

# Identification of a transcriptional regulator that controls intracellular gene expression in *Salmonella* Typhi

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## Summary

*Salmonella enterica* serovar Typhi (*S. Typhi*), the aetiological agent of typhoid fever, is an exclusively human pathogen. Little is known about specific factors that may confer to this bacterium its unique pathogenic features. One of these determinants is CdtB, a homologue of the active subunit of the cytolethal distending toxin, which causes DNA damage leading to cell cycle arrest and distension of intoxicated cells. A unique property of *S. Typhi* CdtB is that it is only synthesized when this bacterium is within an intracellular compartment. Through a genetic screen, we have identified a transcriptional regulatory protein that controls the intracellular expression of *cdtB*. This regulator, which we have named IgeR, is a member of the DeoR family of transcriptional regulatory proteins and is highly conserved in all *S. enterica* serovars. IgeR directly binds the *cdtB* promoter and represses its expression in the extracellular environment. Microarray analysis identified additional IgeR-regulated genes that are involved in virulence. Constitutive expression of *igeR* resulted in the reduction of intracellular expression of *cdtB* by *S. Typhi* and in significant impairment of the virulence of *Salmonella enterica* serovar Typhimurium (*S. Typhimurium*) in mice. We propose that IgeR may co-ordinate gene expression during *Salmonella*'s transition from an extracellular to an intracellular environment.

## Introduction

*Salmonella enterica* serovar Typhi is the cause of typhoid fever, a life-threatening disease of humans (House *et al.*, 2001; Parry *et al.*, 2002). Unlike other *S. enterica* serovars, *S. Typhi* exclusively infects humans. Although its incidence in industrialized nations is low, in developing

countries typhoid fever results in an estimated 200 000 deaths annually (Pang *et al.*, 1998). Despite its importance, little is known about specific factors that confer on this pathogen its unique virulence properties. Comparison of the nucleotide sequences of the genome of *S. Typhi* with those of other *S. enterica* serovars has revealed the presence of an unusually large number of pseudogenes (Parkhill *et al.*, 2001; Deng *et al.*, 2003). This observation has led to the hypothesis that the reduction of its 'functional genome' may have contributed to *S. Typhi*'s exclusive restriction to the human host (Parkhill *et al.*, 2001). In addition to its narrow host range, *S. Typhi* exhibits unique pathogenic features such as the ability to cause systemic infection and persistently colonize the human host, most often by establishing residence within the gall bladder (House *et al.*, 2001; Parry *et al.*, 2002). However, little is known about the genetic bases responsible for these unique virulence attributes. Although the nucleotide sequence of the *S. Typhi* genome has revealed the presence of many genes unique to this serovar, most of these genes are predicted to encode functions related to phages or other mobile genetic elements and therefore may not contribute to its virulence (Parkhill *et al.*, 2001; Deng *et al.*, 2003). However, a number of *S. Typhi* genes are not widely distributed among other *S. enterica* serovars, and thus may contribute to its unique virulence properties. One of these genes is *cdtB*, which encodes a protein homologue of the active subunit of the cytolethal distending toxin (CDT) (Haghjoo and Galan, 2004). This toxin, which is widely distributed among bacterial pathogens, causes DNA damage resulting in cell cycle arrest and cellular distension (Lara-Tejero and Galán, 2000; 2002). In addition to the catalytically active 'A' subunit CdtB, CDTs possess a heterodimeric 'B' subunit that mediates its delivery into target cells (Lara-Tejero and Galán, 2001). However, *S. Typhi* does not encode homologues of the CDT 'B' subunits, and exogenous addition of purified CdtB does not lead to intoxication (Haghjoo and Galan, 2004). Instead, CdtB is delivered into host cells by a unique bacterial invasion-mediated pathway. Consistent with this delivery mode, expression of *cdtB* does not occur when *S. Typhi* is grown in standard culture medium. Instead, its expression is stimulated only once *S. Typhi* has reached a specific intracellular compartment following

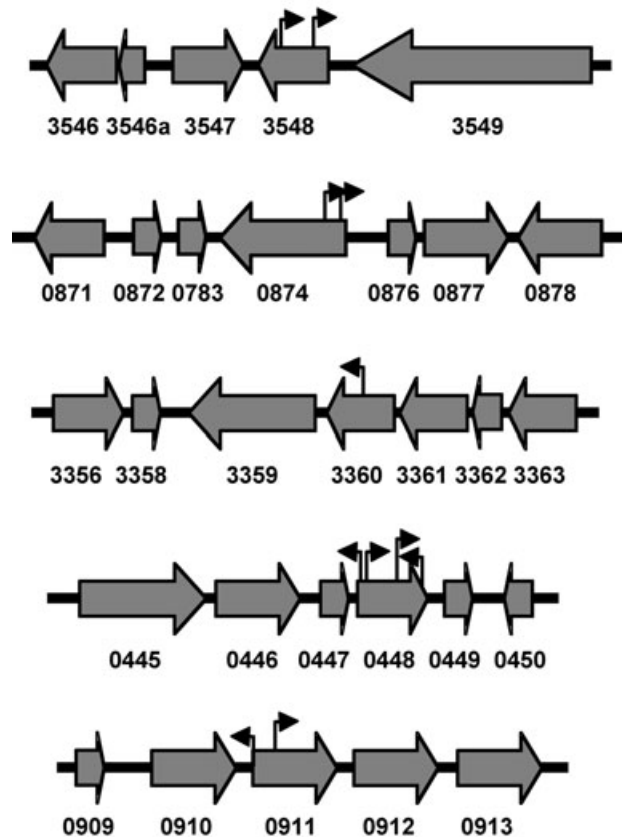
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at least 3 h of infection (Haghjoo and Galan, 2004). Presumably, this regulatory mechanism ensures that expression of the toxin occurs only when the appropriate conditions for its delivery from an intracellular compartment are encountered. We carried out a genetic screen to identify genes involved in the regulation of *cdtB* expression. One of the genes identified, *sty0448*, which we have named *igeR* (for intracellular gene expression regulator), represses the extracellular expression of *cdtB* by directly binding to its promoter sequence. We found that IgeR belongs to the DeoR family of transcription regulators and is widely distributed among other *Salmonella enterica* serovars. We propose that IgeR is involved in the regulation of intracellular gene expression in *S. Typhi* and presumably other *S. enterica* serovars and may co-ordinate gene expression during *Salmonella's* transition from an extracellular to an intracellular environment.

