nature (e.g., within genes and upstream of seemingly unrelated genes) (17–19). To date, ChIP-seq and other genome-scale approaches have been sparingly applied to study the layered regulation of *S.* Typhimurium virulence (19–25).

In this work, we combine ChIP-seq and targeted reporter gene assays to map the regulon of HilD in *S.* Typhimurium and identify regulation upon HilD binding. We identify 11 novel HilD-bound

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TABLE 1 List of strains and plasmids used in this study

Strain or plasmid	Description	Source or reference
Strains		
E. coli AMD054	MG1655 Δ*lacZ*	27
S. Typhimurium		
14028s	Wild type	59
BLP006	14028s *hilD*-$FLAG_3$	31
NR-40655	14028s Δ*hilD*	BEI Resources
Plasmids		
pAMD-BA-*lacZ*	Single-copy *lacZ* expression vector, encodes chloramphenicol resistance	27
pBAD24	Expression plasmid with P_{BAD} promoter	26
pBLP013	pBAD24-*hilD*	This work
pBLP011	pBAD24-*hilC*	This work
pBLP010	pBAD24-*rtsA*	This work
pBLP002	pAMD-BA-*lacZ* with *STM14_1282* upstream region	This work
pBLP003	pAMD-BA-*lacZ* with *STM14_2342* upstream region	This work
pBLP005	pAMD-BA-*lacZ* with *hilD* upstream region	This work
pBLP006	pAMD-BA-*lacZ* with *hilA* upstream region	This work
pBLP008	pAMD-BA-*lacZ* with *siiA* upstream region	This work
pBLP009	pAMD-BA-*lacZ* with *rtsA* upstream region	This work
pAMD144	pAMD-BA-*lacZ* with *lpxR* upstream region	This work
pAMD151	pAMD-BA-*lacZ* with *ytfK* upstream region	This work
pAMD145	pAMD-BA-*lacZ* with *STM14_1613* upstream region	This work
pAMD147	pAMD-BA-*lacZ* with *mcpC* upstream region	This work
pAMD149	pAMD-BA-*lacZ* with *ytfJ* upstream region	This work
pAMD150	pAMD-BA-*lacZ* with *STM14_5291* upstream region	This work
pAMD152	pAMD-BA-*lacZ* with *flhD* upstream region	This work
pBLP001	pAMD-BA-*lacZ* with *sinR* upstream region	This work
pBLP004	pAMD-BA-*lacZ* with *STM14_2343* upstream region	This work
pBLP007	pAMD-BA-*lacZ* with *STM14_5116* upstream region	This work
pAMD148	pAMD-BA-*lacZ* with *STM14_5184* upstream region	This work

regions and four novel HilD-regulated genes. We also show regulation of many HilD target genes by HilC and RtsA. To our knowledge, this is the first study to globally map the regulon of this transcription factor essential to *S.* Typhimurium virulence.

MATERIALS AND METHODS

Strains and plasmids. All strains and plasmids used in this work are listed in Table 1. All oligonucleotides used in this work are listed in Table S1 in the supplemental material.

hilD, *hilC*, and *rtsA* were cloned into pBAD24 (26) for arabinose-inducible expression. PCR products for each gene were generated and cloned into NheI and SphI restriction sites using standard ligation. PCR products were generated with the following oligonucleotides: plasmid pBLP013 (*hilD*) with oligonucleotides JW2401 and JW2402, plasmid pBLP011 (*hilC*) with oligonucleotides JW2399 and JW2400, and plasmid pBLP010 (*rtsA*) with oligonucleotides JW2403 and JW2404.

Regions upstream of candidate HilD-regulated genes were cloned upstream of *lacZ* in the single-copy plasmid pAMD-BA-*lacZ* (27). All upstream regions are described in Fig. S2 and S3 in the supplemental material. PCR products for each upstream region were generated and cloned into SphI and HindIII restriction sites. Cloning was performed using either standard ligation or using an In-Fusion kit (Clontech). PCR products were generated using the primers listed in Table S2 in the supplemental material.

ChIP-qPCR and ChIP-seq. The ChIP methods are based on those described previously (28). Wild-type 14028s or BLP006 (14028s *hilD*-$FLAG_3$) *S.* Typhimurium cells were grown overnight in LB (1% NaCl, 1% tryptone, 0.5% yeast extract), subcultured 1/100 in 20 ml, and grown in screw-cap 50-ml tubes with shaking (250 rpm) to an optical density at 600 nm (OD_{600}) of ~1.0. Expression of SPI-1 genes has been observed previously under similar growth conditions (14). Cells were cross-linked for 20 min with formaldehyde (1% final concentration), pelleted by centrifugation, and washed once with Tris-buffered saline (TBS). Cell pellets were resuspended in 1 ml FA lysis buffer (50 mM HEPES-KOH [pH 7], 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 0.1% sodium deoxycholate, 0.1% SDS) with 2 mg/ml lysozyme and incubated at 37°C for 30 min. Samples were then chilled and sonicated for 30 min in a Bioruptor sonicator (Diagenode) with 30-s-on/30-s-off pulsing at maximum amplitude. Samples were pelleted in a microcentrifuge to remove debris, and supernatants (chromatin) were saved, 1 ml FA lysis buffer was added, and samples were stored indefinitely at −20°C.

