Balance between extracellular matrix production and macrophage survival by a *Salmonella*-specific SPI-2 encoded transcription factor

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Abstract

Cellulose is a major component of the *Salmonella* biofilm extracellular matrix and it is considered an antivirulence factor because it interferes with *Salmonella* survival inside macrophages and virulence in mice. Its synthesis is stimulated by CsgD, the master regulator of biofilm extracellular matrix formation in enterobacteria, which in turn is under the control of MlrA, a MerR-like transcription factor. In this work we identified a SPI-2 encoded *Salmonella*-specific transcription factor homolog to MlrA, MlrB, that represses transcription of its downstream gene, STM1389, also known as *orf319*, and of *csgD* inside host cells. MlrB is induced in laboratory media mimicking intracellular conditions and inside macrophages, and it is required for intramacrophage survival. An increased expression of *csgD* is observed in the absence of MlrB inside host cells. Interestingly, inactivation of the CsgD-controlled cellulose synthase coding-gene, *bcsA*, restored intramacrophage survival to rates comparable to wild type bacteria in the absence of MlrB. These data indicate that MlrB represses CsgD expression inside host cells and in consequence activation of the cellulose synthase. Our findings provide a novel link between biofilm formation and *Salmonella* virulence.

INTRODUCTION

Salmonella enterica success on infection and transmission between hosts relies greatly on its ability to recognize, to evade and to even exploit host defenses in its favor (Behnsen *et al.*, 2015). Critical to *Salmonella* pathogenesis is the coordinated

assembly of two type III secretion systems (T3SS) which are located at specific genome regions called pathogenicity islands (SPI). The *Salmonella* pathogenicity island 1, SPI-1, is associated with the invasion of epithelial cells, whereas SPI-2 is required for intracellular survival (Deiwick *et al.*, 1999; Ellermeier and Slauch, 2007; McGhie *et al.*, 2009; Agbor and McCormick, 2011), although there is certain overlap in their functions (Deiwick *et al.*, 1998; Steele-Mortimer *et al.*, 2002; Brown *et al.*, 2005). Aside from these orthodox virulence traits, it is widely recognized that *Salmonella* resistance in extra-host environments is also an important factor for the attainment of the infectious cycle (Waldner *et al.*, 2012; Maruzani *et al.*, 2019). Essential to the interaction with hosts and the environment is its ability to form biofilms, bacterial communities embedded in an adhesive, self-produced extracellular matrix (EM) with an intricate physiological and structural organization (Serra *et al.*, 2013).

Although accumulated evidence supports the importance of biofilms in environmental conditions, the role of the community lifestyle in *Salmonella* pathogenic processes is far less understood (Maruzani *et al.*, 2019). Most host-generalists involving non-typhoidal serovars preserve their ability to form biofilms *in vitro* (reviewed in MacKenzie *et al.*, 2017). For these pathovars, it has been suggested that the biofilm formation not only favors resistance inside the gastrointestinal tract (MacKenzie *et al.*, 2017), but it also modulates the local inflammatory responses, contributing to the pathogen competitiveness and, therefore, transmission (Thiennimitr *et al.*, 2012). Host-specific typhoidal serovars, on the other hand, are able to adapt the expression and composition of their EM

avoiding host immunity (Gonzalez-Escobedo and Gunn, 2013; Adcox *et al.*, 2016). In a broader context, it is increasingly accepted that biofilms promote long-term, intra-host persistence in a variety of pathogenic bacteria, whereas planktonic lifestyle favors acute infection (Crawford *et al.*, 2010; Desai and Kenney, 2019). In line with the latter, overproduction of cellulose, one of the major *Salmonella* EM components, has been proven to hinder virulence (Pontes *et al.*, 2015; Ahmad *et al.*, 2016). Nevertheless, it has also been found that basal expression levels of cellulose are present when inside macrophages (Pontes *et al.*, 2015). These findings support a role of EM production in the regulation of virulence, although much remains to be explored about the molecular mechanisms relating *Salmonella* community lifestyle and the infectious process.

