**lac Repressor Is an Antivirulence Factor of *Salmonella enterica*: Its Role in the Evolution of Virulence in *Salmonella***

Sandeepa M. Eswarappa, Guruswamy Karnam, Arvindhan G. Nagarajan, Sangeeta Chakraborty, Dipshikha Chakravorty*

Centre for Infectious Disease Research and Biosafety Laboratories, Department of Microbiology and Cell Biology, Indian Institute of Science, Bangalore, Karnataka, India

**Abstract**

The genus *Salmonella* includes many pathogens of great medical and veterinary importance. Bacteria belonging to this genus are very closely related to those belonging to the genus *Escherichia*, *lacZYA* operon and *lacI* are present in *Escherichia coli*, but not in *Salmonella enterica*. It has been proposed that *Salmonella* has lost *lacZYA* operon and *lacI* during evolution. In this study, we have investigated the physiological and evolutionary significance of the absence of *lacI* in *Salmonella enterica*. Using murine model of typhoid fever, we show that the expression of LacI causes a remarkable reduction in the virulence of *Salmonella enterica*. LacI also suppresses the ability of *Salmonella enterica* to proliferate inside murine macrophages. Microarray analysis revealed that LacI interferes with the expression of virulence genes of *Salmonella* pathogenicity island 2. This effect was confirmed by RT-PCR and Western blot analysis. Interestingly, we found that SGB0326 of *Salmonella bongori* is homologous to *lacI* of *Escherichia coli*. *Salmonella bongori* is the only other species of the genus *Salmonella* and it lacks the virulence genes of *Salmonella* pathogenicity island 2. Overall, our results demonstrate that LacI is an antivirulence factor of *Salmonella enterica* and suggest that absence of *lacI* has facilitated the acquisition of virulence genes of *Salmonella* pathogenicity island 2 in *Salmonella enterica* making it a successful systemic pathogen.

**Introduction**

The genus *Salmonella* includes Gram-negative facultative intracellular pathogens that infect reptiles, birds and mammals. This genus includes two species, viz. *Salmonella bongori* (S. bongori) and *Salmonella enterica* (S. enterica). *S. enterica* is subdivided into many subspecies and serovars. In humans, *Salmonella enterica* serovars Typhi causes typhoid fever and serovars Typhimurium and Enteritidis cause food poisoning.

The virulence of *S. enterica* can be attributed largely to horizontally acquired genomic islands termed *Salmonella* pathogenicity islands (SPI) [1,2]. Among them, *Salmonella* pathogenicity island 2 (SPI-2) imparts *S. enterica* an ability to cause systemic infection. SPI-2 is 40 kb in size and can be divided into two distinct parts. One part is 25 kb in size and encodes a type three secretion system (TTSS). This part is essential for the systemic virulence and is present in *S. enterica*, but absent in *S. bongori*. The other part is 15 kb in size and is not essential for the systemic virulence of *S. enterica*. This region encodes the tetrahtionate reductase involved in anaerobic respiration and is present in both *S. bongori* and *S. enterica* [3–5]. Evolutionary significance of *S. bongori* not having the 25 kb part and *S. enterica* having both parts of SPI-2 is not clear.

Bacteria belonging to the genus *Salmonella* are closely related to those belonging to the genus *Escherichia* and they have diverged from a common ancestor about 100 million years ago [6]. Despite their close relationship, *E. coli* has more than 800 genes that are absent in the *S. enterica* genome and more than 1,100 *S. enterica* genes lack their homologues in *E. coli* [7]. The region containing *lacI* and the *lac operon* is one such locus and is present in *E. coli*, but absent in *S. enterica*. Thus, *E. coli* is a lactose fermenter, whereas *S. enterica* is a lactose non-fermenter. Nonetheless, diseases caused by lactose-fermenting *Salmonella* have been reported occasionally and they harbor genes responsible for lactose fermentation in extra-chromosomal genetic elements like plasmids [8–12].

The *lac operon* consists of three genes, *lacZ*, *lacT* and *lacA* which encode β-galactosidase, lactose permease and a transacetylase, respectively. *lacI* which encodes lac repressor (LacI) is located adjacent to *lacZTA*, but is transcribed as a separate message. LacI binds to the operator region of the *lac operon* and prevents the transcription of *lacZTA* genes unless lactose is present in the environment [13]. Expression of the *lac operon* when lactose is absent in the environment is costly for the bacteria [14] and LacI prevents such unnecessary expression of the *lac operon*. Constitutive expression of the *lac operon* lowers the fitness of the bacteria when lactose is not present in the environment [15,16]. Therefore, LacI is very important to maintain the fitness of the bacterium that harbors the *lac operon*. None of the lactose-fermenting *Salmonella* strains (see above) are reported to harbor LacI, except S. Typhi.
strain ST-2 which harbors a mutant LacI with a diminished repressing ability [12]. It has been proposed that S. enterica has lost lac region (lacI and lacZ) during evolution [17]. Many serovars of S. enterica are intestinal pathogens of mammals and like E. coli they are exposed to lactose in the mammalian gut. Then, why has S. enterica lost the ability to ferment lactose? So far, there are no studies which address this issue. In this study, we have investigated the physiological and evolutionary significance behind the loss of (or absence of) lac region in S. enterica. We have chosen LacI for this study because it is very important for a bacterium harboring the lac operon to have lac as unnecessary expression of the lac operon lowers the fitness of the bacteria harboring it [15,16]. Our study demonstrates that the expression of LacI in S. enterica reduces its virulence and suggests that lack of lacI has facilitated S. enterica to gain systemic virulence via SPI-2.