For each immunoprecipitation (IP), 500 μl chromatin was incubated with 300 μl FA lysis buffer, 20 μl protein A-Sepharose slurry (50%) in TBS, and 2 μl M2 anti-FLAG antibody (Sigma) for 90 min at room temperature with gentle mixing on a LabQuake rotisserie rotator (Thermo Scientific). Beads were then pelleted at 1,500 × *g* in a microcentrifuge for 1 min. The supernatant was removed, and the beads were resuspended in 750 μl FA lysis buffer and transferred to a Spin-X column (Corning). Beads were then incubated for 3 min with gentle mixing on a rotisserie rotator before being pelleted at 1,500 × *g* in a microcentrifuge for 1 min. Equivalent washes were performed with FA lysis buffer, high-salt FA lysis buffer (50 mM HEPES-KOH [pH 7], 500 mM NaCl, 1 mM EDTA, 1% Triton X-100, 0.1% sodium deoxycholate, 0.1% SDS), ChIP wash buffer (10 mM Tris-HCl [pH 8.0], 250 mM LiCl, 1 mM EDTA, 0.5% Nonidet P-40, 0.5% sodium deoxycholate), and TE buffer (10 mM Tris-HCl [pH 7.5], 1 mM EDTA). After the TE wash, beads were transferred to a fresh Spin-X column and eluted with 100 μl ChIP elution buffer (50 mM Tris-HCl [pH 7.5], 10 mM EDTA, 1% SDS) for 10 min at 65°C with occasional agitation. Eluted samples were centrifuged at 1,500 × *g* in a microcentrifuge for 1 min. Supernatants were de-cross-linked by boiling for 10 min and cleaned up using a PCR purification kit (Qiagen). For all ChIP-quantitative PCR (qPCR) experiments, 20 μl untreated chromatin was de-cross-linked by boiling for 10 min and cleaned up using a PCR purification kit (Qiagen). This sample served as the "input" control.

For qPCR, ChIP and input samples were analyzed using an ABI 7500 fast real-time PCR machine, as described previously (17). Enrichment of ChIP samples was calculated relative to the control region within *sbcC* and normalized to input DNA. Occupancy units represent background-subtracted fold enrichment. The oligonucleotides used for real-time PCR were JW1495 and JW1496 (*sbcC*), JW2412 and JW2413 (*sinR*), JW2414 and JW2415 (*STM14_1282*), JW2418 and JW2419 (*lpxR*/*STM14_1613*), JW2424 and JW2425 (*STM14_2342*/*3*), JW2430 and JW2431 (*hilC*), JW2434 and JW2435 (*prgH*/*hilD*), JW2436 and JW2437 (*hilA*), JW2442 and JW2443 (*invF*/*H*), JW3454 and JW3455 (*mcpC*), JW3458 and JW3459 (*siiA*), JW2458 and JW2459 (*rtsA*/*STM14_5189*), JW3460 and JW3461 (*ytfJ*/*K*), JW2410 and JW2411 (*ribF*), JW2420 and JW2421 (*STM14_1833*), JW2428 and JW2429 (*sprB*), JW2438 and JW2439 (*sicP*), JW2440 and JW2441 (*invA*), JW2448 and JW2449 (*ygiK*), JW2452 and JW2453 (*STM14_4640*), JW2456 and JW2457 (*STM14_5184*), and JW2462 and JW2463 (*STM14_5405*).

For ChIP-seq, 32 ChIP samples for HilD-$FLAG_3$ cells were pooled (32 were needed to generate enough DNA for a Helicos sequencing run), ethanol precipitated, and resuspended in water to a final volume of 11 μl. For the control sample, 2 μl from each of the 32 chromatin samples was de-cross-linked by boiling for 10 min and cleaned up using a PCR purification kit (Qiagen). DNA from ChIP or control samples was then treated using the NEB quick blunting kit using 1 μl blunting enzyme mix (NEB) according to the manufacturer's instructions. Samples were incubated for 30 min at room temperature, followed by inactivation of the enzyme by

heating samples to 70°C for 10 min. Once cooled, samples were treated with RNase A, incubated for 15 min at 37°C, and purified using the Qiagen PCR purification kit, eluting in 11 μl. DNA (10.8 μl) was then poly(A) tailed by adding 2 μl 2.5 mM $CoCl_2$, 2 μl 10× terminal transferase buffer, heating at 95°C for 5 min, cooling at 4°C, adding 4 μl 50 μM dATP, 0.2 μl bovine serum albumin (BSA), 0.75 μl water, and 0.25 μl terminal transferase and incubating the mixture at 37°C for 1 h and then 70°C for 10 min, followed by cooling to 4°C. Samples were blocked using biotin-ddATP by heating 20 μl poly(A)-tailing reaction mixture at 95°C for 5 min, cooling to 4°C, adding 1 μl 2.5 mM $CoCl_2$, 1 μl 10× terminal transferase buffer, 0.5 μl 200 μM biotin-ddATP, 7.25 μl water, and 0.25 μl terminal transferase, incubating the mixture at 37°C for 1 h and then 70°C for 20 min, followed by cooling to 4°C.

