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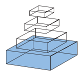

The subtleties and contrasts of the LeuO regulator in *Salmonella* Typhi: implications in the immune response

Carmen Guadarrama*[1], *Tomás Villaseñor*[2] *and Edmundo Calva*[1]

[1] Departamento de Microbiología Molecular, Instituto de Biotecnología, Universidad Nacional Autónoma de México, Cuernavaca, Mexico

[2] Departamento de Medicina Molecular y Bioprocesos, Instituto de Biotecnología, Universidad Nacional Autónoma de México, Cuernavaca, Mexico

Edited by:
Adam F. Cunningham, University of Birmingham, UK

Reviewed by:
Xun Suo, China Agricultural University, China
Heinrich Korner, Menzies Research Institute Tasmania, Australia

****Correspondence:***
Edmundo Calva, Instituto de Biotecnología, UNAM, Av. Universidad 2001, Cuernavaca, Morelos 62210, Mexico
e-mail: ecalva@ibt.unam.mx

Salmonella are facultative intracellular pathogens. *Salmonella* infection occurs mainly by expression of two *Salmonella* pathogenicity Islands (SPI-1 and SPI-2). SPI-1 encodes transcriptional factors that participate in the expression of virulence factors encoded in the island. However, there are transcriptional factors encoded outside the island that also participate in the expression of SPI-1-encoded genes. Upon infection, bacteria are capable of avoiding the host immune response with several strategies that involve several virulence factors under the control of transcriptional regulators. Interestingly, LeuO a transcriptional global regulator which is encoded outside of any SPI, is proposed to be part of a complex regulatory network that involves expression of several genes that help bacteria to survive stress conditions and, also, induces the expression of porins that have been shown to be immunogens and can thus be considered as antigenic candidates for acellular vaccines. Hence, the understanding of the LeuO regulon implies a role of bacterial genetic regulation in determining the host immune response.

Keywords: LeuO, Typhi, OmpS1, OmpS2, H-NS, porins

INTRODUCTION

Salmonella enterica are Gram-negative bacterial pathogens capable of infecting human beings and other vertebrates, and causing substantial morbidity and mortality (1, 2). In human beings, most of *Salmonella* serovars can cause infections in the small intestine and hence gastroenteritis; yet a small percentage of *Salmonella* serovars can cause a systemic infection, such as typhoid fever by the Typhi serovar (3). Control of *Salmonella* infection is difficult, in part due to the capacity of the bacterium to tolerate environmental stress, to its widespread distribution, multiple drug resistance, and adaptability (4). They infect human beings and other animals by the fecal–oral route, via contaminated food and water.

After oral acquisition, *Salmonella* resists low pH in the stomach and colonizes the intestinal tract and some cells can disseminate to cause systemic infection of organs such as liver and spleen (1). *Salmonella* virulence factors as well as host immune responses are determinant in the infectious process developed in the pathology (5). *S. enterica* Typhimurium and Typhi serovars interact with host cells through the activities mainly of two type three secretion systems (TTSS), encoded in two pathogenicity islands, 1 and 2 (SPI-1 and SPI-2) (6, 7). While SPI-1 participates in bacterial cell entry into non-phagocytic epithelial cells, SPI-2 is required for intracellular maintenance of the bacteria in a specialized membranous compartment (8). *Salmonella* internalization is mediated by effectors encoded in SPI-1: SopE, SopE2, and SopB, which activate the Rho family of GTPases Rac1, Cdc42 and RhoG (9, 10). These bacterial effectors promote a transcriptional reprograming in host cells, which in turn leads to the expression of pro-inflammatory cytokines, which could be essential for the initiation of diarrhea, a hallmark of acute *Salmonella* infection. Recently, it has been observed that the expression of the pro-inflammatory cytokine interleukin 22 (IL-22) can be exploited by pathogens, such as *Salmonella*, to suppress the growth of their closest competitors thereby enhancing pathogen colonization of mucosal surfaces (11–13).