## Results

### Identification of *S. Typhi* genes that control *cdtB* expression

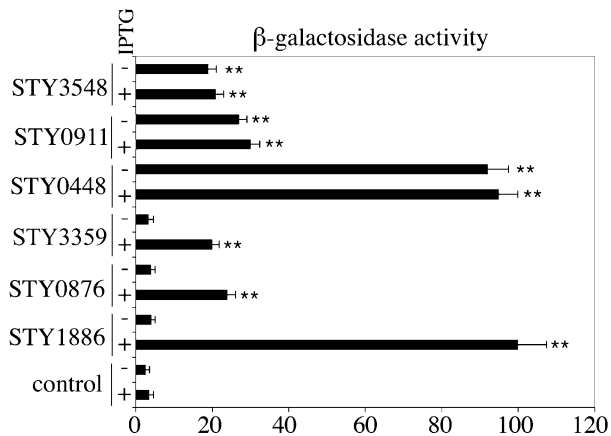
In order to identify genes involved in the regulation of *S. Typhi cdtB* expression, we carried out a screen to isolate mutants that allow the expression of this gene under non-permissive conditions (i.e. extracellularly). We used the transposon TnMod-RKm'-*lacI<sup>q</sup>tacp*, a derivative of Tn5 encoding an IPTG-inducible outward promoter (Maxson and Darwin, 2004). This transposable element allows the identification of insertions whose phenotypes are either due to the insertional inactivation of a given gene, or the IPTG-dependent expression of downstream genes (Maxson and Darwin, 2004). We reasoned that this approach should allow us to identify repressors and activators of *cdtB* expression. The TnMod-RKm'-*lacI<sup>q</sup>tacp* transposon was mobilized into a strain of *S. Typhi* encoding a *cdtB::lacZ* transcriptional reporter gene fusion, and insertion mutants that resulted in *cdtB::lacZ* expression under non-permissive conditions were identified on indicator plates. Twenty-eight mutants of approximately 70 000 random transposon insertions screened showed increased  $\beta$ -galactosidase activity. The positions of the different transposon insertions were determined by nucleotide sequencing. Seventeen insertions were mapped to regions immediately upstream of the *cdtB::lacZ* reporter fusion, which placed *cdtB* expression under the control of the *ptac* promoter present in the transposable element. The remaining 11 mutants mapped to various chromosomal loci and were chosen for further analysis (Fig. 1). To ensure the linkage of the phenotype to the transposon insertion, all mutations were moved into the parent *S. Typhi* strain by  $\lambda$ -red recombination (Datsenko and



**Fig. 1.** Location of TnMod-RKm'-*lacI<sup>q</sup>tacp* insertion mutants resulting in *cdtB* expression under non-inducing conditions. Arrowheads indicate the approximate location and direction of transcription *ptac* promoter of the different transposon insertion mutants that resulted in *cdtB* expression under non-inducing conditions. The different genes are labelled according to the nomenclature used in the annotation of the *Salmonella enterica* serovar Typhi strain CT18 genome sequence and correspond to the STY numbers of each open reading frame.

Wanner, 2000) (see *Experimental procedures*), and the phenotypes of the resulting strains were confirmed on indicator plates. In addition, the growth *in vitro* of all the mutant strains was indistinguishable from that of wild type (data not shown).

We then tested whether the phenotypes of the different insertion mutants were due to inactivation of a gene or to the expression of a downstream gene driven by the *ptac* promoter of the transposable element. All mutant strains were grown in the presence or absence of IPTG, the inducer of the *ptac* promoter, and the  $\beta$ -galactosidase activity resulting from the expression of the *cdtB::lacZ* reporter was measured as indicated in *Experimental procedures*. Predictably, the phenotype of the insertions that mapped immediately upstream of the reporter *cdtB::lacZ* was strictly dependent on the addition of IPTG (Fig. 2). Two other classes of insertions also exhibited an IPTG-dependent phenotype. One of them was due to an insertion

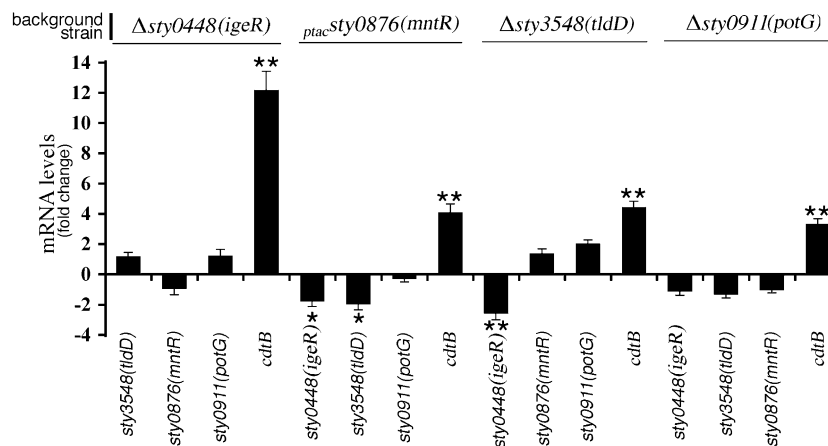


**Fig. 2.**  $\beta$ -Galactosidase activity of the *cdtB* regulatory mutant strains grown in the presence or absence of the inducer IPTG. *S. Typhi* regulatory mutant strains were grown in LB broth in the presence or in the absence of IPTG (as indicated) and the  $\beta$ -galactosidase activity was measured as described in *Experimental procedures*. Values are standardized to the activity of insertions in STY1886 (considered 100), which placed a *ptac* promoter immediately upstream of the *cdtB::lacZ* reporter. Numbers represent the mean  $\pm$  standard deviation of three independent measurements. The asterisks (\*\*) indicate values statistically significantly different ( $P < 0.001$ ) from the control. The control values were obtained by measuring the  $\beta$ -galactosidase activity of the parent strain carrying the *cdtB::lacZ* fusion grown under identical conditions.

within the *sty3360* gene, which placed the promoter in the direction of transcription of the downstream gene *sty3359*. Sty3359 is an orthologue of the *Escherichia coli* ParE protein, which is the B subunit of topoisomerase IV (Kato *et al.*, 1990). Changes in the expression of ParE leads to DNA relaxation, which has been shown to result in the modulation of gene expression and the derepression of

silenced genes (McNairn *et al.*, 1995). We therefore hypothesize that the derepression of *cdtB::lacZ* observed in this mutant strain is due to changes in DNA supercoiling. Two other transposon insertions mapped within *sty0874*. Both insertions placed the transposon-encoded *ptac* promoter in the direction of transcription of the upstream gene *sty0876*. Because their phenotype was dependent on the addition of IPTG, we concluded that the increased expression of *cdtB::lacZ* observed in these mutants is due to the *ptac*-driven expression of the upstream gene *sty0876*. This gene encodes MntR, a metal-dependent transcriptional regulator (Patzner and Hantke, 2001) that controls the expression of the putative *S. Typhimurium* metal transport systems MntH and SitABCD (Kehres *et al.*, 2002; Ikeda *et al.*, 2005). Like *cdtB*, expression of the *sitABCD* transporter is also induced within host cells (Zaharik *et al.*, 2004), which is consistent with the apparent similarity in their regulatory mechanisms.

The transposon insertions that led to the induction of *cdtB::lacZ* transcription in a manner that was independent of the addition of IPTG mapped within the *sty0448*, *sty0911* and *sty3548* loci. Therefore, the phenotype of these insertions is most likely due to the inactivation of the genes in which the transposon inserted or a gene located downstream in the same transcriptional unit. To distinguish between these possibilities, we constructed *S. Typhi* strains carrying in-frame non-polar deletions of these genes and examined the expression of the *cdtB::lacZ* reporter in the resulting strains. Deletion of *sty0448*, *sty0911* or *sty3548* resulted in levels of *cdtB::lacZ* expression that were indistinguishable from those observed in the transposon insertions mutants (Fig. 3). These results indicate that the observed IPTG-



**Fig. 3.** Expression of regulatory genes in the different *S. Typhi* regulatory mutants. The levels of expression of the different genes (as indicated) was determined by quantitative PCR as described in *Experimental procedures*. Strain *ptacsty0876*, which carries an insertion placing the *ptac* promoter upstream of *sty0876*, was grown in the presence of IPTG. Values are mRNA levels standardized as fold changes relative to the wild-type strain and represent the mean  $\pm$  standard deviation of three independent determinations. Values that were statistically different from the control are indicated (\*\* $P < 0.01$ ; \* $P < 0.05$ ). The control values were obtained by measuring the  $\beta$ -galactosidase activity of the parent strain carrying the *cdtB::lacZ* fusion grown under identical conditions.