Results

We used pTrc99A plasmid that harbors lac gene to express LacI in S. enterica [18]. We deleted lacI from this plasmid to get pTrc(-LacI) plasmid and also we mobilized lacI along with its promoter from pTrc99A to pBR322 to get pBR322(+LacI). All these plasmids, viz. pTrc99A, pTrc(-LacI), pBR322 and pBR322(+LacI), were individually transformed into the wild-type (WT) strain of S. enterica serovar Typhimurium (S. Typhimurium). Expression of lacI in the strains harboring pTrc99A and pBR322(+LacI) was confirmed by RT-PCR and Western blot (Fig. S1 A and C). Growth of all these strains in Luria broth (LB) and M9 minimal medium were comparable to that of the parental WT strain (data not shown). Both pTrc99A and pBR322 have colEl replicon and thus, have low to moderate (fifteen to twenty) copy numbers [19].

S. enterica expressing LacI exhibits reduced virulence in murine typhoid fever model

Most of the known S. enterica serovars are pathogenic to one or the other vertebrate species. We were interested to know the effect of LacI on the virulence of S. enterica. We used murine typhoid fever model for this purpose. Mice were infected intra-peritoneally with S. enterica strains harboring different plasmids (see above) and infected mice were monitored for survival for 3 months. All the mice infected with the WT strain and strains harboring either pTrc(-LacI) or pBR322 (+LacI) were dead within 5 days of infection. Interestingly, 60% of the mice infected with strains harboring either pBR322(+LacI) or pTrc99A (+LacI) (plasmids expressing lacI) survived and did not succumb to the infection even after 3 months of infection (Fig. 1A).

Then, we examined the bacterial load in infected mice after 3 days of infection. We observed about 2 to 3 log order reduction in the bacterial load in spleen and mesentric lymph node of the mice infected with strains harboring either pTrc99A or pBR322(+LacI) when compared to the organs of the mice infected with the WT strain (P<0.01). However, the bacterial load in spleen and mesenteric lymph node of the mice infected with strains harboring either pBR322 or pTrc(-LacI) were not different from those of the mice infected with the WT strain (P>0.05) (Fig. 1 B and C). These findings clearly show that LacI suppresses the virulence of S. enterica. LacI-dependent attenuation of Salmonella was also observed when the infection was done via intra-gastric route (Figure S2).

There is a possibility that plasmids may get lost from bacteria during infection and in such case, selecting bacteria on medium containing antibiotic will underestimate the bacterial load. To rule out such possibility, the organ homogenate was plated separately on medium with or without antibiotics. We observed that presence of antibiotic in the medium does not cause any change in the bacterial load estimation indicating that bacteria does not lose plasmid during infection.

To further confirm the effect of LacI on the virulence of S. enterica, we determined the competitive indices (CI) of strains expressing LacI with respect to the WT strain. Determination of CI is a very sensitive assay that compares the virulence of different strains [20]. For this assay, we used the WT strain constitutively expressing green fluorescent protein (GFP) to enable us to distinguish colonies of WT bacteria from the colonies of other strains on LB agar plate. The presence of GFP did not show any significant effect on the virulence of the WT strain (CI = 1.22±0.24; P>0.05). The CI values of strains harboring either pBR322(+LacI) or pTrc99A, determined in both spleen and mesenteric lymph node, were significantly (P<0.001) lesser than those of strains harboring either pBR322 or pTrc(-LacI) (Fig. 2). This result unequivocally demonstrates that LacI suppresses the virulence in S. enterica.

S. enterica expressing LacI cannot proliferate inside murine macrophages

Our next aim was to understand how LacI is suppressing the virulence of S. enterica. To address this, we investigated the effect of LacI on the ability of S. enterica to multiply inside macrophages. Ability to multiply inside host macrophages is vital for the virulence of S. enterica [21]. We observed that strains harboring either pBR322(+LacI) or pTrc99A were unable to proliferate inside RAW 264.7 cells, a murine macrophage-like cell line. In contrast, proliferation of strains harboring either pTrc(-LacI) or pBR322 inside these macrophages were comparable to that of the WT strain (Fig. 3A and B). These finding illustrates that LacI suppresses the ability of S. enterica to multiply inside host macrophages and explains, at least in part, why S. enterica expressing LacI is less virulent. Nevertheless, LacI expression did not affect the entry of bacteria into epithelial (HeLa) cells as demonstrated by an invasion assay (Figure S3).

Our next aim was to investigate the mechanism of action of LacI that leads to the above observed effects in S. enterica; is DNA binding property of LacI responsible for these effects? To address this issue, we used isopropyl-β-D-thiogalactoside (IPTG). IPTG binds with high affinity to LacI and lowers its affinity for the operator DNA, the natural target of LacI [22]. However, IPTG does not affect the binding of LacI to non-operator DNA [23,24]. Interestingly, LacI expressing strains [harboring either pTrc99A or pBR322(+LacI)] did not show any defect in their ability to multiply inside macrophages cultured in the presence of 10 mM IPTG. Proliferation of all the strains, viz. S. enterica having either pTrc99A or pTrc(-LacI) or pBR322 or pBR322(+LacI) were similar to that of the WT strain inside macrophages (Fig. 3C and D). ΔsaV, a mutant strain defective in secretion by the SPI-2 type three secretion system (TTSS), was used as positive control because SPI-2 encoded TTSS is essential for S. enterica to proliferate inside host macrophages [5]. The phenotype of ΔsaV strain was unaffected by IPTG (Fig. 3B and D).