Poly(A)-tailed, ddATP-blocked samples were sequenced using a HeliScope single-molecule sequencer (Helicos Biosciences). Sequence reads were mapped to the *S.* Typhimurium 14028s genome sequence using HeliSphere software (Helicos Biosciences). Putative HilD-bound regions were identified using model-based analysis of ChIP-seq (MACS) (default settings), with both ChIP and input samples (29). The coordinate of the peak center was defined as the position midway between the highest-scoring position on the top strand and the highest-scoring position on the bottom strand. Secondary peaks within two regions were identified by virtue of having >100 mapped sequence reads and being separated from the major peak and other secondary peaks by genome positions with <10% of the number of reads of the major peak.

β-Galactosidase assays. For *Escherichia coli*, 2 to 3 ml of cells were grown in LB ± 0.2% arabinose at 37°C to an OD_{600} of 0.7 to 1.0, and the OD_{600} was recorded. For *S.* Typhimurium, 2 to 3 ml of cells were grown in LB to an OD_{600} of ~1.0. Cells (800 μl) were pelleted at full speed in a microcentrifuge for 1 min. (Eighty microliters was used for strongly active fusions, and this was corrected for at the final calculation step.) Cell pellets were resuspended in 800 μl Z buffer (0.06 M Na_2HPO_4, 0.04 M NaH_2PO_4, 0.01 M KCl, 0.001 M $MgSO_4$) plus 50 mM β-mercaptoethanol (added fresh). Twenty microliters chloroform and 10 μl 0.1% SDS were added to the cells, followed by vortexing for 5 s. Assays were started by addition of 160 μl *o*-nitrophenyl-β-D-galactopyranoside (ONPG) (4 mg/ml in distilled water [dH_2O]) and stopped by addition of 400 μl 1 M Na_2CO_3, upon development of an appropriate yellow color. The reaction time was noted. Samples were centrifuged at full speed in a microcentrifuge to separate the chloroform phase. The OD_{420} of the supernatant was recorded. Assay units were calculated as 1,000 × A_{420}/(A_{600})(total time).

Multiple sequence alignment. Peptide sequences for the HilD, HilC, and RtsA DNA-binding domains were aligned using ClustalW (30).

Microarray data accession number. Raw ChIP-seq data are available through the EMBL-EBI ArrayExpress site under accession no. E-MTAB-1848.

RESULTS

To identify HilD-regulated genes, we mapped the genome-wide association of HilD using ChIP-seq. HilD was C-terminally epitope tagged with three FLAG tags, as described previously (31). We confirmed that FLAG-tagged HilD is fully active by measuring expression from the *hilA* promoter (see Fig. S1 in the supplemental material). We used model-based analysis of ChIP-seq (MACS) (29) to analyze the ChIP-seq data in combination with a control "input" data set from DNA taken prior to immunoprecipitation. Thus, we identified 51 candidate HilD-bound regions (see Table S3 in the supplemental material). We manually subdivided two of these regions (see Materials and Methods), since by visual inspection of aligned sequence reads, it was clear that they included multiple, independent ChIP-seq peaks. To validate the ChIP-seq data, we used ChIP coupled with quantitative real-time PCR (ChIP-qPCR) to measure association of HilD with 21 of the top-scoring ChIP-seq peaks identified by MACS. As a control, we per-