In *Salmonella* and related enterobacteria, transition to the multicellular behavior is primarily controlled by the master regulator CsgD that activates the synthesis of the major EM components, curli and cellulose (Gerstel and Römling, 2003). CsgD expression is modulated by several factors that integrate diverse environmental signals into what is today recognized as one of the most complex bacterial regulation networks known (Gerstel and Römling, 2003; Ogasawara *et al.*, 2011). Among those factors, MlrA, a MerR-like family response regulator, has been identified as a key *csgD* transcriptional activator in *Escherichia coli* and *S. Typhimurium* (Brown *et al.*, 2001). MlrA recognizes a symmetrical dyad sequence, its operator, ~100 bp upstream of the -35 element in the *E. coli csgD* promoter (Ogasawara *et al.*, 2010), and a similar operator sequence is distinguished in the *Salmonella csgD*–*csgB* intergenic region.

In this work, we detected the presence of a *Salmonella*-specific, MerR-like transcription factor, Orf242, which we renamed MlrB for *MerR-like regulator B*, that shows sequence homology to MlrA, specially at the DNA recognition domain. We identified that MlrB acts as a repressor of both *csgD* and the *mlrB* downstream gene, STM1389, also known as *orf319*. MlrB is not only induced in media mimicking intracellular conditions, but it is also required for *Salmonella* intramacrophage survival. An increased expression of *csgD* is observed in the *mlrB* deleted strain indicating that it exerts this function by reducing *csgD* expression inside host cells. These results provide a novel link between biofilm formation and *Salmonella* virulence. Additionally, they allow us to postulate a mechanism by which two close homologues act antagonistically depending on the specific niche.

RESULTS

Identification and genetic context of MlrB, a *Salmonella*-specific MlrA-like transcriptional regulator

STM1390, also known as *orf242*, encodes a putative, *Salmonella*-specific MerR-like family protein of 242 amino acid residues, similar to MlrA, a key regulator controlling *csgD* expression and production of extracellular matrix (Brown *et al.*, 2001). Conservation between these two regulators (40% amino acid sequence identity, and 70% similarity) becomes more evident at their N-terminal domains (Fig. S1A), especially on the residues purportedly involved in DNA recognition (Brown *et al.*, 2001). Sequence divergence between MlrA and MlrB is higher at their C-terminals,

which is a region typically involved in signal recognition among members of the MerR family of transcriptional regulators (Brown *et al.*, 2003). In particular, as demonstrated for *E. coli*, the C-terminal domain of MlrA exerts a regulatory role by interacting with proteins of the c-di-GMP signaling pathway (Lindenberg *et al.*, 2013). Regarding *STM1390* (hereinafter “*mlrB*”), inference of its physiological function through homology search remains elusive, as this region shows no similarity to any known protein domain.

The *mlrB* coding gene is located within the *Salmonella* SPI-2 (Fig. S1B), which is a species-specific, chimeric genomic island with two distinctive DNA fragments acquired by different horizontal transfer events (Hensel, Nikolaus, *et al.*, 1999; Hensel, 2000). One encodes the majority of structural, regulatory and effector factors of the Type III Secretion System (T3SS-2) involved in *Salmonella* intracellular survival during infection (Hensel, 2000). The other encodes the tetrathionate reductase system involved in the outgrowth of *Salmonella* during the intestinal colonization (Hensel, Hinsley, *et al.*, 1999; Winter *et al.*, 2010) as well as various genes of yet unknown function, including *mlrB*.

Considering the similarity between MlrB and MlrA, and *mlrB* location in the genome, we investigated whether MlrB also modulates EM production, and whether it has a role in *S. Typhimurium* virulence.