This result clearly demonstrates that IPTG abolishes the inhibitory effect of LacI on the ability of S. enterica to multiply inside host macrophages. In addition, this result suggests that this inhibitory effect of LacI may be due to its binding to operator DNA and also it rules out the possible role of binding of LacI to a non-operator DNA sequence in this effect. This result also shows that LacI is indeed expressed in S. enterica harboring either pTrc99A or pBR322(+LacI) because the effect of IPTG is due to its binding with high affinity to LacI protein.
Figure 1. *S. enterica* expressing LacI shows attenuated virulence *in vivo*. (A) Survival plots of mice (*n* = 5 in each case) intra-peritoneally infected with *S. enterica* harboring different plasmids (10⁶ bacteria per mouse). Survival of mice infected with *S. enterica* expressing LacI [pTRc99A and pBR322(+LacI)] were compared to that of mice infected with the WT strain to calculate the statistical significance (*P* = 0.0023 for both the strains) using Logrank test. Bacterial load in (B) spleen and (C) mesenteric lymph node of mice (*n* = 5 in each case) intra-peritoneally infected (for 3 days) with *S. enterica* harboring different plasmids (10³ bacteria/mouse) are plotted in (B) and (C). The bacterial loads in organs of mice infected with experimental strains were compared to those of mice infected with the WT strain to calculate statistical significance by student’s t-test. Horizontal lines in (B) and (C) indicate mean values. Graphs are representatives of at least two independent experiments.

doi:10.1371/journal.pone.0005789.g001

Figure 2. *S. enterica* expressing LacI are less competitive with respect to the WT strain. Mice (*n* = 5 in each case) were infected intra-peritoneally with a mixture of the WT strain and the indicated strain at 1:1 ratio (total 10⁸ bacteria/mouse). After 3 days, CI values for all the strains were calculated with respect to the WT strain. CI values determined in spleen (A) and mesenteric lymph node (B) are plotted. Horizontal lines indicate mean values. Only four CI values are shown for the strain harboring pTRc99A in spleen and for the strain harboring pBR322(+LacI) in mesenteric lymph node as the fifth CI values were zero in both cases. Student’s t-test was used to calculate the statistical significance. Graphs are representatives of two independent experiments.

doi:10.1371/journal.pone.0005789.g002
Ability of LacI to bind to the operator sequence is not responsible for its effect on the virulence of S. enterica

In E. coli, LacI binds to the operator sequence present in the lac operon [25]. Mutating Gln60 to Gly and inserting three extra glycines after 60th amino acid of LacI abolishes its ability to bind to the operator sequence without affecting its folding, assembly and inducer (IPTG)-binding property. However, the ability of this mutant LacI(Gly60+3) to bind to a non-operator DNA sequence is similar to that of the wild-type LacI [26]. We utilized this mutant LacI to investigate whether operator-binding property of LacI is responsible for its effect on the virulence of S. enterica. To our surprise, mice infected with S. enterica expressing LacI(Gly60+3) survived longer than those infected with the WT strain (Fig. 4A). Four out of five mice infected with S. enterica expressing LacI(Gly60+3) survived for more than 3 months after infection and did not succumb to infection. There were no detectable bacteria in the organs of mice infected with S. enterica expressing LacI(Gly60+3) after 3 days of infection (Fig. 4B). In addition, S. enterica expressing LacI(Gly60+3) could not proliferate inside macrophages (Fig. 4C). Our search for the presence of operator sequence in S. Typhimurium genome (gi: 16763390) using genomic BLAST program (BLASTN 2.2.18) revealed that there is no sequence that is significantly similar (expect value = 10) to the operator sequence (5'-tgttgttgttgtgagcggataaatactgacagg-3'). These results clearly demonstrate that the effect of LacI on the virulence of S. enterica is independent of its ability to bind to the operator sequence.

S. enterica expressing LacI is sensitive to mice serum

Pathogenic bacteria like S. enterica that are capable of causing systemic disease are resistant to serum-mediated killing and this
A

Percent survival

\( P = 0.0031 \)

Number of days after infection

B

CFU per g. of mesenteric lymph node

\( 10^4 \)  \( 10^5 \)  \( 10^6 \)  \( 10^7 \)

WT Lacl WT Lacl

(Gly^{59+3})  (Gly^{52+3})

Mesenteric lymph node Spleen

C

Fold intracellular multiplication

\( P < 0.001 \)
property is important for their virulence [27]. Interestingly, the growth of S. enterica harboring pTrc99A was significantly diminished in mouse serum (Fig. 5). S. enterica expressing LacI thus appears to be sensitive to serum-mediated killing; this might also contribute to the diminished virulence of S. enterica expressing LacI.

LacI inhibits the expression of SPI-2 inside macrophages

Our next aim was to find out the possible targets of LacI in S. enterica. We used microarray technique for this purpose. Microarray expression profiling was done using RNA isolated from the WT strain and the strains harboring either pTrc99A or pTrc(-LacI) plasmids during mid-log phase growth in M9 minimal medium. The genes whose expression was affected in the strain harboring pTrc99A but not in the strain harboring pTrc(-LacI) were taken into consideration. We found 75 genes that were significantly down-regulated and 147 genes that were significantly up-regulated (>2-fold and P<0.02) in the strain harboring pTrc99A (Table S1 and Table S2). Among them, we looked for the genes that can influence the virulence of S. enterica. To our interest, 4 genes (ssaK, ssaF, steel and orf245) belonging to SPI-2 and phoP were down-regulated in the strain harboring pTrc99A but not in the strain harboring pTrc(-LacI). Both SPI-2 and PhoP are essential for the virulence of S. enterica [3,28]. This result encouraged us to look for the expression profile of these genes in the strain expressing LacI inside macrophages.

In addition to these genes, microarray analysis showed up-regulation of some flagella-related genes (fliC, fliT, fliA, cheY, cheW, and fltC) and genes belonging to SPI-1 (spaP, spaK, spaT, spaO and invJ). We did not pursue these genes further because up-regulation of these genes does not explain attenuation of virulence in S. enterica.