Upon infection of intestinal epithelial cells, early transcriptional host responses occur characteristically after the stimulation of the innate immune receptors (14). However, the *Salmonella*-induced responses are unique in that this pathogen is capable of stimulating them independently of innate immune receptors (12), which are largely inactive in the intestinal epithelial cells due to robust negative regulatory mechanisms (15–17). After internalization in epithelial cells, bacteria traverse the intestinal epithelium and can invade M-cells overlying Peyer's patches, as well as being captured by dendritic cells directly from the intestinal lumen (18).

Systemic infection requires intracellular survival and replication, while *Salmonella*-macrophage interactions are essential for bacterial virulence, disease, pathology and chronic infection (19–21). Immunity to intra-macrophage pathogens (i.e., *Salmonella*) requires the infected host to generate a robust and sustained CD4 Th1 response (22). *Salmonella* infection of inbred mouse strains induces a robust $CD4^+$ T-cell response that is essential toward protective immunity to secondary infection (23–27). *Salmonella* also induces $CD8^+$ T-cells and antibody responses that can contribute to the resolution of infection (25, 27, 28). The first study to successfully characterize *Salmonella*-specific $CD4^+$ T-cell clones identified the target antigen of these T-cells as an I-Ak epitope within the central hypervariable portion of bacterial flagellin encoded by the FliC gene (29). Subsequently, additional MHC class II epitopes were identified in the same protein and thus flagellin remains the most thoroughly defined target antigen in the *Salmonella* infection model (30, 31). Additional studies have

shown that immunization with flagellin provides a modest degree of protective immunity to *Salmonella* infection, usually defined by slightly lower bacterial counts or a delay in time to death after infection. Thus, flagellin is a well-defined target antigen of CD4$^+$ T-cells during *Salmonella* infection and this response contributes modestly to protective immunity *in vivo* (32, 33). Among other antigens, the outer membrane proteins (OMPs) are particularly important. In a murine model, the highly abundant OmpC and OmpF porins (34) can induce long-term antibody responses with high bactericidal capacity, and they even confer protection against challenge with *Salmonella* Typhi (35, 36).

THE LeuO GLOBAL REGULATOR IS AN LTTR

LeuO is part of the LysR-type transcriptional regulators (LTTRs), the largest family of transcriptional regulators in prokaryotes. In consequence, they regulate a wide variety of genes that are involved in a diversity of cellular functions such as biosynthesis of amino acids, catabolism of aromatic compounds, antibiotic resistance, oxidative stress response, nitrogen fixation, quorum sensing and virulence (**Figure 1**) (37–40). Many structural studies have shown an organization of an N-terminal DNA-binding domain (DBD) with a winged Helix-Turn-Helix (wHTH) motif; and a long linker helix (LH) involved in dimerization that connects the DBD with the C-terminal effector binding domain (EBD) or regulatory domain (RD) (37, 41–43). These regulators are proteins between 300 and 350 residues, mostly acting as transcriptional activators that bind to A–T rich DNA sequences in similar positions.

In the classical model of action, LTTRs activate the transcription of a divergent gene and repress their own transcription, independently of the presence of a co-inducer or effector (small signal molecule); although there are exceptions where no co-inducer is required and in most of these cases they act as repressors (37). Therefore, the members of the family have been described as dual regulators (44). Nevertheless, there are examples where the LTTR positively autoregulates its expression; and some LTTRs can have more gene targets that they activate or repress, involved in different cellular process, different from those divergently located with respect to the gene for the regulator (39). Even more, as addressed below, LeuO is an interesting case due to the fact that it can act as derepressor, and has been shown to have complex DNA-binding sites (45, 46).