independent phenotypes of the transposon insertion mutants are due to the inactivation of the genes they interrupted and not to polar effects on downstream genes. *sty0911* encodes PotG, a component of a putrescine uptake system (Pistocchi *et al.*, 1993). It is unclear why inactivation of PotG may lead to derepression of *cdtB*, although in all likelihood this effect is indirect. *sty3548* encodes a homologue of TldD. *tldD* was originally identified as a suppressor of the activity of LetD (CcdB), which inhibits chromosomal partitioning presumably by modulating the activity of DNA gyrase (Murayama *et al.*, 1996). We hypothesize that similar to the phenotype observed by the overexpression of ParE, inactivation of *tldD* may lead to chromosomal DNA relaxation and subsequent derepression of *cdtB*. *sty0448* encodes a protein of unknown function with significant amino acid sequence similarity to the DeoR family of transcriptional regulatory proteins (Hammer-Jespersen and Munch-Petersen, 1975) (Fig. S1, *Supplementary material*). Inactivation of *sty0448* resulted in the strongest derepression of *cdtB::lacZ* expression. In fact, expression of *cdtB::lacZ* in this mutant strain was equivalent to the levels of expression resulting from transposon insertions that placed a *ptac* promoter immediately upstream of the reporter. We have named this gene *igeR* for intracellular gene expression regulator.

Taken together, a transposon mutagenesis screen identified five genes potentially involved in the regulation of *cdtB* expression. Two of these genes, *sty3359* (*parE*) and *sty0876* (*mntR*) can be operationally defined as 'activators' because their ectopic expression driven by the *ptac* promoter resulted in increased *cdtB* expression. The other three genes, *sty0448* (*igeR*), *sty0911* (*potG*) and *sty3548* (*tldD*), can be operationally defined as 'repressors' because their inactivation led to increased *cdtB* expression.

#### Hierarchy in the regulation of *CdtB* expression

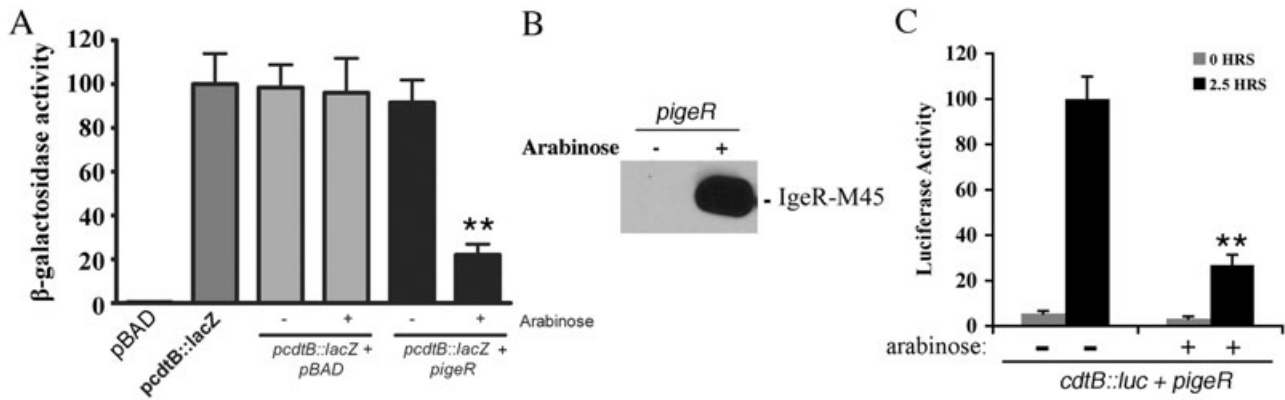
To investigate whether the regulatory genes identified in the screen operate on the same regulatory pathway or constitute independent regulatory loops in the control of *cdtB* expression, we examined their transcriptional levels in the different *cdtB* regulatory mutant strains. In-frame deletions of *sty0911* (*potG*) and *sty0448* (*igeR*) had no effect on the expression of the other regulatory genes, suggesting that these two genes may not exert their effect on *cdtB* through regulation of the other regulators (Fig. 3). However, *sty0448* (*igeR*) transcription was reduced both in the  $\Delta$ *sty3548* (*tldD*) as well as in the strain in which the expression of *sty0876* (*mntR*) was driven by the *ptac* promoter in the presence of IPTG (Fig. 3). These results suggest the possibility that both these regulatory genes may exert their effect on *cdtB* expression at least in part through the modulation of *sty0448* (*igeR*) expression.

Moreover, constitutive expression of *sty0876* (*mntR*) led to a slight reduction in the levels of *sty3548* (*tldD*) transcripts (Fig. 3), suggesting that Sty0876 (MntR) may also act upstream of *sty3548* (*tldD*) in the control of *cdtB* gene expression. Consistent with the results obtained with the reporter fusion, expression of *cdtB* was significantly increased in the  $\Delta$ *sty3548* (*tldD*),  $\Delta$ *sty0911* (*potG*), and  $\Delta$ *sty0448* (*igeR*) deletion strains, as well as in the strain with a transposon insertion immediately upstream of *sty0876* (*mntR*) when grown in the presence of IPTG (Fig. 3). Also consistent with results obtained with the reporter fusion, expression of *cdtB* was the highest in the  $\Delta$ *sty0448* (*igeR*) mutant strain (Fig. 3). Taken together, these data suggest that there are different regulatory pathways controlling the expression of *cdtB*, and that Sty0448 (IgeR) may play a more direct role in the control of *cdtB* expression.

#### *IgeR* can repress *cdtB* gene expression in an heterologous host and during infection of intestinal cells

The experiments described above suggest that IgeR acts as a repressor of *cdtB* expression. To directly test this hypothesis we examined the expression of *cdtB* in *E. coli* in the presence or absence of IgeR. Using the promoter-probe vector pRS414 (Simons *et al.*, 1987), we constructed a plasmid in which transcription of *lacZ* is controlled by the *cdtB* promoter. An *E. coli* strain carrying this reporter plasmid showed high levels of  $\beta$ -galactosidase activity, which indicates that the *cdtB* promoter can function in a heterologous host under conditions that are not permissive for expression in its native host *S. Typhi* (Fig. 4). To test the effect of expression of IgeR on the expression of *cdtB::lacZ* in *E. coli*, we introduced a plasmid that expresses *igeR* under an arabinose-inducible promoter (Guzman *et al.*, 1995) (Fig. 4). The levels of  $\beta$ -galactosidase activity after growth in the presence or absence of arabinose were measured as an indicator of *cdtB* transcription. When the *E. coli* reporter strain was grown in the presence of arabinose, the levels of *cdtB* expression were markedly reduced, which indicates that expression of IgeR alone can repress the activity of the *cdtB* promoter in an heterologous host (Fig. 4).

We then tested whether ectopic expression of IgeR in *S. Typhi* would be sufficient to repress the expression of *cdtB* under inducing conditions (i.e. when *S. Typhi* is within cultured intestinal epithelial cells). We used an *S. Typhi* strain carrying a *cdtB::luc* reporter fusion in the chromosome and a plasmid in which expression of *igeR* is controlled by an arabinose-inducible promoter. Cultured intestinal Henle-407 cells were infected with this strain in the presence or absence of arabinose. The luciferase activity was determined at the time of infection or 2.5 h after infection, when the expression of the *cdtB* gene was



**Fig. 4.** Constitutive expression of *igeR* represses the expression of *cdtB* in a heterologous bacterial host and within host cells.