The expression profile of SPI-2 genes in the strain harboring pTrc99A inside macrophages was investigated using RT-PCR. RNA was isolated from intracellular bacteria and subjected for reverse transcription followed by PCR. We chose three virulence genes, viz. ssaK, sseB and spaC which represent three different operons of the 25 kb part of SPI-2 that encodes a TTSS. Expression of ssaK, sseB and spaC was reduced in the strain harboring pTrc99A inside macrophages. This reduction was not observed in the strain harboring pTrc(-LacI) indicating that LacI interferes with the expression of these genes. However, the expression of phoP was unaffected (Fig. 6A). These results were confirmed using quantitative real-time PCR analysis (Fig. S4).

To further confirm the effect of LacI on SPI-2 expression, we looked at Ssb protein level in intracellular bacteria recovered from macrophages by Western blotting. We observed a remarkable reduction in Ssb protein level in the strain harboring pTrc99A but not in the strain harboring pTrc(-LacI) inside macrophages (Fig. 6B). Expression of SPI-2 can be induced in vitro by using F medium [29]. This medium mimics intra-phagosomal environment as it is low in magnesium and phosphorous and acidic in pH. When grown in F medium for 2 h, there was significant decrease in the Ssb protein level in the strain harboring pTrc99A but not in the strain harboring pTrc(-LacI) (Fig. 6C).

These results unequivocally demonstrate that LacI interferes with the expression of SPI-2 genes inside macrophages explaining, at least in part, why S. enterica expressing LacI are unable to proliferate inside macrophages and are attenuated in virulence in vivo. As some of the SPI-2 mutants are shown to be sensitive to serum mediated killing [30], this result also explains the serum sensitivity of S. enterica expressing LacI.

SBG0326 of S. bongori is homologous to lac

S. bongori, the only other Salmonellaceae species, is found as a commensal of cold-blooded animals and it cannot cause systemic infections in mammals. 25 kb region of SPI-2 that encodes TTSS is absent in S. bongori, but is present in S. enterica [5]. This region is essential for the systemic virulence of S. enterica. These facts prompted us to look for the presence of lacI homologues/s in S. bongori genome. Interestingly, we observed that SBG0326 of S. bongori is homologous to lacI of E. coli. The predicted amino acid sequences of protein encoded by SBG0326 and LacI share 77% identity and 88% similarity (Fig. 7A). However, we did not find any transcript corresponding to lacI in S. bongori suggesting that SBG0326 is probably a pseudogene (Fig. S1 A and B).

Our search also revealed that SBG0324 of S. bongori is homologous to lacZ of E. coli. The predicted amino acid sequences of protein encoded by SBG0324 and LacZ share 79% identity and 87% similarity. There were no genes that are homologous to lacY and lacA in S. bongori genome. We observed that ppbE and hemB are contiguous in S. enterica. However, these two genes are separated by four genes in S. bongori two of which are SBG0324 (lacZ) and SBG0326 (lacI). In E. coli,
lac Repressor and Salmonella

A

|   | WT | pTrc99A | pTrc(-LacI) |
|---|----|---------|-------------|
| rpoD | ![Image](image1) | ![Image](image2) | ![Image](image3) |
| 165 rRNA | ![Image](image4) | ![Image](image5) | ![Image](image6) |
| phoP | ![Image](image7) | ![Image](image8) | ![Image](image9) |
| ssaK | ![Image](image10) | ![Image](image11) | ![Image](image12) |
| sseB | ![Image](image13) | ![Image](image14) | ![Image](image15) |
| spiC | ![Image](image16) | ![Image](image17) | ![Image](image18) |

Without RT (ssaK)

|   | WT | pTrc99A | pTrc(-LacI) |
|---|----|---------|-------------|
| 25 cycles | ![Image](image19) | ![Image](image20) | ![Image](image21) |
| 30 cycles | ![Image](image22) | ![Image](image23) | ![Image](image24) |
| 35 cycles | ![Image](image25) | ![Image](image26) | ![Image](image27) |

B

|   | WT | pTrc99A | pTrc(-LacI) |
|---|----|---------|-------------|
| SseB | ![Image](image28) | ![Image](image29) | ![Image](image30) |
| Rrf | ![Image](image31) | ![Image](image32) | ![Image](image33) |

C

|   | 'F' Medium | LB medium |
|---|------------|-----------|
|   | WT | pTrc99A | pTrc(-LacI) | WT | pTrc99A | pTrc(-LacI) |
| SseB | ![Image](image34) | ![Image](image35) | ![Image](image36) | ![Image](image37) |
| Rrf | ![Image](image38) | ![Image](image39) | ![Image](image40) | ![Image](image41) |
Figure 6. LacI interferes with SPI-2 expression. (A) The effect of LacI on the expression of virulence genes inside macrophages was analyzed by RT-PCR. cDNA was synthesized from the RNA isolated from intracellular bacteria and ssaK, sseB, spiC (representatives from SPI-2 island) and phoP were amplified by PCR. (B) Analysis of SseB expression by Western blot using total protein extracted from intracellular bacteria. (C) Analysis of SseB expression by Western blot using total protein extracted from bacteria grown in SPI-2-inducing (F medium) medium or LB for 2 h. Ribosomal recycling factor (Rrf) served as internal control. Results are representatives of at least two independent experiments.

doi:10.1371/journal.pone.0005789.g006

Figure 7. SBG0326 of S. bongori is homologous to lacI of E. coli. (A) Alignment of predicted amino acid sequences of SBG0326 and LacI (E. coli K12). Both of them share 77% identity and 88% similarity. Identical residues are underlined. (C) Schematic diagram showing organization of prpE to hemB locus in S. bongori, S. Typhimurium and E. coli (K12). Each arrow represents indicated gene. Genes belonging to lac region are shown in darker shade. In case of S. bongori and S. Typhimurium, the names of the homologous genes in E. coli are given below the arrows. Arrows are not to scale.