LeuO HISTORY

The first report of the LeuO regulator was by the localization of the *leuO* gene between the *leuABCD* and *ilvIH* operons; upon which it was included in the LysR family due to its amino acid sequence

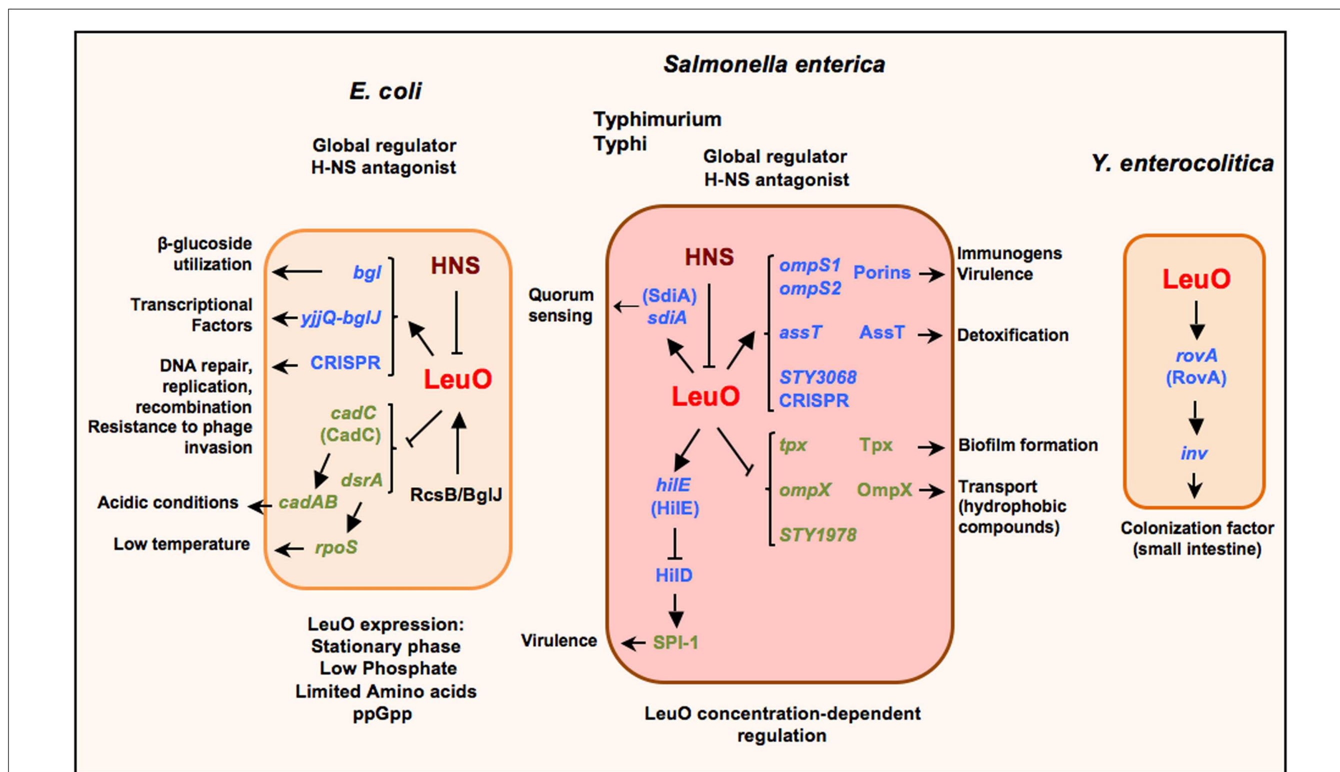

FIGURE 1 | Schematic representation of the LeuO regulon in *Escherichia coli, S. enterica* serovars Typhimurium and Typhi, and *Yersinia enterocolitica*. LeuO is a dual regulator that can induce the expression of several genes (arrows) and also is capable of repressing gene expression (lines). When acting as a repressor it has been suggested to function as a backup for H-NS; nevertheless in several cases LeuO acts as a derepressor of gene expression by displacement or prevention of H-NS repression. Recently, LeuO has been denominated as a global antagonist of H-NS in *E. coli* and in *S. enterica* serovar Typhimurium. The expression of *leuO* is repressed by H-NS, although there are some stress conditions when LeuO can be detected in *E. coli*. Also, in *Salmonella* it has been described as an interesting case of differential control of transcriptional regulation, which depends on LeuO concentration. Parentheses depict the proteins coded by the indicated genes. Small arrows denote the several functions for the LeuO-regulated genes.

similitude with other members of the family (47, 48). Based on the localization of its gene, LeuO was presumed to be a *leuABCD* regulator, although Leu auxotrophy was not observed in a *leuO* mutant strain (49).