MlrA, rather than MlrB promotes biofilm formation and *csgD* transcription under low osmolarity

We first analyzed a potential role of MlrB in EM production by evaluating biomass adhesion to an abiotic surface using crystal violet-binding quantification (see Materials and Methods, and Pitts *et al.*, 2003). Biomass adherence of strains deleted in either *mlrA*, *mlrB* or the *mlrA mlrB* double mutant was compared after incubation at 28°C for 48 h in LB broth lacking sodium chloride (SLB). These conditions were reported to promote *in vitro* biofilm formation in *Salmonella* via activation of *csgD* expression (Kader *et al.*, 2006). Regardless the sequence similarity between these two regulators, the $\Delta mlrB$ strain showed no difference from the wild-type strain either in the amount of adhered biomass (Fig. 1A) or in *csgD* expression (Fig. 1B). A possible explanation for this result is that MlrB deficiency in the $\Delta mlrB$ strain could be masked by the presence of MlrA. However, the low and similar *csgD* expression observed in both $\Delta mlrA$ and $\Delta mlrA \Delta mlrB$ mutants (Fig 1B), along with the lack of adhesion of the $\Delta mlrA$ strain (Fig 1A) suggest otherwise. More likely, the conditions in which the experiments were carried out might not be optimal for MlrB activity, and thus a dominant role of MlrA is observed. In sum, under low osmolarity, MlrA exerts a predominant role in controlling of biofilm formation and *csgD* expression over its homologue MlrB.

***mlrB* is induced in SPI-2 *in vitro* conditions and inside macrophages**

mlrB is located immediately adjacent to and downstream of *ssrB* (Fig. S1B), the gene coding for the response regulator of the SsrAB two-component system that controls the transcription of the *Salmonella* T3SS-2 (Deiwick *et al.*, 1999; Walther *et al.*,

2007). We examined whether *mlrB* expression is coordinated with the rest of the T3SS-2 genes and whether this depends on SsrB. *mlrB* transcription increases under SPI-2-inducing conditions, i.e., PCN, InSPI-2 (Kröger *et al.*, 2013) or LPM (Coombes *et al.*, 2004), compared to LB (Fig. 2A and Fig. S2A) whereas *mlrA* transcription showed an opposite pattern (Fig. 2A and Fig. S2B). Using the strains harboring the *mlrB*::3xFLAG and *mlrA*::3xFLAG alleles tagged at their 3' end, we determined that MlrB levels also had a 2.4-fold increment in InSPI-2 medium while MlrA had a 3-fold decrease, compared to LB (Fig. 2B). Although our results showed that the expression of MlrB follows that of T3SS-2 genes, deletion of *ssrB* had no effect on *mlrB* transcription (Fig. S3A) indicating that *mlrB* is neither co-transcribed with *ssrB*, nor is under SsrB transcriptional control. Conversely, deletion of MlrB had no effect on the transcription of *sseA* (Fig. S3B), a SsrA/SsrB regulated gene (Hensel, Nikolaus, *et al.*, 1999; Hensel, 2000), suggesting that MlrB does not influence the SsrA/ssrB regulon.

We then determined the relative abundance of the 3xFLAG-tagged MlrB and MlrA from *Salmonella* cells grown inside RAW 264.7 macrophages, using anti IgA as control. In accordance to the previous results we observed that MlrB increased almost 6-fold at 18 h.p.i. while we did not detect a substantial modification in MlrA concentration (Fig. 2C and S4).

***orf319* is a SPI-2 encoded- MlrB-repressed gene**

We searched for putative MlrB-regulated genes in the *Salmonella* genome taking advantage of the similarity between MlrB and MlrA DNA-binding domains (Fig. S1A). Using the reported MlrA-recognized sequences in *E. coli* (Ogasawara *et al.*, 2010), a positional weight matrix was generated with the MEME tool (Bailey *et al.*, 2009). The resulting motif was confronted to the *S. Typhimurium* LT2 genome using the FIMO tool (Grant *et al.*, 2011), also part of MEME suite. A sequence similar to the MlrA-consensus operator was identified by this method in the *csgD-csgB* intergenic region, 149 bp upstream of the predicted *csgD* transcription start site (Fig. S5). In addition, we detected a second putative MlrA operator, located 113 bp upstream of the *csgD* transcription start site (Fig. S5). Additionally, an MlrA-like operator was detected in *orf319* promoter region, a SPI-2 gene coding for a protein of unknown function, located immediately downstream of *mlrB* (Fig. S1B). This sequence was located at only 6 bp upstream of the predicted *orf319* transcriptional start site, overlapping the -10 and -35 promoter elements (Fig. S5B).