doi:10.1371/journal.pone.0005789.g007
Introduction of either absent in Shigella, Salmonella enterica type 1 fimbriae, is observed in nonpathogenic and uropathogenic E. coli [31]. Some bacteria have selectively inactivated genes that are horizontally acquired genetic elements encoding virulence factors. For example, Salmonella has many horizontally acquired pathogenicity islands [1]. In some cases, virulence results from allelic differences in homologous genes. For example, genetic variation of fimH, a gene that encode adhesin of type I fimbriae, is observed in nonpathogenic and uropathogenic E. coli [31]. Some bacteria have selectively inactivated genes that are no longer compatible with the pathogenic lifestyle of the bacteria either by point mutation, insertion, or deletion. Such genes are called ‘antivirulence genes’ [32]. For example, cadA and ompT are absent in Shigella, but are present in nonpathogenic E. coli. Introduction of either cadA or ompT attenuates virulence in Shigella [33,34]. zrTS, gvaA and pegL are the antivirulence genes reported in S. enterica [35–37]. However, all these genes are present in S. enterica and modulate its virulence. So far, there is no report of an antivirulence gene of S. enterica that has been lost during evolution. Our study unequivocally demonstrates that lacI is an antivirulence gene of S. enterica which has been lost from it, but present in closely related S. bongori and E. coli.

We recognize the fact that the observed remarkable inhibitory effect of LacI on the virulence of S. enterica may be an exaggerated effect of LacI expression using plasmids having low to moderate copy numbers (fifteen to twenty). pTrc99A plasmid has operator region that can quench a part of LacI which reduces the amount of free LacI and decreases the effect of copy number of the plasmid on freely available LacI. Nevertheless, a slight inhibitory effect on the expression of virulence gene is sufficient to drive a genetic trait (if dispensable) towards negative selection.

Plasmids lacking LacI (pTrc99A-LacI) and pBR322 express beta-lactamase and tetracycline resistance proteins which are foreign to Salmonella, but they do not affect the virulence of S. enterica. Moreover, constitutive expression of green fluorescent protein also did not affect the virulence of S. enterica. Therefore, the effect of LacI on the virulence of S. enterica is unlikely to be just an artifact caused by the expression of a foreign protein.

There are two possibilities by which LacI can inhibit the virulence of S. enterica: either by binding to DNA or by binding to a protein. With respect to DNA binding, IPTG and Gly^{80V3} mutation in LacI have same effect on the function of LacI. Both of them inhibit the LacI binding to the operator DNA without affecting its binding to non-operator sequence. IPTG could reverse the inhibitory effect of LacI on the ability of S. enterica to proliferate inside macrophages, whereas S. enterica expressing LacI mutant (Gly^{80V3}) was unable to proliferate inside macrophages. These results indicate that the effect of LacI on the virulence of S. enterica is not because of its DNA binding property. The other possibility is the binding of LacI to a protein. As LacI can form homodimer and heterotetramer, there is a possibility of LacI forming heterodimer or heterotetramer with other transcriptional factors or any proteins present in S. enterica and IPTG might inhibit such protein-protein interactions. All these possibilities need to be investigated.

Our study demonstrates that the antivirulence property of LacI in S. enterica is, at least in part, because of its interference with the expression of SPI-2. Acquisition of SPI-2 by S. enterica is a landmark event in the evolution of Salmonella. SPI-2 has enabled S. enterica to survive and multiply inside macrophages resisting their antimicrobial activities [38,39]. This ability is essential for S. enterica to cause systemic infection [3,5]. Thus, acquisition of virulence genes of SPI-2 has made it possible for S. enterica to extend its niche beyond intestine, whereas absence of the same has made S. bongori a mere commensal of cold-blooded animals. Based on these facts and our results, we propose that presence of SBG0326 (lacI) has probably hindered the acquisition of virulence genes of SPI-2 in S. bongori, whereas absence of lacI has facilitated the same in S. enterica making it a successful systemic pathogen. Thus, lacI has played a remarkable role in the evolution of virulence in Salmonella.

Discussion

The evolution of bacterial pathogens from their nonpathogenic ancestors is an exciting saga which involves many kinds of fascinating genetic events. This is central to the understanding of both the origin of infectious diseases and the emergence of new pathogens. Some bacteria have horizontally acquired genetic elements encoding virulence factors. For example, Salmonella has many fascinating genetic events. This is central to the understanding of the evolution of bacterial pathogens from their nonpathogenic ancestors is an exciting saga which involves many kinds of fascinating genetic events. This is central to the understanding of the evolution of bacterial pathogens from their nonpathogenic ancestors is an exciting saga which involves many kinds of fascinating genetic events.

Materials and Methods

Bacterial strains, plasmids and culture conditions

Salmonella enterica serovar Typhimurium strain NCTC 12023 was used as wild-type (WT) in all experiments. This was the parental strain for all other strains. ΔasaV and S. bongori IH4 strains were kind gifts from Prof. Michael Hensel. Various plasmids used in this study are listed in Table S3. pTrc(-LacI) was constructed by digesting pTrc99A plasmid with BssHII to remove 872 nucleotide region encoding LacI and self-ligating the remaining plasmid. The deleted region included the promoter of lacI. lacI, including its promoter region, of pTrc99A was mobilized into EcoRI and HindIII sites of pBR322 to get pBR322(+LacI) plasmid. Primers used for constructing different plasmids are listed in Table S4. All plasmids were individually transformed to WT S. enterica. Bacteria were routinely cultured in Luria broth (LB) at 37°C. Optical density of the bacterial culture at 600 nm was measured every hour till stationary phase reached in order to monitor the growth of all the strains in LB or M9 minimal medium.