A. Levels of  $\beta$ -galactosidase activity were measured in *E. coli* carrying a *cdtB::lacZ* reporter plasmid (*pcdtB::lacZ*) along with a plasmid expressing *igeR* (*pigeR*) under an arabinose-inducible promoter or the vector control (pBAD), and grown in the presence or absence of arabinose as indicated. The relative levels of  $\beta$ -galactosidase activity were standardized based on the values of the strain that carries the *cdtB::lacZ* reporter plasmid alone, which was considered 100. Values represent the mean  $\pm$  standard deviation of three independent determinations. The asterisks (\*\*) indicate values statistically significantly different ( $P < 0.001$ ) from the control. The control values were obtained by measuring the  $\beta$ -galactosidase activity of the strain encoding plasmid-born *cdtB::lacZ* and *igeR* grown in the absence of arabinose.

B. Western immunoblot of cell lysates of *E. coli* carrying a plasmid used in the studies shown in this figure expressing epitope tagged IgeR after growth in the presence or absence of arabinose.

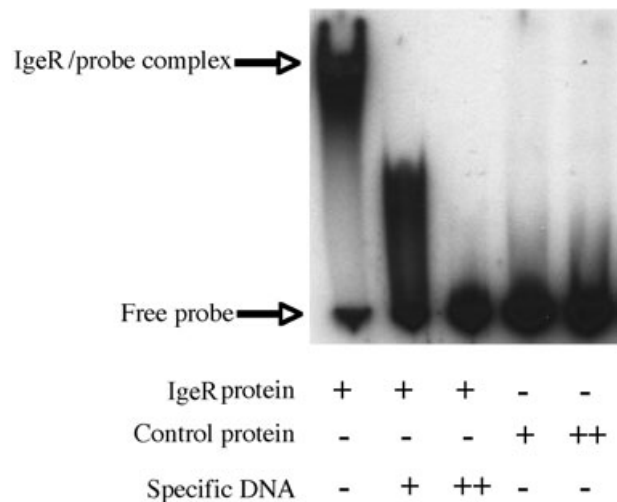
C. Cultured intestinal Henle-407 cells were infected with a *S. Typhi* *cdtB::luc* reporter strain that expresses *igeR* under the control of an arabinose-inducible promoter. Bacterial growth and infections were carried out in the presence of arabinose. Determination of luciferase activity was carried out as described in *Experimental procedures*. Values have been standardized to the luciferase level in the absence of arabinose measured 2.5 h after infection, which was considered 100. Numbers represent the mean  $\pm$  standard deviation of three independent determinations. The asterisks (\*\*) indicate values statistically significantly different ( $P < 0.001$ ) from those obtained 2.5 h after infection in the absence of arabinose.

expected to be fully induced. The expression of *cdtB::luc* by intracellular bacteria was drastically reduced in the presence of arabinose (Fig. 4), which further supports the hypothesis that IgeR can directly repress *cdtB* expression. Taken together, these results indicate that IgeR is a repressor of *cdtB* expression and may be largely responsible for the control of its expression under non-inducing conditions.

#### *IgeR binds the cdtB promoter region*

The results shown above indicate that IgeR can repress the expression of *cdtB* and strongly suggest that it may do so by directly binding the *cdtB* promoter. To confirm this hypothesis, we investigated whether IgeR could bind the *cdtB* promoter region directly by performing electrophoretic gel mobility shift assays. A radiolabelled 500 bp DNA fragment comprising the region immediately upstream of *cdtB* was incubated with purified His-tagged IgeR in the presence or absence of specific competitors. Addition of purified His-tagged IgeR (but not a control protein) to the radiolabelled probe resulted in a significant retardation of the mobility of the probe (Fig. 5), which indicates an interaction between the probe and IgeR. The interaction of CdtB with the radiolabelled probe could be selectively competed in a concentration-dependent manner by addition of unlabelled probe (Fig. 5), which further demonstrates the

specificity of the interaction. Taken together, these results indicate that IgeR can bind directly to the *cdtB* promoter region, which is consistent with its proposed function as a transcriptional repressor of this gene.



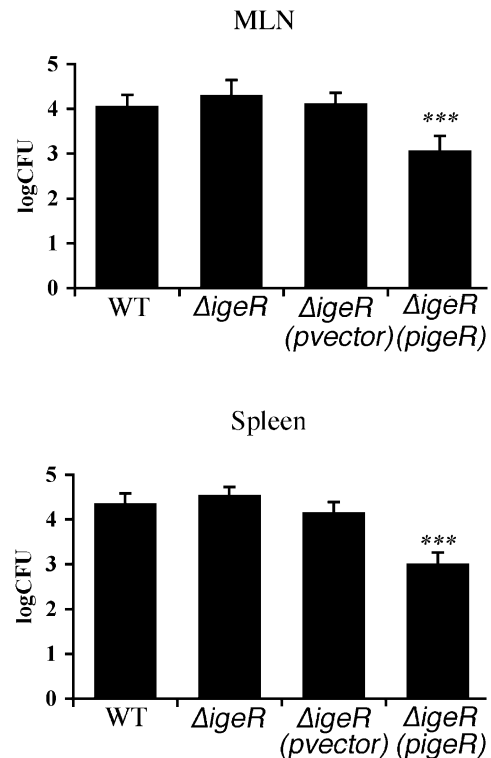
**Fig. 5.** IgeR binds the *cdtB* promoter region. Gel retardation assays performed as described in *Experimental procedures* revealed binding of IgeR to the *cdtB* promoter region. A radioactively labelled probe encompassing the *cdtB* promoter region was incubated with IgeR or a control protein in the presence or absence of increasing concentrations of unlabelled DNA, as indicated.

### Constitutive expression of IgeR impairs *S. Typhimurium* virulence

The observation that IgeR is involved in controlling the expression of a gene that is selectively induced within host cells suggested the possibility that IgeR may have a broader role in the control of intracellular gene expression and hence *Salmonella* virulence. Indeed IgeR is highly conserved in all *S. enterica* serovars, even those that do not encode *cdtB* such as *S. Typhimurium*, whose IgeR homologue, Stm0410, exhibits 100% amino acid identity with the *S. Typhi* protein. To test the potential role of IgeR in virulence we turned to *S. Typhimurium*, because there is no suitable animal model to address this issue in *S. Typhi*. We constructed a  $\Delta$ *igeR* *S. Typhimurium* mutant strain as well as a strain that constitutively expresses *igeR* from a *plac* promoter. Mice were orally infected with these mutant strains and 5 days post infection, the number of colony-forming units (cfu) in different organs was determined as indicated in *Experimental procedures*. Deletion of *igeR* did not affect the ability of *S. Typhimurium* to colonize either the spleen or mesenteric lymph nodes (Fig. 6). In contrast, constitutive expression of IgeR significantly ( $P < 0.001$ ) reduced the ability of *S. Typhimurium* to colonize deep tissues of orally infected mice (Fig. 6). Although the regulatory targets of IgeR in *S. Typhimurium* are currently unknown, by analogy with the observations in *S. Typhi*, we hypothesize that constitutive expression of IgeR prevents the intracellular expression of some genes that are required for these bacteria to display wild-type virulence in the mouse model of infection. Taken together, these data support the hypothesis that IgeR is an important regulator of virulence gene expression in *Salmonella enterica*.