We tested whether *orf319* is under the transcriptional control of either MlrA or MlrB. Although both regulators repressed *orf319* transcription when *Salmonella* was grown in LB (Fig. S6), and their simultaneous deletion further increased the transcription of the target gene, only MlrB affected *orf319* transcription under SPI-2 inducing conditions (InSPI-2, Fig. 3). Furthermore, in InSPI-2 conditions, the $\Delta mlrB \Delta mlrA$ double mutant showed an *orf319* transcription level similar to that displayed by the $\Delta mlrB$ strain. This correlates well with the reduced expression of MlrA as well as the increased expression of MlrB in this condition (Fig. 2 and S2).

MlrB is required for intramacrophage survival

MlrB increased expression inside macrophages and under SPI-2 inducing conditions, prompted us to study whether this regulator is required for intramacrophage survival.

Similar to mutants in the T3SS-2 system (Cirillo *et al.*, 1998), the $\Delta mlrB$ strain had a defect in replication inside RAW 264.7 macrophages compared to the WT (Fig. 4A). Interestingly, MlrB overexpression from a multi-copy plasmid increased survival compared to the WT strain (Fig. 4B). Conversely, the $\Delta mlrA$ strain showed higher survival than the wild-type strain (Fig. 4A), and its overexpression decreased *Salmonella* intramacrophage survival (Fig. 4B). Consistent with the opposite effect of both regulators on intracellular survival, the $\Delta mlrA \Delta mlrB$ double mutant strain showed a survival behavior closed to the wild-type (Fig. 4A).

orf319 was not an intermediary in the MlrB-mediated intracellular survival, since its deletion did not alter the survival of either the wild-type or the $\Delta mlrB$ mutant (Fig. 5).

In view of the above results, and aware that MlrB did not affect *csgD* expression under laboratory conditions (Fig. 1), we hypothesized that MlrB could exert its function by modulating CsgD expression in a different context, i.e., within macrophages.

MlrB promotes intramacrophage survival by repressing *csgD* expression

We determined intramacrophage *csgD* transcription from a $P_{csgD}::gfp$ reporter at 0 (inoculum) and 18 h.p.i. A marked reduction of *csgD* transcription at 18 h.p.i. was observed in the WT strain but not in the $\Delta mlrB$, which maintained similar values to that of the inoculum (Fig. 6A), indicating that, during *Salmonella* intramacrophage growth, MlrB is capable of negatively regulating *csgD* expression.

The repressive effect of MlrB on CsgD during intracellular survival suggests the involvement of MlrB in the control of EM production. This would explain the macrophage survival defect of the *mlrB* mutant. In particular, increased cellulose production, but not curli, has been reported to be detrimental for intracellular survival (Pontes *et al.*, 2015). Indeed, deletion of the cellulose synthase coding gene, *bcsA*, restored wild-type intramacrophage replication levels of the $\Delta mlrB$ mutant (Fig. 6B).

Altogether these results indicate that MlrB, a SPI-2 encoded *Salmonella*-specific MlrA-like transcriptional regulator, links macrophage survival to extracellular matrix production.

DISCUSSION

Salmonella biofilm formation is considered relevant for extra-host environments (Gerstel and Römling, 2003; Simm *et al.*, 2014) or during gallbladder colonization in chronic infections (Crawford *et al.*, 2010). Furthermore, although cellulose has been shown to be synthesized by *Salmonella* inside macrophages, its increased production is detrimental for survival within these cells and for systemic infection in

mice (Pontes *et al.*, 2015; Ahmad *et al.*, 2016). Nevertheless, the biological significance of cellulose production by intra-macrophage bacteria remains to date poorly understood.