Mice infections

All procedures with animals were carried out in accordance with institutionally approved protocols. Six- to eight-week-old BALB/c mice were infected intra-peritoneally with S. enterica in all the experiments. A dose of 10^3 bacteria per mouse was used to investigate the bacterial load in spleen and mesenteric lymph node. After 3 days of infection, spleen and mesenteric lymph node of infected mice were harvested and homogenized. Bacteria were enumerated after plating a dilution series onto LB agar. A dose of 10^4 bacteria per mouse was used to follow the survival of the infected mice for 3 months. To calculate the competitive indices (CI) of S. enterica harboring different plasmids, mice were infected with equal amounts of WT S. enterica constitutively expressing green fluorescent protein (pFPV25.1) and the experimental strain (a ratio of 1:1). A total of 10^3 bacteria per mouse were inoculated. After 3 days, spleen and mesenteric lymph node were harvested, homogenized and the homogenate was plated onto LB agar. Bacterial colonies with (WT strain) or without (experimental strain) green fluorescence were enumerated. The CI was defined as the ratio of the experimental strain to the WT strain recovered from the mice divided by their ratio in the initial inoculum [20]. Presence of green fluorescent protein did not affect the virulence of S. enterica.

Serum sensitivity assay

Blood collected by retro-orbital bleeding of mice was used to extract the serum. Healthy, Six- to eight-week-old BALB/c mice (that were to be sacrificed for some other purpose) were used for retro-orbital bleeding. Bacteria in exponential phase were seeded
in a 96-well plate at a concentration of 2 to $5 \times 10^4$ bacteria per well in 50 μl of a solution containing 0.5% tryptone and 0.5% sodium chloride. 50 μl of mouse serum was added to each well and incubated at 37°C in shaking conditions for 4 h. The samples were then plated at different dilutions on LB plates. Fold multiplication was calculated by dividing the number of bacteria recovered after 4 h by the number of bacteria in the inoculum.

**Eukaryotic cell culture and intracellular proliferation assays**

RAW 264.7 cells were grown routinely in Dulbecco’s Modified Eagle’s Medium (DMEM; Sigma-Aldrich, USA) supplemented with 10% fetal calf serum (Sigma-Aldrich, USA) at 37°C in 5% CO₂. RAW 264.7 cells (3×10⁵ per well) were seeded in 24-well plates 12 to 24 h prior to infection. These cells were infected with overnight culture of a specific strain of *S. enterica* as described earlier at a multiplicity of infection (MOI) of 1:1 [40]. To check the expression of SPI-2 genes by RT-PCR and western blot, an MOI of 50:1 was used. Fold intracellular multiplication was calculated by dividing the intracellular bacterial load at 16 h by the intracellular bacterial load at 2 h.

**Microarray analysis**

The WT strain and the strains harboring either pTrc99A or pTrc(-LacI) were grown in M9 minimal medium for 9 h (mid-log phase). After 9 h, total RNA was isolated from equal number of bacteria of each strain using ‘RNaseasy Mini Kit’ (QIAGEN, USA). ‘RNAprotect Bacteria Reagent’ (QIAGEN, USA) was used to stabilize RNA before bacterial cells were lysed. The quality of the RNA was checked using “Agilent - 2100 Bioanalyzer” (Agilent Technologies, USA). RNA was amplified using ‘MessageAmp II-Bacteria RNA Amplification kit’ (Ambion, USA) and labeled using ‘Low Input RNA amplification kit’ (Agilent Technologies, USA). Hybridization was done using ‘In situ Hybridization kit’ (Agilent Technologies, USA). Dye-swap was included in the hybridization plan. Hybridization plan was as follows: WT Cy5 vs. pTrc99ACy3, WT Cy3 vs. pTrc99ACy5, WT Cy3 vs. pTrc(-LacI)Cy5, WT Cy5 vs. pTrc(-LacI)Cy3, Slides were scanned at 5 μ resolution using Agilent scanner. Automated feature extraction was done using Agilent’s Feature Extraction Software. Analysis of feature extracted data was done using Agilent’s GeneSpring GX V 7.3.1 software. The normalization was done using GeneSpring GX using the recommended Per Chip and Per Gene Data Transformation. Genes with ratio of 2 and above were considered as up-regulated and with ratio 0.5 and below were considered down-regulated. P value was calculated by GeneSpring GX for each gene on the basis of replicate probes to indicate statistical significance. P value less than 0.02 was taken as statistically significant. Raw data of microarray experiment is provided in Table S5. All microarray data reported in the manuscript is described in accordance with MIAME guidelines and the data from the experiments are deposited in GEO (accession no. GSE15580 and GPL8320).

**RT-PCR**

RNA was extracted from an overnight bacterial culture to check the expression of lacI and from intracellular bacteria (isolated from RAW 264.7 cells) to check the expression of SPI-2 genes. RNA (2 μg) was treated with RNase-free DNase (Fermentas, USA) and then reverse transcribed using a reverse transcription system (Promega, USA). The cDNA thus generated was amplified using specific primers designed for lacI, phoP, ssk, sedB, spoC, rpoD and 16S rRNA. The nucleotide sequences of all primers are given in Table S4.

**Western blot**

To check the expression of SPI-2, total protein was extracted from intracellular bacteria (isolated from RAW 264.7 cells) and also from bacteria grown in F or LB medium for 2 h. The F medium was prepared as described previously [29]. Protein was electrophoresed and transferred to a polyvinylidene difluoride membrane (Millipore, USA). The membrane was then probed with SbeB or Rrf antibody (a kind gift from Prof. Hensel and Prof. Varshney, respectively) and respective horse radish peroxidase-(HRP) conjugated secondary antibody (Bangalore Genei, India). Immune complexes were detected using an enhanced chemiluminescence reagent (PerkinElmer, USA).