### Identification of IgeR-regulated genes by microarray analysis

In an effort to identify additional IgeR-regulated genes in *S. Typhi*, we compared the gene expression profiles of wild-type and  $\Delta$ *igeR* mutant strains after growth in Luria-Bertani (LB) broth. Growth under these conditions does not lead to *cdtB* expression in wild-type *S. Typhi* but allows its full expression in the absence of IgeR. Therefore, we reasoned that this strategy could identify other genes that might be coregulated with *cdtB* and hence may play a role in the intracellular life cycle of *S. Typhi*. Our analysis identified 45 genes whose expression was altered (i.e. more than twofold change in gene expression in two separate array experiments) in the  $\Delta$ *igeR* mutant strain. Thirteen of these genes, like *cdtB*, showed increased expression in the mutant strain (Table 1), while 32 genes showed reduced expression in comparison to wild type (Table 2). The expression of a subset of these



**Fig. 6.** Constitutive expression of *igeR* attenuates *S. typhimurium* virulence. Mice were orally infected with  $10^7$  cfu of wild-type *S. typhimurium*, its isogenic  $\Delta$ *igeR* mutant, a  $\Delta$ *igeR* mutant carrying a plasmid encoding *igeR* under the control of a constitutive *lac* promoter, or the same mutant carrying the empty plasmid vector. The bacterial loads in the mesenteric lymph nodes (MLN) and the spleen were determined 5 days after infection. Numbers are cfu recovered from the indicated tissues and represent the mean  $\pm$  standard deviation (five mice per condition). The asterisks (\*\*\*) indicate that the difference in the number of cfu (vs. any of the other strains) was statistically significant ( $P < 0.001$ ).

genes was verified by quantitative real-time polymerase chain reaction (qPCR) (Table 3). In addition to *cdtB*, another previously characterized virulence gene, *pagC*, showed increased expression in the  $\Delta$ *igeR* mutant strain. *pagC* was originally identified as a PhoP/PhoQ-regulated gene required for *S. Typhimurium* virulence in a mouse model and for its survival within macrophages (Miller *et al.*, 1989). Similarly to *cdtB*, the expression of *pagC* is induced when *S. Typhimurium* reaches an intracellular environment (Hohmann *et al.*, 1995). However, unlike *pagC*, *cdtB* is not regulated by the PhoP/PhoQ two-component system. Furthermore, the expression of the *phoP/phoQ* system was not altered in the  $\Delta$ *igeR* *S. Typhi* mutant, which indicates that the influence of IgeR on the expression of *pagC* is not exerted through this two-component regulator. Another gene that showed increased expression in the  $\Delta$ *igeR* mutant was *yciG*, which is the first gene of the *yciGEF-katN* operon. Originally identified in a screen for RpoS-regulated genes

**Table 1.** Genes with increased expression in the *S. typhi*  $\Delta$ *igeR* mutant strain.

Locus	Gene	Description	Fold change <sup>a</sup>	P-value
STY0239	<i>rpsB</i>	30S ribosomal protein S2	2.14	0.006
STY1323	<i>yciG</i>	Unknown function; expression altered by bile	2.55	< 0.001
STY1777	<i>infC</i>	Translation initiation factor IF-3	2.14	0.002
STY1878	<i>pagC</i>	PhoP/PhoQ activated gene required for virulence	2.22	0.003
STY1886	<i>cdtB</i>	Cytolethal distending toxin active subunit	7.15	0.003
STY2204	<i>cspB</i>	Cold shock protein	2.01	0.002
STY4369	<i>rplX</i>	Ribosomal subunit protein L24	2.24	0.003
STY4372	<i>rpsH</i>	30S ribosomal subunit protein S8	2.20	< 0.001
STY4373	<i>rplF</i>	50S ribosomal subunit protein L6	2.06	0.004
STY4375	<i>rpsE</i>	30S ribosomal subunit protein S5	2.14	0.004
STY4382	<i>rpsD</i>	30S ribosomal subunit protein S4	2.12	0.003
STY4689	<i>groES</i>	Chaperonin	2.62	0.006
STY4690	<i>groEL</i>	Chaperonin	2.36	0.0152

a. Fold change in the expression levels relative to wild type calculated as indicated in *Experimental procedures*.

(Ibanez-Ruiz *et al.*, 2000), the *yci* operon encodes proteins of unknown function.

Several flagellar genes exhibited decreased expression in the  $\Delta$ *igeR* mutant although this strain is motile (data not

shown), which indicates that even in the absence of this regulator there is adequate flagellar gene expression to support motility. The effect of *IgeR* on flagellar gene expression appears to be exerted upstream of its specific

**Table 2.** Genes with decreased expression in the *S. typhi*  $\Delta$ *igeR* mutant strain.

Locus	Gene	Description <sup>a</sup>	Fold change <sup>b</sup>	P-value <sup>c</sup>
STY1120	<i>pipC</i>	SPI-5-encoded virulence protein	5.27	0.0013
STY1121	<i>sopB</i>	SPI-1 TTSS effector protein	6.78	< 0.0001
STY1211	<i>flgM</i>	Regulator of flagellar gene expression	2.81	0.005
STY1222	<i>flgK</i>	Flagellar structural component	2.43	0.015
STY1908	<i>ychH</i>	Conserved putative membrane protein of unknown function	3.25	< 0.0001
STY2134	<i>flhD</i>	Regulator of flagellar gene expression	2.41	0.0008
STY2158	<i>sdiA</i>	LuxR-family transcriptional activator	2.38	< 0.0001
STY2163	<i>fliZ</i>	Regulator of flagellar and SPI-1 TTSS gene expression	2.62	0.0041
STY2167	<i>fliC</i>	Flagellin	5.61	< 0.0001
STY2168	<i>fliD</i>	Flagellar protein	4.50	0.0012
STY2169	<i>fliS</i>	Flagellar protein	3.10	0.0001
STY2170	<i>fliT</i>	Flagellar protein	2.28	0.0051
STY2749	n/a	Unique <i>S. typhi</i> protein of unknown function	3.36	0.0049
STY2990	<i>orgB</i>	SPI-1 TTSS component	2.59	0.0049
STY2991	<i>orgA</i>	SPI-1 TTSS component	3.02	0.0009
STY2992	<i>prgK</i>	SPI-1 TTSS component (Needle complex component)	5.42	< 0.0001
STY2993	<i>prgJ</i>	SPI-1 TTSS component (inner rod protein)	7.13	0.0162
STY2994	<i>prgI</i>	SPI-1 TTSS component (needle protein)	11.49	0.0052
STY2995	<i>prgH</i>	SPI-1 TTSS component (Needle complex component)	3.43	0.0017
STY2996	<i>sprA/hilD</i>	SPI-1 TTSS transcription regulator	4.58	0.0013
STY3007	<i>sipC</i>	SPI-1 TTSS component (translocase)	6.26	< 0.0001
STY3008	<i>sipB</i>	SPI-1 TTSS component (translocase)	3.25	0.0021
STY3009	<i>sicA</i>	SPI-1 TTSS chaperone	6.90	0.0134
STY3015	<i>invJ</i>	SPI-1 TTSS component (needle complex assembly regulator)	2.15	0.0086
STY3016	<i>spaM</i>	SPI-1 TTSS component	3.76	0.002
STY3018	<i>spaK</i>	SPI-1 TTSS component	5.41	0.0053
STY3019	<i>invA</i>	SPI-1 TTSS component	2.09	0.0036
STY3021	<i>invG</i>	SPI-1 TTSS component (Needle complex component)	2.24	0.0127
STY3022	<i>invF</i>	SPI-1 TTSS transcription regulator	4.43	0.0085
STY3073	<i>sopD</i>	SPI-1 TTSS effector protein	2.43	0.0008
STY3309	n/a	Putative methyl accepting protein of unknown function	2.46	0.0099
STY4609	<i>sopE</i>	SPI-1 TTSS effector protein	4.12	0.0009

a. Abbreviations: SPI-1, *Salmonella* pathogenicity island 1; TTSS, type III secretion system.

b. Fold change in the expression levels relative to wild type calculated as indicated in *Experimental procedures*. n/a, not applicable.

c. A P-value for each ORF was calculated by a two-tailed Welch's unpaired *t*-test comparison of the microarray replicates for each strain. Relative changes in signal intensity of an ORF between strains were calculated as the following ratio: average *S. typhi* *igeR* signal intensity/average wild-type *S. typhi* signal intensity. Only ORFs with relative changes of at least two and a P-value less than or equal to 0.05 and called present on both microarrays were considered significant.