In this work we demonstrated that MlrB, a SPI-2 encoded MlrA-like transcription factor, is required for *Salmonella* intramacrophage survival. Further, our results indicate the MlrB negatively controls *csgD* expression and activation of the cellulose biosynthesis pathway when inside macrophages. We showed that, opposite to MlrA, MlrB expression increased under SPI-2 inducing condition (Fig. 2 and S2A). Consequently, we observed a 6-fold increase in MlrB levels in intramacrophages-grown *Salmonella* (Fig. 2C and Fig S4). Using *E. coli* MlrA target operators (Ogasawara *et al.*, 2010) we identified another SPI-2 gene, *orf319*, as a MlrB- as well as MlrA-regulated gene (Fig. 3, S5 and S6), and showed that control of MlrB or MlrA over *orf319* depends on the growth conditions. For example, both MlrB and MlrA were active in repressing *orf319* transcription in LB (Fig. S6), but only MlrB repressed *orf319* under SPI-2 inducing conditions (Fig. 3).

On the other hand, and while only MlrA activated *csgD* expression in laboratory conditions (Fig. 1B), we showed an increased expression of *csgD* inside macrophages in the absence of MlrB (Fig. 6) indicating that in this environment MlrB acts as a repressor of the biofilm master regulator. In this sense, we propose a counteracting role between MlrB and MlrA inside macrophages. This is further supported by the increased survival of the $\Delta mlrA \Delta mlrB$ double mutant strain compared with the mutant deleted in *mlrB* (Fig. 4A).

Our results sustain a MlrB-activating condition inside macrophages rather than a consequence of a balance between MlrB and MlrA concentrations because there was no MlrB-dependent effect on *csgD* expression either in LB or in InsPI-2 inducing conditions (Fig. 1B and Fig. S7). Nevertheless, this is under current investigation at the lab. Certainly, as the effector-binding C-terminal domains of MlrA and MlrB share low sequence identity (Fig. S1A), it is expected that each regulator would be modulated by different signals and therefore respond to different environmental cues.

The prediction of two MlrA-like operators in the *csgD* promoter may also account for the opposite role between MlrB and MlrA in controlling CsgD expression. Whether these regulators are interacting and recognizing the same sequence or they prefer one over the other needs to be investigated. In marked contrast, the only operator found in the promoter region of the MlrB- and MlrA-repressed gene *orf319* overlaps this gene's -10 and -35 elements (Fig. S5B). It is feasible that binding of either MlrB or MlrA would hinder the RNA-polymerase from its productive interaction with the promoter. If this is the case, the proposed mechanism of action of these regulators differs from other *Salmonella* MerR-regulators like GolS or CueR (Checa *et al.*, 2007; Pérez Audero *et al.*, 2010; Humbert *et al.*, 2013). Not only these canonical regulators do not impede the binding of RNA-polymerase, but they favor it (Pezza *et al.*, 2016).

The deficiency in macrophage survival of the *mlrB* mutant strain can be attributed to an increased cellulose production, as the deletion of the cellulose synthase gene *bcsA* restored wild-type survival of the Δ *mlrB* strain (Fig. 6B) while the absence of Orf319, the other MlrB-controlled gene product, did not affect *Salmonella* survival

inside macrophages (Fig. 5). This results is in concordance with the observation that overproduction of cellulose is the cause of the virulence deficiency of a $\Delta mgtC$ strain as the inactivation of *bcsA* restored wild-type virulence of the *mgtC* mutant (Pontes *et al.*, 2015). MgtC is a virulence protein located in the inner membrane that controls ATP synthesis membrane potential by interacting with the α subunit of the F_1F_0 ATP synthase (Pontes *et al.*, 2015), and phosphate uptake by inducing the PhoB/PhoR regulatory system (Lee *et al.*, 2014). MgtC controls *bcsA* expression and also c-di-GMP intracellular concentration, which is an allosteric cellulose synthase activator, although the mechanisms involved in this regulation are still not known (Pontes *et al.*, 2015).

Why cellulose production affects intramacrophage growth it is not known, although one possible explanation could be related to the glucose consumption that its production requires, that otherwise could be redirected to intramacrophage growth (Petersen *et al.*, 2019). Cellulose production during *Salmonella* intramacrophage survival was associated to a persister subpopulation (Petersen *et al.*, 2019). It would be interesting to know whether this slow replicating population is maintained as a safeguard reservoir and produces the EM component as a means of self-protection. In this sense, we propose that MlrB would act as a rheostat to balance intracellular growth versus persisters' reservoir.