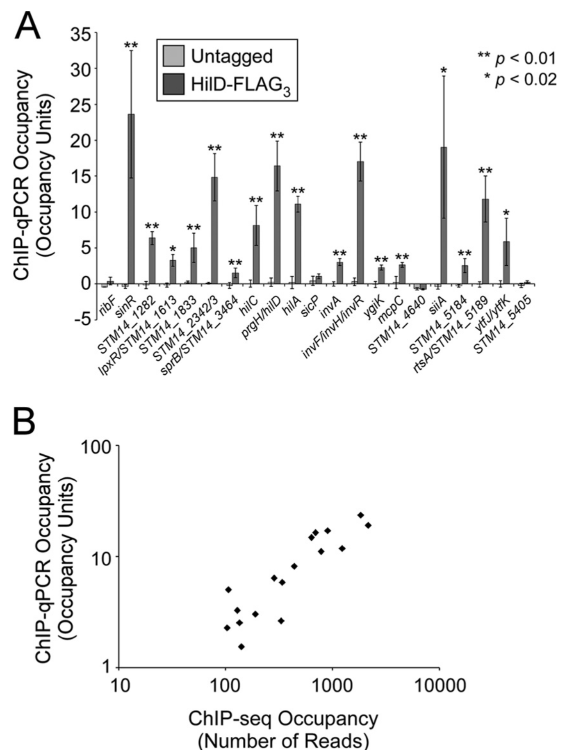

FIG 1 Sites of HilD association identified by ChIP-seq. (A) Validation of putative *S.* Typhimurium HilD target regions identified by ChIP-seq. Data are from ChIP of untagged 14028s or FLAG-tagged HilD, followed by quantitative real-time PCR. Occupancy units represent background-subtracted fold enrichment relative to a control genomic region within the *sbcC* gene. Error bars represent 1 standard deviation from the mean, based on three independent biological replicates, with the exception of *lpxR/STM14_1613*, for which only two replicates were performed. Asterisks indicate a significant difference between the occupancy unit values for tagged and untagged strains (**, $P < 0.01$; *, $P < 0.02$). (B) Comparison of ChIP-seq and ChIP-qPCR data for HilD-$FLAG_3$. Each data point represents a HilD target region identified by ChIP-seq and confirmed by ChIP-qPCR. ChIP-seq scores are the sum of the read counts on both strands at the highest-scoring position. ChIP-qPCR scores are in occupancy units.

formed ChIP-qPCR with an untagged strain. We confirmed association of HilD with 17 of the 21 regions, with significantly greater enrichment observed for the ChIP sample than for the sample from the untagged strain (Fig. 1A) (*t* test, $P < 0.02$ in all cases). Our data suggest that regions identified by MACS with a score of <600 (there are 32 such regions) are likely to be false positives. One of the highest-scoring regions from the MACS analysis showed no significant enrichment in the targeted ChIP-qPCR region. Closer inspection of this region revealed a long, inverted repeat, which is likely to result in anomalous sequence read mapping. Consistent with this, the vast majority of sequence reads mapped to only one of the two DNA strands in this region.

The 17 experimentally confirmed targets are listed in Table 2, and ChIP-seq data are shown for each corresponding MACS region in Fig. S2 in the supplemental material. Six of the confirmed HilD-bound regions are associated with genes that have been previously described as HilD targets: *hilC* (*STM14_3465*), *hilD* (*STM14_3474*), *hilA* (*STM14_3475*), *invF* (*STM14_3498*)/*invR* (ChIP-seq peak too broad to separate two distinct bound regions), *siiA* (*STM14_5117*; regulation was shown only for the down-

TABLE 2 List of ChIP-seq peaks for HilD[a]

Genome coordinate[b]	Nearby gene(s)[c]	ChIP-seq score[d]	ChIP-qPCR score[e]
349261	*sinR* (*STM14_0358*)	1,830	23.6
1172313	***STM14_1282***	284	6.4
1418290	***lpxR*** (***STM14_1612***)/*STM14_1613*	129	3.3
1603868	*STM14_1833*	106	5.0
2034044	*flhD* (*STM14_2341*)/**STM14_2342**/*STM14_2343*	636	14.8
3031987	*sprB* (*STM14_3463*)/*STM14_3464*	140	1.5
3033327	***hilC*** (***STM14_3465***)	440	8.2
3037906	*prgH* (*STM14_3473*)/***hilD*** (***STM14_3474***)	694	16.4
3039616	***hilA*** (***STM14_3475***)	785	11.1
3059473	***invA*** (***STM14_3495***)	190	3.0
3065086	***invF*** (***STM14_3498***)/invH (*STM14_3499*)/***invR***	898	17.0
3352436	*ygiK*	103	2.3
3400740	*mcpC* (*STM14_3893*)	330	2.6
4490820	*STM14_5116*/***siiA*** (***STM14_5117***)	2,156	19.0
4571735	*STM14_5184*	135	2.5
4574788	***rtsA*** (***STM14_5188***)/*STM14_5189*	1,231	11.8
4656019	*ytfJ* (*STM14_5290*)/*STM14_5291*/***ytfK*** (***STM14_5292***)	339	5.9

[a] SPI-1 regions are shaded in gray.
[b] Position (bp) in the genome of the ChIP-seq peak.
[c] Genes confirmed to be regulated by HilD in this or previous studies are indicated in bold text.
[d] Number of sequence reads at the genome coordinate with the most signal in the region.
[e] Occupancy units.

stream gene *siiE*), and *rtsA* (*STM14_5188*) (6, 15, 32, 33). We did not identify HilD binding in regions corresponding to the described HilD targets *dsbA* (*STM14_4806*), *slrP* (*STM14_0928*), and *ssrAB* (*STM14_1687-STM14_1686*) (14, 33, 34). The remaining 11 regions have not previously been linked to HilD, and 9 are located outside SPI-1. Most ChIP-seq peaks are located close to the start of an annotated gene, with the exception of peaks that fall well within the *STM14_1833*, *invA* (*STM14_3495*), and *ygiK* (*STM14_3843*) genes. The levels of association determined by ChIP-qPCR correspond well with those determined by ChIP-seq (Fig. 1B and Table 2). We attempted to identify enriched DNA sequence motifs at the confirmed sites of HilD association using MEME (default settings) (35), but were unable to detect any significant hits, suggesting that HilD binds to a degenerate sequence motif.