Nevertheless, since the first report of LeuO as a transcriptional regulator, it was shown to be involved in the regulation of genes important for bacterial survival in stringent conditions (**Figure 1**). Thereby, when LeuO was overexpressed in *E. coli* it was found to repress *cadC*: this was the result of searching for genes that can complement an H-NS mutant strain, thus providing an insight about a relationship between LeuO and H-NS (50). CadC activates the *cadAB* operon, an important system expressed under acidic conditions (51). H-NS is a global regulator that acts as a nucleoid protein (52, 53). Later, LeuO was determined to reduce *rpoS* translation (which encodes S sigma factor) by repression of the small regulatory DsrA-RNA, who positively regulates *rpoS* translation, mainly at low temperature (54). Both *cadC* and *dsrA* are repressed by H-NS (55, 56). Interestingly, in both cases, LeuO indirectly represses the *cadAB* operon expression and RpoS translation.

According with a LeuO-dual role regulator, it was found to be a positive regulator of *bgl* and *yjjQ-bglJ* operons in *E. coli*. Later, it was demonstrated that LeuO counteracts H-NS repression (49, 57, 58). The *bgl* operon is involved in the utilization of some β-glucosides as salicin and arbutirin; and the *yjjQ-bglJ* genes encode for a transcriptional factor belonging to the LuxR family. These operons are repressed by H-NS in a wild type genotype (59) (**Figure 1**).

In several studies in *Salmonella* Typhimurium, a model called cis-acting promoter relay mechanism has been described that involves LeuO and DNA local supercoiling in a complex regulatory interplay, in a strain with a mutated promoter of *leuABCD* (p*leuO*-500), and a suppressor mutation in *topA* (60–62). In this complex regulatory mechanism, the Leucine-responsive regulator protein (Lrp) elicits changes in local DNA supercoiling by *ilvIH* promoter activation, exposing the *leuO* regulatory region upon which *leuO* can be transcribed (63–65). Also, there are H-NS binding sites in the regulatory region of *leuO*: hence the system appears to be repressed by changes in local supercoiling and LeuO prevents a cis-spreading of H-NS enhancing positive autoregulation and permits *leuABCD* transcription (66–69).

THE LeuO REGULATOR IN OTHER GRAM-NEGATIVE BACTERIA

Studies in *S. enterica* serovar Typhi (**Figure 1**) have shown that overexpression of LeuO induces the expression of two quiescent genes that encode for the OmpS1 and OmpS2 porins (70, 71). An interesting observation was that the LeuO concentration differentially affects *ompS1* and *ompS2* expression. The *ompS2* gene is expressed at lower concentrations of LeuO, whereas *ompS1* is expressed at higher concentrations where *ompS2* expression is repressed. Moreover, for the first time, in a detailed study of *ompS1* expression, LeuO was shown to exert an antagonist role toward H-NS (71). The relevance of this observation is that such function had not been reported for other LTTRs members until now. Interestingly, members of other transcriptional regulators families such as VirF (AraC/XilS), RovA (SlyA/Hor), and Ler (H-NS/StpA) have been described as antagonists of H-NS mainly on genes involved in virulence (72–74).

In a subsequent study to pursue more targets in *Salmonella* Typhi, LeuO was found to also positively regulate *assT* and STY3070; and negatively *ompX*, *tpx* and STY1978 (**Figure 1**). These genes are involved in a variety of cellular functions (75). AssT is a putative arylsulfate sulfotransferase that has been proposed to be involved in detoxification by transforming toxic phenolic derivatives into non-toxic compounds (76). The global regulators H-NS and LeuO regulate the *assT-dsbL-dsbI* cluster expression negatively and positively, respectively, and this regulation depends on specific growth conditions (77). STY3070 in *Salmonella* was later determined to be the *casC* gene of the CRISPR/Cas system; and its repression was found to depend also on Lrp, and its expression induced in minimal media independent of LeuO (78).