It has been shown that *Salmonella* serovars involved in systemic infection suffered an elevated genome degradation compared with the restricted gastrointestinal serovars although genome degradation is observed in all serovars analyzed (Nuccio and Bäumlér, 2014). This correlates with the requirement of particular traits in each

case, showing an increased loss of genes encoding Type III secreted effectors, fimbrial adhesins, and motility and chemotaxis genes in extraintestinal serovars compared to gastrointestinal pathovars (Nuccio and Bäumler, 2014).

Despite the lack of information about the gene-repertoire modulated during persistence either in the gallbladder or in *Salmonella*-containing granulomas, genes encoding biofilm formation components and its regulators appear to be conserved in both groups (Nuccio and Bäumler, 2014), suggesting that this trait is relevant for common processes.

In sum, our work describes the function of MlrB, a novel SPI-2-encoded, MlrA-like transcription factor required for *Salmonella* intramacrophage survival. Our genetic and biochemical studies allowed us to propose a plausible mechanism for this regulation, in which MlrB modulates extracellular matrix production when inside macrophages to regulate pathogenicity. Divergent patterns of expression and activities suggest that MlrB, which showed no obvious effect on regulating biofilm-formation *in vitro*, antagonizes MlrA *in vivo*. This not only illustrates how two closely related transcription factors counteract in response to specific environmental signals, but also contributes to the increasingly accepted notion that biofilm-formation is a complex process that affects both intra- and extra-host lifestyles.

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AUTHOR CONTRIBUTIONS

MLE, NRF and FCS designed the experiments and wrote the manuscript. MLE, NRF, LVH and MGP performed the experiments. MLE, NRF, LVH, MGP, FGdP and FCS analyzed the data.

MATERIALS & METHODS

Bacterial strains and growth conditions

S. enterica serovar Typhimurium strains and plasmids used in this study are listed in Table S1. Oligonucleotides are listed in Table S2. Cells were routinely grown at 37°C in Luria–Bertani (LB broth) or on LB-agar plates, except when indicated. Ampicillin, tetracycline, kanamycin, and chloramphenicol were used when necessary

at 100, 15, 50, and 20 $\mu\text{g ml}^{-1}$, respectively. Culture media that emulate the intravacuolar environment used were PCN, InSPI-2 (Kröger *et al.*, 2013) and LPM (Coombes *et al.*, 2004).

All reagents and chemicals were from Sigma, except the Luria-Bertani culture medium that was from Difco. Oligonucleotides and enzymes were purchased from Life Technologies.

Genetic and molecular biology techniques

The strains carrying gene deletions, chromosomal *lacZ* reporter fusions or 3xFLAG tags were generated by Lambda Red-mediated recombination followed by P22-mediated transduction using previously described protocols (Pérez Audero *et al.*, 2010; Ibáñez *et al.*, 2013; Pezza *et al.*, 2016; López *et al.*, 2018). When necessary, antibiotic resistance cassettes inserted at the deletion points were removed using FLP-mediated recombination (Datsenko and Wanner, 2000). DNA fragments as well as plasmids were introduced into bacterial cells by electroporation. All constructs were verified by DNA sequencing.

The Plasmid carrying the transcriptional fusion of the native *Salmonella* P_{csgD} promoter to *gfp* (Table S1) was constructed by cloning the PCR-amplified promoter into the SmaI site of pPROBE(NT) using previously described protocols (Miller *et al.*, 2000).