**Bioinformatic analysis**

The complete genome sequence of *S. bongori* 12419 ATCC 43975 available in Welcome Trust Sanger Institute website (http://www.sanger.ac.uk/Projects/Salmonella/) and the nucleotide sequences of lacI and lacZYA genes of *E. coli* K12 were used to search for the presence of lac region in *S. bongori*. ‘BLAST 2 sequences’ tool was used for this analysis [41]. ExPASy-Translate tool was used to predict amino acid sequences [42]. ClustalW program was used for amino acid sequence alignment [43].

**Statistical methods**

Logrank test was used to analyze the survival curves of mice infected with different strains of *S. enterica*. Student’s t-test was used to analyze the bacterial load in the organs of infected mice and also to analyze the ability of different strains of *S. enterica* to multiply inside macrophages and in the presence of mouse serum.

**Supporting Information**

**Table S1** List of genes which are down-regulated in the strain having pTrc99A but not in the strain having pTrc(-LacI) 
Found at: doi:10.1371/journal.pone.0005789.s001 (0.16 MB DOC)

**Table S2** List of genes which are up-regulated in the strain having pTrc99A but not in the strain having pTrc(-LacI) 
Found at: doi:10.1371/journal.pone.0005789.s002 (0.30 MB DOC)

**Table S3** Plasmid constructs used in this study 
Found at: doi:10.1371/journal.pone.0005789.s003 (0.03 MB DOC)

**Table S4** Primers used in this study 
Found at: doi:10.1371/journal.pone.0005789.s004 (0.03 MB DOC)

**Table S5** Raw data of microarray experiment 
Found at: doi:10.1371/journal.pone.0005789.s005 (8.59 MB XLS)

**Figure S1** Analysis of expression of LacI. (A) RT-PCR analysis of lacI expression in different strains of Salmonella and *E. coli*. **Iac Repressor and Salmonella**
RNA isolated from overnight culture of bacteria was treated with RNase-free DNase and reverse transcribed. cDNA thus generated was used for PCR amplification. (B) PCR amplification of lac from the genomic DNA of bacteria. In both (A) and (B), PCR was done using lac-specific primers. rpoD was used as internal control. (C) Western blot analysis of LacI expression in different strains of Salmonella. Equal number of bacteria from overnight culture was lysed and the lysate was used for Western blot analysis. LacI antibody was from Abcam.

Figure S2 Effect of LacI on the proliferation of Salmonella in mice infected via intra-gastric route. The graph shows the bacterial load of the spleens of mice infected with Salmonella. mice infected via intra-gastric route. The graph shows the bacterial load of the spleens of mice infected with Salmonella.

Figure S3 Expression of LacI in Salmonella does not affect invasion. HeLa cells were infected as described in Materials and Methods with log-phase bacteria with an MOI of 1. Cells were lysed at 1 h after infection to recover the intracellular bacteria. Percentage of entry was calculated by comparing the number of entered bacteria with the number of bacteria in the pre-inoculum.