The CRISPR/Cas system in *Escherichia coli* has been involved in DNA repair, replication and recombination and is proposed to confer resistance to phage invasion in bacteria and archaea, thus the suggestion that it is an ancient defense mechanism (79). Interestingly, LeuO was shown to be an antagonist of H-NS in the CRISPR-system in *E. coli* (80). OmpX is an OMP that is homolog to PagC and Rck and Ail proteins of *Salmonella* and *Yersinia*, respectively. When overexpressed, it has been observed to increase sigma E activity; and the lack of *ompX* increased the tolerance to sodium dodecyl sulfate and antibiotics, thus appearing to affect the transport of hydrophobic compounds across the membrane (81–84). Tpx is a thiol peroxidase that codes for a periplasmic antioxidant enzyme that is induced during the exponential growth phase and during biofilm formation (85). It is important to notice that LeuO down-regulates proteins that are involved in the resistance to different pH conditions (83). Another down-regulated gene was STY1978, which codes for a hypothetical protein without an association to any cellular process until now. In this report, LeuO was denominated as a global regulator and opened the possibility that LeuO could have more targets depending on the growth conditions (75).

In *Y. enterocolitica*, LeuO was found to positively regulate *rovA* and, in turn, H-NS also negatively regulates its expression (86) (**Figure 1**). RovA is a MarA/SlyA type regulator that regulates *inv* gene expression in response to temperature and growth phase (87).

In *E. coli*, by SELEX screening, LeuO was found to regulate genes involved in sulfa drug sensitivity and to increase its own expression during transition into stationary phase and after a week of culture, where H-NS concentration decreased (**Figure 1**). Even more, a global antagonistic interplay between H-NS and LeuO was proposed, acting on some genes involved in stress response, such as cryptic chaperone/usher-type fimbriae. In addition, mutants in *leuO* and in some fimbrial genes were defective or altered in biofilm formation (88, 89).

In *S. enterica* serovar Typhimurium, LeuO was reported to increase *sdiA* expression in low levels (90) (**Figure 1**). SdiA is proposed to respond to signals produced by other organisms (91, 92) and recently was found to be active in gut in response to AHLs (*N*-acyl homoserine lactones) a quorum sensing signal produced by other species (93–96).

In a genomic study in *S. enterica* serovar Typhimurium, using ChIP-chip, the LeuO regulon members were extended to include SPI-1 (**Figure 1**) and SPI-2 genes. In addition, the differential binding of LeuO and regulation of genes was observed depending on the concentration of LeuO. Another important observation was the intragenic binding; hence opening the possibility that LeuO could act as a negative regulator preventing the progress of transcription or as nucleoid structure protein. The finding of LeuO co-binding at various sites with H-NS and RNA polymerase confirms the notion of the antagonist role of LeuO, although they could likely be acting together to regulate a large number of genes. Moreover, the possible interaction with RNA polymerase and H-NS would suggest another mechanism of LeuO regulation (45, 46).

In this respect, the structural properties of LeuO as an LTTR member have been initially explored: finding that it is active as a tetramer, that the mechanisms for induction and repression of gene expression appear to be different, and that there are relevant interactions between the N- and C-termini (97).

LeuO EXPRESSION CONDITIONS

In the *Salmonella* Typhi and *E. coli* wild type genomic backgrounds, LeuO expression is silenced by H-NS (unpublished data). Nevertheless, in *E. coli* and *Salmonella* Typhimurium, *leuO* expression has been detected when grown under stress conditions, especially in the stationary phase under nutrient limitation. Nevertheless, *leuO* is not under the control of *rpoS*; although its expression requires the presence of ppGpp in stationary phase (54, 63, 98, 99). Interestingly, LeuO was shown to be essential to restore cellular growth, after a 2-h delay in a media lacking isoleucine, valine, and leucine (100).