Quantification of biofilm adhesion to abiotic surfaces

To evaluate the adhesion to polystyrene microplates, a Crystal Violet (CV) binding and quantification protocol was implemented (Pitts *et al.*, 2003) . For this, 150 µl of 1:100 dilutions of saturated cultures of the strains to be tested were deposited in 96-well polystyrene plates. The inoculated plates were incubated at the temperatures and times indicated. After incubation, the cultures were discarded and the wells were washed 4 times with distilled water by immersion and allowed to dry at room temperature. Then 200 µl of a 1% (w/v) aqueous solution of CV was added to each well and incubated for 20 minutes at room temperature. Subsequently, unbound CV was washed out by thorough immersion in distilled water. After drying the plates at room temperature, 200 µl of an ethanol:acetone mixture 80:20 (v/v) was added to each well. Desorption of the dye was allowed for one hour at room temperature on a shaking platform. Finally, the absorbance at 562 nm was recorded using a spectrophotometer (BioTek ELx808).

β-galactosidase activity assays

Measurements of β-galactosidase activity of strains carrying transcriptional fusions to the *lacZY* genes were made following a modification of the protocol proposed by Miller (Miller, 1972), and essentially as described in (Pérez Audero *et al.*, 2010).

Western blot analysis

Western blot analyses of 3xFLAG-tagged proteins or IgaA were carried out as described previously (Pontel and Soncini, 2009; Pérez Audero *et al.*, 2010) with mouse anti-FLAG monoclonal (Sigma-Aldrich) or rabbit polyclonal anti-IgaA antibodies. Quantification of individual bands by densitometry was performed using

the Image J Program, using the IgaA band as a load control (Lobato-Márquez *et al.*, 2015).

Eukaryotic cells culture conditions and gentamicin protection assay

Salmonella survival in RAW 264.7 macrophages was tested as described (Thompson *et al.*, 2011). Briefly, macrophages were cultured in 24-well plates containing Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS) and were infected at a multiplicity of infection (MOI) of 10 bacteria per cell. The macrophage medium was supplemented with IPTG (1 mM) or Arabinose 0,05% (v/v) when these cells were infected with *Salmonella* strains harboring plasmid pUHE-21–2lacIq, pBAD30 or its derivatives. After infection, plates were incubated at 37°C for 30 min and then fresh D-MEM 10% FBS medium supplemented with gentamicin 100 µg/ml was added. After 1 h at 37°C, infected cells were incubated with medium containing gentamicin at a concentration of 30 µg/ml for a total of 18 h. At indicated time points, cells were washed and lysed with 0,1% Triton X-100 in PBS. Lysates were recovered and serially diluted. CFUs were determined on LB agar plates and the intracellular survival (fold of change) was determined.

For large-scale experiments needed to monitor protein production by intracellular bacteria, RAW 264.7 macrophages were incubated in 500 cm² plates as described previously (Núñez-Hernández *et al.*, 2013). Briefly, cells were seeded in BioDish-XL 500-cm² plates until they reached confluence. Then they were infected with *Salmonella* at a MOI of 10 bacteria per cell. After 40 min, cells were washed tree

times with PBS and then were incubated with fresh medium containing 100 µg/ml of gentamicin. After 1 h at 37 °C, infected cells were incubated with medium containing gentamicin at a concentration of 30 µg/ml until 18 h post infection. At indicated time points, infected macrophages were washed with PBS and lysed in a solution containing 4% SDS, 1% acidic phenol, and 19% ethanol in water. After 30 min of incubation at 4°C, intracellular bacteria were collected by centrifugation (27,500 g, 4°C, 30 min) and washed three times with 1 ml of a, 19% ethanol RNase free solution. For western blot analyses, intracellular bacteria were processed as described previously (Pontel and Soncini, 2009; Pérez Audero *et al.*, 2010). Immunodetection was carried out using mouse anti-FLAG monoclonal (Sigma-Aldrich) or rabbit polyclonal anti-IgaA antibodies (Lobato-Márquez *et al.*, 2015).