References
1. Henkel M (2004) Evolution of pathogenicity islands of Salmonella enterica. Int J Med Microbiol 294: 93–102.
2. Marcus SL, Brunnell JH, Pfeifer CG, Finlay BB (2000) Salmonella pathogenicity islands: big virulence in small packages. Microbes Infect 2: 143–156.
3. Shear JE, Henkel M, Gleson C, Holden DW (1996) Identification of a virulence locus encoding a second type III secretion system in Salmonella typhimurium. Proc Natl Acad Sci U S A 93: 2593–2597.
4. Henkel M, Hinley AP, Nikolau S, Sawers G, Berks BC (1999) The genetic basis of tetrathionate respiration in Salmonella typhimurium. Mol Microbiol 32: 275–287.
5. Henkel M (2000) Salmonella pathogenicity island 2. Mol Microbiol 36: 1015–1023.
6. Doolittle RF, Feng DF, Tsang S, Cho G, Little E (1996) Determining divergence times of the major kingdoms of living organisms with a protein clock. Science 271: 470–477.
7. McClelland M, Sanders KE, Spieth J, Clifton SW, Latreille P, et al. (2001) Complete genome sequence of Salmonella enterica serovar Typhimurium LT2. Nature 413: 852–856.
8. Falcao DP, Trabulsi LR, Hickman FW, Farmer JJ 3rd (1975) Unusual Enterobacteraiceae: lactose-positive Salmonella typhimurium which is endemic in Sao Paulo, Brazil. J Clin Microbiol 2: 349–353.
9. Kohbata S, Takahashi M, Yabuuchi E (1983) Lactose-fermenting, multiple antibiotic resistant Salmonella typhi strains isolated from a patient with postoperative typhoid fever. J Clin Microbiol 19: 920–925.
10. Coleman SL, Wylie BA, Soska A, Koornhof HJ (1987) Bacteremia caused by a lactose-fermenting, multiply resistant Salmonella typhi strain in a patient recovering from typhoid fever. J Clin Microbiol 25: 1316–1318.
11. Kunz LJ, Ewing WH (1960) Laboratory Infection with a Lactose-Fermenting Strain of Salmonella Typhi. J Bacteriol 90: 1629.
12. Falkow S, Baron LS (1962) Episomic Element in a Strain of Salmonella Typhosa. J Bacteriol 84: 561–569.
13. Wilson C, Zhan H, Swint-Kruse L, Matthews KS (2007) The lactose repressor system: paradigms for regulation, allosteric behavior and protein folding. Cell 4: 107–111.
14. Fields PJ, Swanson RV, Haidaris CG, Helfron F (1986) Mutants of Salmonella typhimurium that cannot survive within the macrophage are avirulent. Proc Natl Acad Sci U S A 83: 5189–5193.
15. Riggs AD, Newby RF, Bourgeois S (1970) lac repressor—perator interaction. II. Effect of galactosides and other ligands. J Mol Biol 51: 303–314.
16. Lin S, Riggs AD (1973) The general affinity of lac repressor for E. coli DNA: implications for genes regulation in procaryotes and eucaryotes. Cell 4: 107–111.
17. Lin SY, Riggs AD (1972) Lac repressor binding to non-operator DNA: detailed studies and a comparison of equilibriam and rate competition methods. J Mol Biol 72: 671–690.
18. Fields PJ, Swanson RV, Haidaris CG, Helfron F (1986) Mutants of Salmonella typhimurium that cannot survive within the macrophage are avirulent. Proc Natl Acad Sci U S A 83: 5189–5193.
19. Mouslim C, Hilbert F, Huang H, Groisman EA (2002) Conflicting needs for a transcription factor and a small molecule regulator in Salmonella enterica. Mol Microbiol 43: 383–395.
20. Sokurenko EV, Chesnokova V, Dykhuizen DE, Ofek I, Wu XR, et al. (1998) Pathogenic adaptation of Escherichia coli by natural variation of the FimH adhesin. Proc Natl Acad Sci U S A 95: 8922–8926.
21. Fields PJ, Swanson RV, Haidaris CG, Helfron F (1986) Mutants of Salmonella typhimurium that cannot survive within the macrophage are avirulent. Proc Natl Acad Sci U S A 83: 5189–5193.
22. Riggs AD, Newby RF, Bourgeois S (1970) lac repressor—perator interaction. II. Effect of galactosides and other ligands. J Mol Biol 51: 303–314.
23. Lin S, Riggs AD (1973) The general affinity of lac repressor for E. coli DNA: implications for genes regulation in procaryotes and eucaryotes. Cell 4: 107–111.
24. Lin SY, Riggs AD (1972) Lac repressor binding to non-operator DNA: detailed studies and a comparison of equilibriam and rate competition methods. J Mol Biol 72: 671–690.
25. Gilbert W, Muller-Hill B (1967) The lac operator is DNA. Proc Natl Acad Sci U S A 58: 2415–2421.
26. Falcon CM, Matthews KS (1999) Glycine insertion in the hinge region of lactose repressor alters DNA binding. J Biol Chem 274: 30849–30857.
27. Joiner KA (1988) Complement evasion by bacteria and parasites. Annu Rev Microbiol 42: 201–230.
28. Miller SI, Kukral AM, Mekalanos JJ (1989) A two-component regulatory system [phoP phoQ] controls Salmonella typhimurium virulence. Proc Natl Acad Sci U S A 86: 5054–5058.
29. Chakravortty D, Rohde M, Jager L, Deiwick J, Henkel M (2005) Formation of a novel surface structure encoded by Salmonella Pathogenicity Island 2. Emb J 24: 2043–2052.
30. Henkel M, Shear JE, Raupach B, Monack D, Falkow S, et al. (1997) Functional analysis of ssaJ and the ssaK/U operon, 13 genes encoding components of the type III secretion apparatus of Salmonella Pathogenicity Island 2. Mol Microbiol 24: 153–167.
31. Sokurenko EV, Chesnokova V, Dykhuizen DE, Ofek I, Wu XR, et al. (1998) Pathogenic adaptation of Escherichia coli by natural variation of the FimH adhesin. Proc Natl Acad Sci U S A 95: 8922–8926.
32. Maurelli AT (2007) Black holes, antivirulence genes, and gene inactivation in the evolution of bacterial pathogens. FEMS Microbiol Lett 267: 1–8.
33. Nakata N, Tohe T, Fukushima T, Komatsu K, et al. (1993) The Absence of a surface protease, OmpT, determines the intercellular spreading ability of Shigella: the relationship between the ompT and kpaA loci. Mol Microbiol 9: 459–466.
34. Mauzerall AT, Fernandez RE, Bloch CA, Rode CK, Fasano A (1998) “Lack holes” and bacterial pathogenicity: a large genomic deletion that enhances the virulence of Shigella spp. and enteroinvasive Escherichia coli. Proc Natl Acad Sci U S A 95: 3943–3949.
35. GadMore O, Gibson DL, Bulata D, Vallance BA, Finlay BB (2000) A novel secretion pathway of Salmonella enterica acts as an antivirulence modulator during salmonolosis. PLoS Pathog 4: e1000056.
36. Ho TD, Slauch JM (2001) Characterization of gvaA, an antivirulence gene on the ssaK-ssaL phase in Salmonella enterica serovar typhimurium. J Bacteriol 183: 611–620.
37. Mouslim C, Hilbert F, Huang H, Groisman EA (2002) Conflicting needs for a Salmonella hypervirulence gene in host and non-host environments. Mol Microbiol 45: 1019–1027.
38. Chakravortty D, Hansen-Wester J, Henkel M (2002) Salmonella pathogenicity island 2 mediates protection of intracellular Salmonella from reactive nitrogen intermediates. J Exp Med 195: 1155–1166.
39. Vazquez-Torres A, Xu Y, Jones-Carson J, Holden DW, Lucia SM, et al. (2000) Salmonella pathogenicity island 2-dependent evasion of the phagocyte NADPH oxidase. Science 287: 1655–1658.
40. Eswarappa SM, Panguluri KK, Henzel M, Chakravortty D (2008) The yejABEF operon of Salmonella confers resistance to antimicrobial peptides and contributes to its virulence. Microbiology 154: 666–678.
41. Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ (1990) Basic local alignment search tool. J Mol Biol 215: 403–410.
42. Gasteiger E, Gattiker A, Hoogland C, Ivanyi I, Appel RD, et al. (2003) ExPASy: The proteomics server for in-depth protein knowledge and analysis. Nucleic Acids Res 31: 3784–3788.
43. Larkin MA, Blackshields G, Brown NP, Chernya R, McGettigan PA, et al. (2007) Clustal W and Clustal X version 2.0. Bioinformatics 23: 2947–2948.