ChIP-seq identifies sites of association with DNA but provides no direct information on regulation; for most HilD-bound regions, the likely target gene was unclear from ChIP-seq data alone. Hence, we selected one or more candidate genes that are located close to each confirmed ChIP-seq peak. In total, we identified 17 candidate genes located within or near the confirmed HilD-bound regions and constructed translational fusions of their upstream regions to a *lacZ* reporter gene (see Fig. S2 and S3 in the supplemental material). We then determined the expression of *lacZ* from the 17 constructs in *Escherichia coli* for cells containing either empty vector or plasmid expressing *hilD* from an arabinose-inducible promoter under inducing conditions. We reasoned that measuring expression in *E. coli* with heterologous expression of HilD would avoid the potential cross talk and redundancy that occur in *S.* Typhimurium between HilD and other regulators, such as HilC, RtsA, and HilA. For 9 of the 17 constructs, we detected no effect of HilD on their expression (see Table S4 in the supplemental material). For the remaining 8 constructs, we detected HilD-dependent transcription activation (Fig. 2; see Table S4). In all 8 cases, transcription activation required both the inducible HilD plasmid and the inducer, arabinose. The level of induction by HilD varied from <2-fold for *hilD* itself to 146-fold for *hilA*. We conclude that these 8 genes are direct regulatory targets of HilD. The 8 genes include four described HilD-activated genes: *hilD*, *hilA*, *siiA*, and *rtsA*. We did not test the other described HilD-activated genes within SPI-1, *hilC* and *invF*. Four of the direct regulatory targets of HilD have not been described previously: *STM14_1282*, *STM14_2342*, *lpxR* (*STM14_1612*), and *ytfK* (*STM14_5292*).

HilC and RtsA are homologues of HilD with 62% and 61% identity with HilD, respectively, in their DNA-binding domains (Fig. 3A). HilD, HilC, and RtsA are members of the AraC family of transcription factors. The crystal structure of MarA, another AraC family member, has been solved in its DNA-bound state. By aligning the sequences of HilD, HilC, and RtsA DNA-binding domains with that of MarA using Pfam (36), we predicted the amino acids that make base-specific contacts with DNA. Strikingly, all but one of these positions are identical in all three proteins (Fig. 3A), suggesting similar cognate DNA-binding sites. Consistent with this, HilD, HilC, and RtsA have been shown to directly regulate several of the same target genes, including *hilA* (33, 34, 37, 38), *invF* (33, 39), *dsbA* (34), and *slrP* (33). Hence, we predicted that the HilD-regulated genes are also regulated by HilC and RtsA. To test this hypothesis, we measured expression of the *lacZ* fusion constructs in *E. coli* containing either plasmid expressing *hilC* or *rtsA* from an arabinose-inducible promoter under inducing and noninducing conditions. We detected positive regulation of 7/8 genes by both HilC and RtsA (Fig. 3B and C; raw data, including for empty vector controls, shown in Table S4 in the supplemental material). The levels of induction by HilD, HilC, and RtsA were similar for all genes tested (c.f. Fig. 2 and Fig. 3B

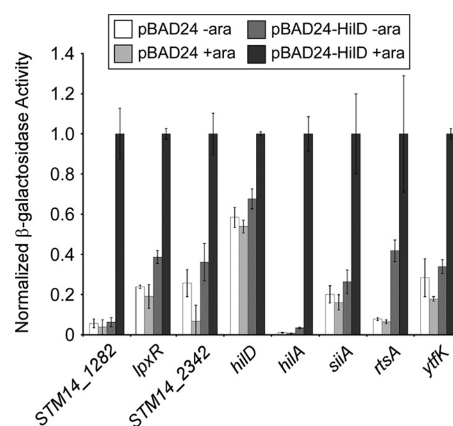

FIG 2 β-Galactosidase assay showing HilD activation of target genes. β-Galactosidase assay of fusions of the indicated upstream regions fused translationally to a *lacZ* reporter gene on a single-copy plasmid. Assays were performed with *E. coli* strain AMD054 containing either empty pBAD24 plasmid or pBLP013 (*hilD*), under noninducing (no arabinose) or inducing (with arabinose) conditions, as indicated. Data are shown normalized to the values in the presence of pBLP013 (*hilD*) and arabinose. Error bars represent 1 standard deviation from the mean, based on at least three independent biological replicates.