**Table 3.** Confirmation of the pattern of expression of selected genes that showed altered expression in the microarray analysis.

Locus	Gene	Fold change <sup>a</sup> (microarray)	Fold change <sup>a</sup> (qPCR)	P-value <sup>b</sup> (qPCR)
STY3022	<i>invF</i>	-4.43	-5.6	0.0023
STY3007	<i>sipC</i>	-6.26	-4.3	0.0043
STY3019	<i>invA</i>	-2.09	-3.1	0.0032
STY1886	<i>cdtB</i>	+7.15	+11.7	0.0007

**a.** Fold change in the expression levels relative to wild type calculated as indicated in *Experimental procedures*. qPCR, quantitative polymerase chain reaction. Negative or positive values indicate reduced or increased expression in the  $\Delta$ *cdtR* *S. typhi* mutant strain.

**b.** P-values determined by two-tailed Student *t*-test from three independent experiments.

regulatory cascade because the expression of *flhD*, which is at the top of the regulatory hierarchy (Macnab, 1992), was affected in the  $\Delta$ *igeR* mutant. Another gene that showed decreased expression in the  $\Delta$ *igeR* mutant is *sdiA*, which encodes a LuxR-family of transcriptional activator proteins involved in quorum sensing. As *Salmonella enterica* does not encode the inducer of this regulator, it has been proposed that its function is to detect mixed microbial communities in the intestinal environment (Michael *et al.*, 2001). Several genes encoding components or substrates of the type III secretion system (TTSS) encoded within the pathogenicity island 1 (SPI-1) also showed decreased expression in the  $\Delta$ *igeR* mutant strain. However, expression of this system in the  $\Delta$ *igeR* mutant was still adequate to sustain bacterial entry into host cells, which is strictly dependant on this system. Absence of IgeR did not affect the expression of HilA, HilC/SprA or HilD, which are three specific regulators located at or near the top of the SPI-1 TTSS regulatory cascade (Lucas and Lee, 2000; Galán, 2001). However, the expression of InvF, an AraC-family regulatory protein encoded within SPI-1 (Kaniga *et al.*, 1994), was decreased in the  $\Delta$ *igeR* mutant. Consistent with this observation, several effector proteins regulated by InvF (Eichelberg and Galán, 1999) also showed decrease expression in the  $\Delta$ *igeR* mutant. It is unclear how IgeR regulates the expression of flagellar or SPI-1 TTSS genes, although most likely the effect is indirect. However, in contrast to *cdtB*, both the flagellar and SPI-1 TTSS systems are switched off once *Salmonella* reaches an intracellular environment of intestinal epithelial cells (Bergman *et al.*, 2005). Therefore, the positive effect of IgeR on the regulation of these systems is consistent with their pattern of expression (Boddicker and Jones, 2004).

## Discussion

The co-ordination of virulence gene expression is essential for bacterial pathogenesis (Cotter and DiRita, 2000).

Virulence gene regulation is presumably especially important for bacterial pathogens, which must transit through very different environments during its infection cycle. Consequently, these pathogens have evolved complex mechanisms to ensure the appropriate temporal and spatial deployment of the virulence factors required for a specific environment. In the case of *Salmonella enterica*, which has evolved the ability to gain access to host cells, life in the intracellular environment requires specific adaptations, whose deployment must be carefully co-ordinated. Therefore, a number of genes have been identified, largely in *S. Typhimurium*, which are only expressed intracellularly (Eriksson *et al.*, 2003; Drecktrah *et al.*, 2006; Faucher *et al.*, 2006; Shi *et al.*, 2006; Thompson *et al.*, 2006). Expression of these genes must be controlled by specific activators and repressors, which co-ordinate the deployment of virulence factors at the appropriate time and in the appropriate environment. Despite its public health significance, very little is known about *S. Typhi* specific virulence factors and their regulation. One virulence factor largely unique to *S. Typhi* is CdtB, which is responsible for a cytolethal distending toxic activity, characterized by pronounced cell distension and cell cycle arrest of the infected cells (Haghjoo and Galán, 2004). A unique feature of *cdtB* is that it is not expressed when *S. Typhi* is outside the cell, and it is dramatically induced once *S. Typhi* has reached a specific location within the host cell. In an effort to understand the regulation of genes that are expressed within host cells, we have investigated the regulatory mechanisms that control *S. Typhi* *cdtB* gene expression.

Through a genetic screen we have identified activators and repressors that influence *cdtB* expression. We found two genes whose constitutive expression led to the expression of *cdtB* under non-permissive (i.e. extracellular) conditions. One of these genes encodes the *S. Typhi* homologue of ParE, the B subunit of DNA topoisomerase IV (Kato *et al.*, 1990). Constitutive expression of DNA topoisomerase IV significantly decreases the overall levels of DNA superhelicity, which alters the expression of genes whose promoters are sensitive to the levels of DNA supercoiling. For example, overexpression of DNA topoisomerase IV in *Shigella flexneri* results in significant changes in virulence gene expression (McNairn *et al.*, 1995). We hypothesize that changes in the degree of DNA superhelicity due to over expression of *parE* leads to derepression of *cdtB*. In fact, another insertion mutation identified in this screen, which mapped to *tldD*, may cause the derepression of *cdtB* by a similar mechanism. *tldD* has been shown to regulate the activity of *letD*, which itself controls the activity of DNA gyrase and hence the level of DNA superhelicity (Murayama *et al.*, 1996). Therefore, these observations indicate that DNA superhelicity may be important in the control of *cdtB* expression.

The other gene whose constitutive expression led to *cdtB* expression under non-permissive conditions is *mntR*. MntR belongs to the DtxR family of transcriptional metaloregulatory proteins that control the expression of the metal transporters MntH and SitABCD (Kehres *et al.*, 2002; Ikeda *et al.*, 2005). In the presence of Mn(2+), MntR represses the expression of *mntH* and *sitABCD*, through direct binding of specific sites within the promoter regions of these genes. Interestingly, like *cdtB*, *sitABCD* is also induced upon *Salmonella* internalization into host cells suggesting the possibility that similar cues are required for the induction of these two loci (Zaharik *et al.*, 2004). Whether the levels of Mn(2+) or other metals could be such cue is unknown. The mechanisms by which MntR regulates *CdtB* gene expression are not known although it is unlikely that this regulator exerts its effect directly on the *cdtB* promoter. Instead, as MntR has been shown to work as a repressor, MntR may exert its effect by repressing the expression of a repressor of *cdtB* expression such as IgeR. Consistent with this hypothesis constitutive expression of *mntR* resulted in reduced levels of *igeR* expression.