Also, LeuO expression was detected in a phosphate-restricted media (98); and recently it was shown that the expression of the *leuO* gene can be activated by the RcsB and BglJ regulators (58, 101)

LeuO HAS SEVERAL FUNCTIONS *IN VIVO*

Even though LeuO is expressed at very low level in standard laboratory conditions, it seems that *in vivo* it has a role in bacterial survival. In this manner, in a mouse and in a *Caenorhabditis elegans* model of infection, a *S. enterica* serovar Typhimurium *leuO* mutant showed to be attenuated in virulence. Also, in *Vibrio cholera*, biofilm formation was reduced in a deleted *leuO* strain (102–104).

Virulence attenuation in a murine model was reported for the *ompC ompF* double mutant (105). In addition, it has been observed that the OmpC and OmpF porins induced long-term antibody response with bactericidal capacity and conferred protection against challenge with *Salmonella* Typhi (35, 36). Nevertheless, these major porins are expressed at very high levels in standard laboratory conditions. In addition, strains lacking *ompS1* and *ompS2* are attenuated for virulence, suggesting that besides lacking the LeuO regulator the absence of OmpS1 and OmpS2 porins affected bacterial survival (103). Virulence attenuation of mutated strains in *leuO* and *ompS1* and *ompS2* quiescent genes offers evidence that they are expressed *in vivo*. Even though the specific role of these porins in *Salmonella* virulence is not clear, it has been shown that the major porins are passive diffusion channels of solutes, nutrients and toxins through the outer bacterial membrane that might allow bacteria to grow in different environments and to be resistant to drugs (106).

Recently it was found that OmpS1 and OmpS2 induced a strong immune response in the mouse, and a single dose conferred a significant protection against *Salmonella* Typhi. The immunostimulatory properties of OmpS1 and OmpS2 porins further reinforce the notion that they could be expressed following host infection. These studies are relevant because they open the possibility of using these porins as antigens for the development of vaccines against typhoid fever and other non-typhoidal salmonellosis (107).

Moreover, in a recent report it was shown that the activation of *leuO* transcription in *S. enterica* serovar Typhimurium represses expression of pathogenicity island 1 (SPI-1) and inhibits invasion of epithelial cells (108). Two different modes of action were found: the major one that involves the induction of *hilE* transcription by LeuO (**Figure 1**) and another one that was HilE-independent. HilE is a regulator encoded outside SPI-1 that represses *hilD* expression. HilD is one of the transcriptional factors encoded in SPI-1 that positively controls the expression of other genes in the island (109, 110). It has been suggested that LeuO repression of SPI-1 genes may occur under growth conditions where H-NS, for unknown reasons, has failed to perform such repression.

The possibility of LeuO acting as a backup for H-NS has two implications: one is that it could allow *Salmonella* to confront the hostile free-living conditions where SPI-1 gene expression has a high cost in bacterial growth; and two, it might ensure the specific, sequential, and appropriate level of SPI-1 gene expression in the intestine (111, 112). Due to the fact that H-NS in *Salmonella* is considered as a genome sentinel that silences horizontally acquired genes (113–115), LeuO could be acting as a backup regulator for H-NS, highlighting the subtleties and contrasts of the LeuO mode of action. Thus, the proposed role of LeuO as an activator or as a repressor depending on its concentration could explain this differential gene regulation.

LeuO is an example of a global regulator whose level of expression is an important issue, since this has an effect on its many regulated genes that are involved in a variety of cellular functions, such as virulence and bacterial survival. The levels of expression could thus have spatial and temporal consequences as well. In addition, knowledge of LeuO-regulated genes has been important in the study of the immune response induced by *Salmonella*, such as that elicited by the quiescent porins, which are protein components of the outer membrane. This has opened the possibility for the development of typhoid fever vaccines and perhaps as adjuvants for others vaccines.

It is intriguing that conditions known at present for LeuO expression are extreme and that in many studies it has to be over-expressed to analyze its function. Furthermore, no co-inducer of LeuO is known until now. These are some of the subjects that pose challenges for the future.

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