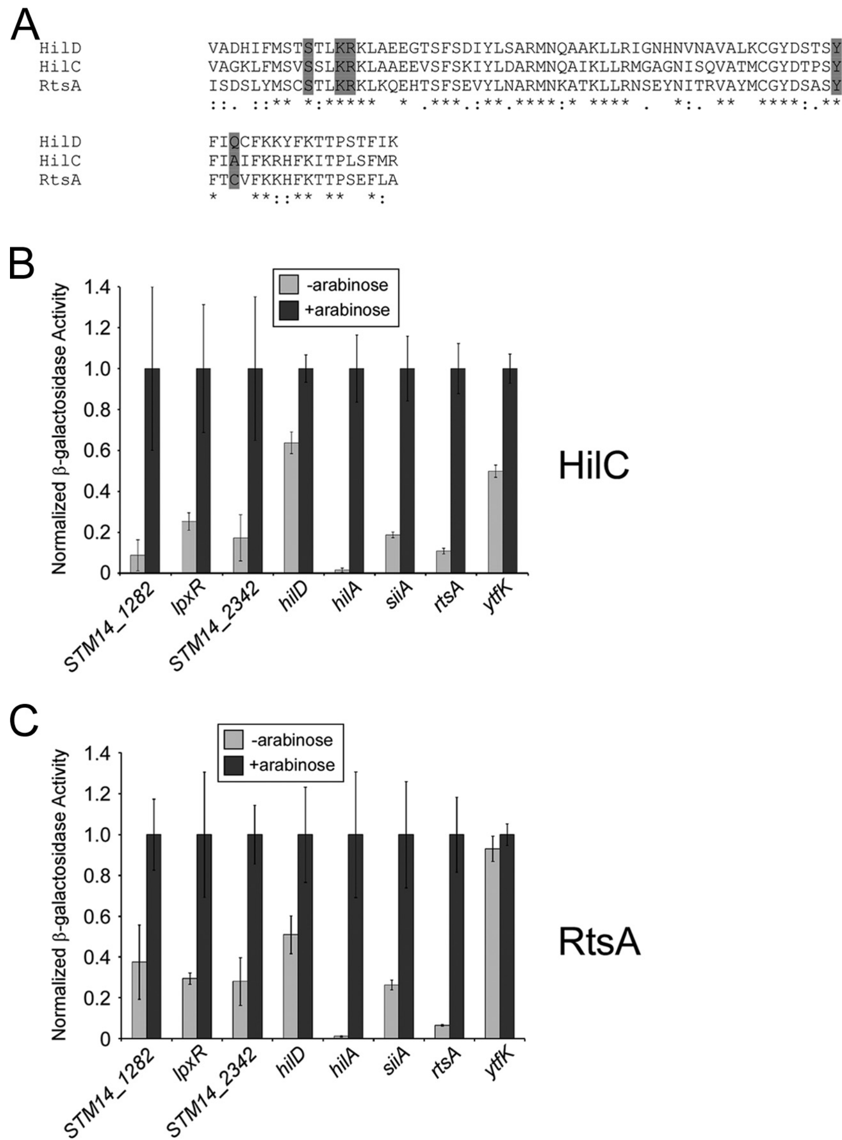

FIG 3 β-Galactosidase assay showing activation of HilD target genes by HilC and RtsA. (A) Sequence alignment of predicted DNA-binding domains of HilD, HilC, and RtsA, shown in the CLUSTAL format (30). Shaded residues indicate those predicted to make base-specific contacts with DNA. (B) β-Galactosidase assay of fusions of the indicated upstream regions fused translationally to a *lacZ* reporter gene on a single-copy plasmid. Assays were performed with *E. coli* strain AMD054 containing pBLP011 (*hilC*), under noninducing (no arabinose) or inducing (with arabinose) conditions, as indicated. Data are shown normalized to the values for cells grown in the presence of arabinose. Error bars represent 1 standard deviation from the mean, based on at least three independent biological replicates. (C) As described above, but with pBLP010 (*rtsA*) used in place of pBLP011 (*hilC*).

and C). Transcription of one gene, *ytfK*, was activated by HilC but not RtsA (Fig. 3B and C).

DISCUSSION

Much of the HilD regulon is outside SPI-1. The known HilD regulon, as determined in this and previous studies, is summarized in Fig. 4. Using a combination of ChIP-seq and targeted expression analysis, we have identified four novel HilD-activated genes: *STM14_1282*, *STM14_2342*, *lpxR*, and *ytfK*. In addition, we confirmed association of HilD upstream of previously described targets *hilC*, *hilD*, *hilA*, *invF*, *siiA*, and *rtsA* (Fig. 1), and we confirmed regulation of previously described targets *hilD*, *hilA*, *siiA*, and *rtsA* by HilD (Fig. 2). We did not detect HilD upstream of the previously described target *ssrAB*, the two-component regulatory system of SPI-2. This could be due to condition-specific binding of HilD upstream of *ssrA*, since *ssrAB* expression is induced by HilD in the late stationary phase, well beyond the time point of our ChIP-seq experiment (14). We also did not detect HilD binding upstream of previously described regulatory targets *dsbA* and *slrP*. Although direct association of HilD with these regions has not been demonstrated, transcription activation of both *dsbA* and *slrP* by HilD has been observed in strains containing deletions of both *hilC* and *rtsA* (33, 34), ruling out the possibility of indirect regulation through cross talk between HilD and HilC/RtsA. We propose that binding of HilD upstream of these genes and regulation by HilD occur in a condition-specific manner.