IgeR belongs to the DeoR family of transcriptional repressor proteins. Inactivation of *igeR* led to the strongest derepression of *cdtB*. Our data indicate that IgeR represses the expression of *cdtB* by directly binding its promoter region and that other regulators identified in this screen may exert their effect at least in part by influencing the expression of IgeR. A recent study using selective capture of transcribed sequences to identify *S. Typhi* genes differentially expressed within cells showed that the expression of *sty0448* (*igeR*) is drastically reduced inside cells (Faucher *et al.*, 2006). Therefore, downregulation of *igeR* within cells may be largely responsible for the induction of *cdtB* expression. IgeR may therefore play a central role in the regulation of expression of genes whose function is exclusively required within an intracellular environment. Consistent with this hypothesis our microarray analysis identified additional IgeR-regulated genes such as *pagC* whose expression is induced intracellularly. However, our analysis also identified IgeR-regulated genes that are expressed during *Salmonella*'s extracellular stage such as the gene encoding the quorum sensing regulator SdiA and genes encoding flagella or components and effectors of the invasion-associated TTSS. However, in sharp contrast with *cdtB* and *pagC*, the levels of *sdiA* and the SPI-1 TTSS and flagellar genes were lower in the  $\Delta$ *igeR* mutant, indicating that IgeR is a positive regulator of these genes. Therefore, we hypothesize that IgeR may be involved in co-ordinating *Salmonella*'s transition from an extracellular to an intracellular location to ensure that the appropriate virulence factors are deployed in these vastly different environments. In this context, it is intriguing that we found similarities between

the genes affected by IgeR with those affected by the addition of bile salts in *S. Typhimurium*. For example, the expression of *pagC* and the *yciGEF-katG* operon were upregulated in response to bile salts while the expression of the flagellar and SPI-1 TTSS-related genes was significantly reduced (Prouty *et al.*, 2004). It is therefore possible that bile salts may constitute one of the cues that help *Salmonella* co-ordinate its transition from an extracellular to an intracellular environment and that IgeR is an important element of this regulation. Although *cdtB* is encoded by very few *S. enterica* serovars, its regulator *igeR* is widely distributed in all serovars. This suggests that IgeR may play a much broader role in virulence. Consistent with this hypothesis, constitutive expression of *igeR* in *S. Typhimurium*, which does not encode *cdtB*, resulted in a significant virulence reduction in a mouse model of infection. Our results also indicate that a unique *S. Typhi* gene, *cdtB*, presumably acquired through horizontal gene transfer, has evolved to be under the regulation of ancestral regulatory networks.

The importance of positive regulation of virulence genes expression has been extensively documented (Cotter and DiRita, 2000). Our results indicate that, although less-well documented, transcriptional repressors also play an important role in ensuring the appropriate temporal and spatial deployment of virulence factors.

## Experimental procedures

### Bacterial strains and growth conditions

The strains used in this study are listed in Table 4. All *S. Typhi* strains were derived from the wild-type strain ISP2825 (Galán and Curtiss, 1991) and the *S. Typhimurium* strains from the wild-type strain SL1344 (Hoiseh and Stocker, 1981). Strains were grown in LB broth or on LB agar plates at 37°C. For infection and gene expression experiments, bacterial strains were grown in LB broth (supplemented with 0.3 M sodium chloride for infection experiments) in a culture tubes placed on a rotating wheel to an optical density measured at 600 nm (OD<sub>600</sub>) of ~0.9. When appropriate, antibiotics were added at the following concentrations: ampicillin, 100 µg ml<sup>-1</sup>; chloramphenicol, 25 µg ml<sup>-1</sup>; kanamycin, 50 µg ml<sup>-1</sup>; tetracycline, 12.5 µg ml<sup>-1</sup>; streptomycin, 100 µg ml<sup>-1</sup>.

### Strain and plasmid construction

DNA purification, molecular cloning and PCR were performed according to standard procedures (Ausubel *et al.*, 1994). Plasmids were introduced in the different strains by electroporation. Non-polar deletions in *sty0448*, *sty3548*, *sty0911* were created using lambda Red mutagenesis procedure as previously described (Datsenko and Wanner, 2000). Briefly, primers were designed to amplify a *kan* cassette encoded in plasmid pKD4, which is flanked by FLP recombinase target (FRT) sites (Datsenko and Wanner, 2000). PCR products were generated by using pairs of ~70 nt long

Strain	Relevant genotype	Reference/source
<i>S. Typhi</i>		
ISP2825	Wild-type isolate	Galán and Curtiss (1991)
SB1438	<i>cdtB::lac</i>	Haghjoo and Galan (2004)
SB1580	<i>cdtB::lacZ</i>	This study
SB1581	$\Delta$ <i>sty0448</i>	This study
SB1582	$\Delta$ <i>sty3548</i>	This study
SB1583	$\Delta$ <i>sty0911</i>	This study
SB1584	<i>sty0874::TnMod-RKm'-lacI<sup>q</sup>tacp</i>	This study
SB1585	<i>sty3548::TnMod-RKm'-lacI<sup>q</sup>tacp</i>	This study
SB1586	<i>sty0911::TnMod-RKm'-lacI<sup>q</sup>tacp</i>	This study
SB1587	<i>sty3360::TnMod-RKm'-lacI<sup>q</sup>tacp</i>	This study
SB1593	<i>sty0448::TnMod-RKm'-lacI<sup>q</sup>tacp</i>	This study
SB1588	<i>cdtB::TnMod-RKm'-lacI<sup>q</sup>tacp</i>	This study
<i>S. Typhimurium</i>		
SB300	Mouse isolate of SL1344	Hoiseh and Stocker (1981)
SB1590	$\Delta$ <i>stm0410</i>	This study

Table 4. Bacterial strains used in this study.

primers that included 50 nt flanking sequences to the gene to be deleted (i.e. *sty0448*, *sty3548* or *sty0911*) and 20 nt priming sequences (P1, 5'-gtgtaggctggagctgcttc and P2, 5'-catatgaatctctccttag) for the pKD4 template, so as to be able to recombine the cassette into the *S. Typhi* genome and generate a complete deletion of the target gene. The resulting PCR products were purified from agarose gels, treated with DpnI, and introduced into *S. Typhi* ISP2825 carrying the plasmid pKD46 (which encodes the lambda red recombinase system under the control of an arabinose-inducible promoter) by electroporation to generate the  $\Delta$ *sty0448::kan*,  $\Delta$ *sty3548::kan* and  $\Delta$ *sty0911::kan* strains. The antibiotic cassettes were resolved by introducing pCP20, a temperature sensitive plasmid that encodes the FLP recombinase (Datzenko and Wanner, 2000). The resulting deletion strains were verified by PCR and cured of the different plasmids by growth at 42°C in the absence of antibiotics. A *S. Typhi* strain encoding a *cdtB::lacZ* transcriptional fusion was constructed as follows. A 500 bp DNA fragment containing the *cdtB* promoter region was amplified by PCR and cloned into the plasmid vector pRS414, which encodes a promoter-less *lacZ* (Simons *et al.*, 1987). The resulting gene fusion was cloned into the suicide vector pGP704 (Miller and Mekalanos, 1988) and mobilized into *S. Typhi* by conjugation. The correct integration of the fusion was verified by PCR and direct sequencing from chromosomal DNA.

#### Transposon mutagenesis of *S. Typhi*

Transposon mutagenesis using the mini Tn5 transposon derivative TnMod-RKm'-*lacI<sup>q</sup>tacp*, which carries an outward facing *tac* promoter, was carried out as previously described (Maxson and Darwin, 2004). Briefly, pAJD428, which is a suicide plasmid with an R6K origin of replication that encodes TnMod-RKm'-*lacI<sup>q</sup>tacp* and *lacI<sup>q</sup>*, was mobilized into *S. typhi* by conjugation using the *E. coli* strain S17-lambda *pir* (Simon *et al.*, 1983). Transconjugants were plated on LB agar plates containing 50 µg ml<sup>-1</sup> kanamycin, 1 mM IPTG and 50 µg ml<sup>-1</sup> of Xgal (5-bromo-4-chloro-3-indolyl-beta-D-galactopyranoside). Transposon insertion that led to expression of *cdtB::lacZ* under non-permissive conditions were identified as blue colonies. The precise location of the transposon inser-

tions was determined by direct nucleotide sequencing from chromosomal DNA using a primer complementary to sequences in the transposon (5'-TCGGCTCGTATAATGTGTGG-3') (Maxson and Darwin, 2004).