The function of novel HilD-regulated genes. Most previously described HilD targets are located within SPI-1 (6). In contrast, all

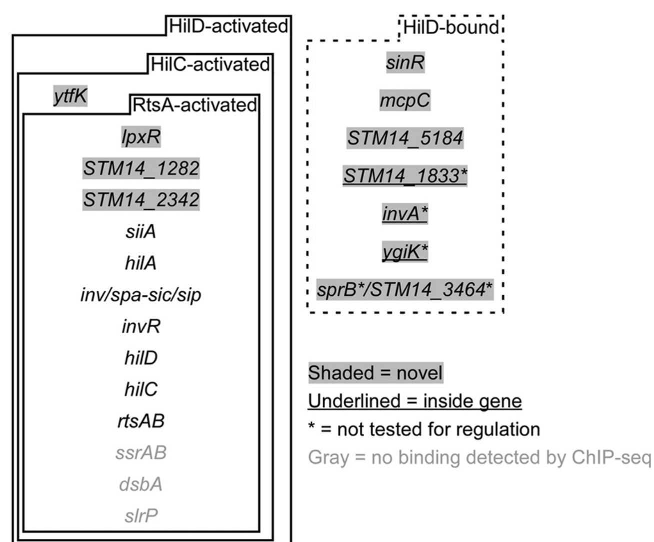

FIG 4 Summary of HilD-bound regions and HilD-regulated genes. Shown are genes identified as being directly regulated by HilD and regions identified as being bound by HilD, based on this and previous studies. Genes given in gray text were not identified as being HilD bound in this study. Genes with gray shading have not previously been described as being HilD regulated or bound. The dashed box indicates genes (i) that are not regulated by HilD or for which regulation has not been tested and (ii) whose upstream region is bound by HilD or for which HilD binds within the gene. (Underlined text indicates intragenic binding.) Asterisks indicate HilD-associated regions for which regulation by HilD was not tested in this or previous studies.

but two of the novel HilD targets are located outside SPI-1. In fact, the majority of HilD-bound regions are outside SPI-1, and the same is true for HilD-activated genes. Thus, HilD coordinates expression of SPI-1 and non-SPI-1 genes. *lpxR*, a novel HilD-regulated gene, encodes an outer membrane protein that removes the 3′-acyloxyacyl group of lipid A, the hydrophobic anchor of lipopolysaccharide (LPS) (40). Modification of lipid A by LpxR increases the ability of *S.* Typhimurium to evade the innate immune response (41) and promotes survival within macrophages (42). LpxR is conserved in other pathogenic bacterial species, and *lpxR* mutants of *Yersinia enterocolitica* are attenuated for virulence in a mouse model (43). (Note that *lpxR* in *Y. enterocolitica* is known as *sfpA*.) Regulation of *lpxR* by HilD, HilC, and RtsA likely coordinates the timing of lipid A deacylation with epithelial cell invasion, suggesting a selective advantage for acylated lipid A prior to epithelial cell invasion. Three of the novel HilD-activated genes identified in this study, *STM14_1282*, *STM14_2342*, and *ytfK*, have no known or predicted function. Given that all other described HilD-activated genes have an established connection to virulence, regulation of *STM14_1282*, *STM14_2342*, and *ytfK* by HilD implicates these genes in virulence-associated processes.

HilD binding that is not associated with detectable regulation. Three of the HilD-bound regions we identified by ChIP-seq can be unambiguously associated with nearby genes that are not detectably regulated by HilD: *mcpC* (*STM14_3893*), *sinR* (*STM14_0358*), and *STM14_5184*. *mcpC* is a member of the flagellar regulon (44) and encodes a protein involved in chemotaxis (44, 45). *sinR* encodes a LysR family transcription factor and is conserved only in serovars of *Salmonella* that infect warm-blooded hosts (46). The function of *STM14_5184* is unknown. None of these genes has been previously linked to SPI-1 gene regulation. However, SinR has a virulence-associated function since *sinR* mutants are defective in survival within macrophages (47). Furthermore, *sinR* is located within a horizontally acquired segment of DNA that encodes a fimbrial apparatus (46). The level of association of HilD upstream of *sinR* is greater than with any other genomic location. We speculate that *mcpC*, *sinR*, and *STM14_5184* are regulatory targets of HilD and possibly also of HilC and RtsA, but require additional transcription factors that are not present or not expressed in *E. coli* under the conditions used in our expression assays. This model suggests that HilD functions cooperatively with one or more additional transcription factors that may be condition or species specific.

HilD binding within genes. Three of the HilD-bound regions we identified by ChIP-seq fall well within annotated genes, far from the start of the overlapping gene or any other nearby gene (Fig. 4; see Fig. S2 in the supplemental material). These include a peak within a SPI-1 gene, *invA*. We did not test regulation of these overlapping genes. Little is known about the function of transcription factor binding sites within genes, although there are a few examples of such binding sites that have been shown to serve as roadblocks to elongating RNA polymerase (48–50). Recent ChIP-chip and ChIP-seq studies have identified numerous intragenic binding sites for some transcription factors (17, 51, 52), including in *S.* Typhimurium (19), suggesting an important regulatory function for these sites.

Cross talk between HilD, HilC, and RtsA. HilD, HilC, and RtsA are homologous AraC family transcription factors. Their DNA-binding domains are each ~60% identical, with the predicted base-specific contacts being largely conserved (Fig. 3A). Previous studies have identified shared regulatory targets of HilD, HilC, and RtsA, and it has been suggested that these transcription factors bind their targets *in vivo* as heterodimers (7). HilD, HilC, and RtsA all activate transcription of *hilA*, *hilC*, *hilD*, *dsbA*, *invF*, *slrP*, *rtsA*, and genes in SPI-4 (6, 15, 32, 33). Furthermore, HilD and HilC bind the same DNA sites upstream of *hilD* and *hilC* (38). Our data confirm a large degree of overlap between the HilD, HilC, and RtsA regulons and extend this overlap to include the novel HilD targets; we observed transcription activation of all 8 of the HilD-activated genes by HilC and all but one by RtsA (Fig. 3). It is important to note that these assays were performed in *E. coli*, in which only one of the three regulators is expressed in any given experiment; there are no close homologues of HilD, HilC, or RtsA in *E. coli*. Hence, regulation by HilD, HilC, and RtsA must be direct. We conclude that HilD, HilC, and RtsA bind very similar DNA sites, although the failure of RtsA to regulate *ytfK* (Fig. 3C) indicates that there are subtle differences in the sequence specificities of HilD, HilC, and RtsA. Consistent with this, RtsA has been shown to be a substantially stronger activator of *slrP* than HilD or HilC (33). Furthermore, a previous study suggested that although HilD and HilC have similar consensus DNA-binding sites, HilD is able to bind only a subset of HilC sites (38).

Overlap of the HilD, HilA, and SprB regulons. In addition to HilD and HilC, three other transcription factors are encoded within SPI-1: HilA, SprB, and InvF. Unlike HilC and RtsA, these three regulators are not homologues of HilD; hence, they are expected to bind DNA with different sequence specificities. The HilD targets we identified have no overlap with the known targets of InvF (53). However, there is overlap of the HilD targets with those of HilA and SprB. HilA has been shown to directly regulate the previously described HilD target *invF* (21, 54). A HilA ChIP-

chip study also identified a HilA binding site in the vicinity of the SL1344 homologue of *STM14_2342*. However, microarray analysis suggested that binding of HilA at this site regulates expression of the downstream gene *flhD* (21), for which we did not detect regulation by HilD (see Table S4 in the supplemental material).

We observed direction regulation of *siiA* by HilD. *siiA* is the first gene in *Salmonella* pathogenicity island 4 (SPI-4), and it is likely to be the first gene in an operon that includes all SPI-4 genes. SPI-4 genes encode a nonfimbrial adhesin and an associated type I secretion system (55) that are required for maximal virulence of *S*. Typhimurium in some, but not all, hosts (56, 57). Regulation of SPI-4 genes by HilD, HilC, and RtsA has been described previously (15, 32, 33) and was suggested to be dependent upon HilA (15, 33). However, modest HilA-independent regulation by HilD and HilC was observed (15), consistent with our data. HilA has been shown by ChIP-chip to bind upstream of *siiA* (21), suggesting that HilD/HilC/RtsA and HilA bind to neighboring sites. Thus, regulation of SPI-4 by HilD, HilC, and RtsA likely coordinates expression of SPI-4 and SPI-1 genes, which are required for attachment to and invasion of epithelial cells, respectively. SprB has been shown to activate transcription of *siiA* (58), although the same study showed no regulation of *siiA* by HilA or HilD, in contrast to our data. We propose that *siiA* is regulated by HilD, HilC, RtsA, HilA, and SprB, which would indicate that SPI-1 and SPI-4 gene expression is tightly linked. Intriguingly, our ChIP-seq analysis identified a weak HilD binding site upstream of *sprB* (Fig. 4; see Fig. S2 in the supplemental material), suggesting an added layer of feedback regulation.

Conclusions. Our data demonstrate that the HilD regulon extends well beyond SPI-1, strengthening links to SPI-4 regulation and adding a novel connection between invasion and lipid A modification. Our findings also broaden the regulatory overlap between HilD and its homologues, HilC and RtsA. The impact of this work on our understanding of invasion gene regulation in *S*. Typhimurium highlights the utility of genome-scale investigation of transcription factor binding, particularly in the context of layered regulatory networks.

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