#### $\beta$ -Galactosidase assay and luciferase assays

To examine the levels of  $\beta$ -galactosidase in bacterial cultures, overnight cultures of the different strains were diluted 1:25 in LB broth with or without 1 mM IPTG and grown at 37°C until an OD<sub>600</sub> of ~0.6. Cultures were immediately chilled on ice and cells were collected by centrifugation. The  $\beta$ -galactosidase enzyme activity was determined using a chemiluminescent assay kit according to the manufacturer's instructions (Roche). Values were determined from at least three independent cultures. Luciferase measurements were carried out as previously described (Haghjoo and Galan, 2004) using a commercial kit (Luciferase Reporter Assay System, Promega). Briefly, Henle-407 cells were infected with the different reporter strains and at different times after infection, cells were washed with PBS and lysed and the firefly luciferase activity in lysates was measured using a luminometer as recommended by the manufacturer (Promega). Cells processed and infected in parallel and in identical fashion were lysed with 0.1% deoxycholic acid, and dilutions of lysates were plated onto LB agar plates to determine the number of intracellular bacteria.

#### Western blot analysis

Analysis of protein expression in whole cell lysates were performed as follows. Bacterial lysates were loaded and run on 10% polyacrylamide electrophoresis gels, and transferred to a nitrocellulose membrane. The membranes were blocked for 1 h with 5% milk in PBS-T (PBS plus 0.1% Tween-20). The blots were then incubated at room temperature with 1:1000 dilution of the monoclonal M45 antibody (Obert *et al.*, 1994) in 5% Milk-PBS-T followed by three washes of 10 min at room temperature in PBS-T. The membranes were then incubated with 1:10 000 dilution of the secondary anti-mouse HRP antibody for an additional hour in 5% Milk-PBS-T. Blots

were then washed in PBS-T (3 ×, 10 min each) and developed by enhanced chemiluminescence as recommended by the manufacturer (Supersignal West Pico, Pierce).

#### Tissue culture cell invasion assays

Growth of Henle-407 intestinal epithelial cells and their infection by *S. Typhi* were carried out as previously described (Galán and Curtiss, 1989; Haghjoo and Galan, 2004).

#### Gel shift assays

A 500 bp fragment directly upstream of the *S. Typhi cdtB* open reading frame (ORF) was amplified by PCR using DNA from the *S. Typhi* strain ISP2825 as template. The PCR product was gel purified and digested with *NcoI* and gel purified again. Approximately 200 ng of digested fragment was labelled at 30°C for 15 min with 1 U of the Klenow enzyme and 20 µCi of [ $\alpha^{32}$ P]-dATP and the labelling reaction was terminated by a 10 min incubation at 75°C. After removal of unincorporated labelled nucleotides, approximately 0.2 ng of probe (containing 10 000 cpm) and 0, 45 and 90 pmol of purified IgeR-His6 or the unrelated *S. Typhi* protein Sty 1890-His6 identically purified were incubated in a 20 µl volume at room temperature for 20 min. Purification of IgeR-His6 and Sty 1890-His6 was carried out by affinity chromatography over nickel columns as previously described (Akeda and Galan, 2004). The binding buffer used for protein–DNA interactions was 20 mM Tris, pH 7.4, 5 mM MgCl<sub>2</sub>, 50 mM KCl, containing 500 ng bovine serum albumin and 150 ng poly(dI-dC). In competition assays an approximately three- and sixfold excess of unlabelled specific DNA was added. Samples were run on 4% acrylamide gels and after electrophoresis the gel was dried and autoradiographed.

#### Microarray gene expression profiling

Wild-type *S. Typhi* ISP2825 and its  $\Delta$ *sty0448* ( $\Delta$ IgeR) mutant were grown in LB medium and incubated with aeration until an OD<sub>600</sub> of 0.6. Bacterial RNA was then isolated using Qiagen RNeasy Midi/Maxi Kit (Qiagen, Valencia, CA) and treated with DNase according to the manufacturer's instructions. Isolated RNA was resuspended in nuclease free water at a concentration of 1 mg ml<sup>-1</sup> and OD<sub>260/280</sub> of at least 1.7, and submitted for hybridization, data processing, and normalization to NimbleGen Systems (NimbleGen Systems, Madison, WI). Microarray analysis was conducted in duplicate, and two independent experiments were performed to generate reproducible and statistically significant data. The data were analysed by NimbleGen Systems, and only genes that exhibited twofold changes in gene expression or higher ( $P \leq 0.05$ ) for the statistical methods employed were considered significant. Data were processed using tools available through the Bioconductor project (<http://bioconductor.org>) using quantile normalization (Bolstad *et al.*, 2003), a comparison of normalization methods for high-density oligonucleotide array data based on bias and variance (Irizarry *et al.*, 2003). Gene calls were generated using the multichip average (RMA) algorithm (Bolstad *et al.*, 2003).

#### Quantitative real-time PCR

The quantitative real-time PCR was performed in the iCycler iQ system (Bio-Rad, Hercules, CA), using iQ SYBR Green Supermix (Bio-Rad, Hercules, CA) according to the manufacturer's instructions. DNA was synthesized using Superscript II Reverse Transcriptase (Invitrogen, Carlsbad, CA) according to manufacturer's instructions. To confirm PCR specificity, the PCR products were subjected to a melting curve analysis. Experiments were run in parallel and the fold-change values were calculated after normalization of each gene to the internal control 16S rRNA, using the comparative threshold method (Livak and Schmittgen, 2001). The primers used in these assays are listed in Table 1.

#### Mouse infections

All animal experiments were conducted according to protocols approved by Yale University's Institutional Animal Care and Use Committee. Overnight cultures of bacteria were freshly inoculated in LB Broth containing 0.3M NaCl and grown to an OD<sub>600</sub> of ~0.8. The number of bacteria used in infections was estimated based on a predetermined calibration curve and confirmed by viable bacterial counts on LB agar plates. Groups of age- and sex-matched C57BL/6 *nramp+/-* mice (M. Lara-Tejero and J. E. Galan, unpublished) were infected at 8–12 week of age. After 8 h of fasting, mice were administered by intragastric gavage 100 µl of 10% bicarbonate solution followed by 10<sup>7</sup> cfu of the different strains in 100 µl of PBS. Mice were monitored daily and 6 days post infection, mice were euthanized and organs were harvested. Spleens and mesenteric lymphnodes were removed and mechanically homogenized in 5 ml of sterile PBS containing 0.05% sodium deoxycholate. Bacterial loads were determined by plating 10-fold serial dilutions of homogenates on LB broth agar plates containing streptomycin and incubated overnight at 37°C. Colonies were counted and the number of total cfu recovered was calculated.

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### Supplementary material

The following supplementary material is available for this article:

**Fig. S1.** Amino acid sequence alignment of IgeR with members of the DeoR family of transcription regulators. The amino acid sequences of different DeoR protein-family members from *Salmonella enterica* were aligned using CLUSTALW (<http://www.ebi.ac.uk/clustalw/>) and the output was processed for display using BOXSHADE 3.1 ([http://www.ch.embnet.org/software/BOX\\_form.html](http://www.ch.embnet.org/software/BOX_form.html)). Identical residues are indicated with black boxes and conserved residues by grey boxes.

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