***csgD* intracellular expression**

Measurement of *csgD* transcription from intracellular bacteria was performed using WT/pPromcsgD-gfp or Δ mlrB/pPromcsgD-gfp strains. A gentamicin protection assay was performed as described above, with modifications, as follows. RAW 264.7 macrophages were cultured in 6-well plates until they reached confluence. WT/pPromcsgD-gfp or Δ mlrB/pPromcsgD-gfp strains were grown ON at 37°C. At the indicated time point, invaded cells were washed four times with PBS and lysed with 0,1% PBS-Triton X-100. Lysates were collected, centrifuged 5 min at 6500 rpm and resuspended in PBS. Two hundred microliters of each sample were used to measure GFP fluorescence in a Synergy 2 Microplate Reader (Biotek) spectrophotometer (λ_{exc} =485 nm, λ_{em} =528 nm). One hundred microliters of each sample were used to determine the number of intracellular viable bacteria (CFU/ml) by serial dilution and

plating on LB. Transcriptional induction values were expressed as fluorescence units per CFU.

***In silico* analyses**

A positional matrix of weights was generated through the MEME program (Bailey *et al.*, 2009) to screen for MlrA targets in *S. Typhimurium* genome, using the reported *E. coli* MlrA-binding sequences in the promoters of *csgD*, *rplU-ispB*, *yrbA*, *dppB* and *cadC* (Ogasawara *et al.*, 2010). This consensus was later confronted with the genome of *S. Typhimurium* LT2 using the FIMO tool (Grant *et al.*, 2011).

Statistical analysis

To test for statistical differences between means, one-way analysis of variance (ANOVA) and the Tukey multiple comparison test with an overall significance level of 0.05 were used. Calculations were performed with GraphPad Prism statistical software.

REFERENCES

Adcox, H.E., Vasicek, E.M., Dwivedi, V., Hoang, K. V, Turner, J., and Gunn, J.S. (2016) Formation and Gallbladder Colonization. **84**: 3243–3251.

Agbor, T.A., and McCormick, B.A. (2011) Salmonella effectors: important players modulating host cell function during infection. *Cell Microbiol* **13**: 1858–1869
<https://www.ncbi.nlm.nih.gov/pubmed/21902796>.

454 Ahmad, I., Rouf, S.F., Sun, L., Cimdins, A., Shafeeq, S., Guyon, S. Le, *et al.* (2016) BcsZ inhibits
455 biofilm phenotypes and promotes virulence by blocking cellulose production in *Salmonella enterica*
456 serovar Typhimurium. *Microb Cell Fact* **15**: 177
457 <http://microbialcellfactories.biomedcentral.com/articles/10.1186/s12934-016-0576-6>.

458 Bailey, T.L., Boden, M., Buske, F.A., Frith, M., Grant, C.E., Clementi, L., *et al.* (2009) MEME SUITE :
459 tools for motif discovery and searching. **37**: 202–208.

460 Behnsen, J., Perez-Lopez, A., Nuccio, S.-P., and Raffatellu, M. (2015) Exploiting host immunity: the
461 *Salmonella* paradigm. *Trends Immunol* **36**: 112–120 <https://pubmed.ncbi.nlm.nih.gov/25582038>.

462 Brown, N.F., Vallance, B.A., Coombes, B.K., Valdez, Y., Coburn, B.A., and Finlay, B.B. (2005)
463 *Salmonella* pathogenicity island 2 is expressed prior to penetrating the intestine. *PLoS Pathog* **1**:
464 e32–e32 <https://pubmed.ncbi.nlm.nih.gov/16304611>.

465 Brown, N.L., Stoyanov, J. V., Kidd, S.P., and Hobman, J.L. (2003) The MerR family of transcriptional
466 regulators. *FEMS Microbiol Rev* **27**: 145–163.

467 Brown, P.K., Dozois, C.M., Nickerson, C.A., Zuppardo, A., Terlonge, J., and Curtiss, R. (2001) MlrA,
468 a novel regulator of curli (AgF) and extracellular matrix synthesis by *Escherichia coli* and *Salmonella*
469 *enterica* serovar Typhimurium. *Mol Microbiol* **41**: 349–363.

470 Checa, S.K., Espariz, M., Audero, M.E.P., Botta, P.E., Spinelli, S. V, and Soncini, F.C. (2007)
471 Bacterial sensing of and resistance to gold salts. **63**: 1307–1318.

472 Cirillo, D.M., Valdivia, R.H., Monack, D.M., and Falkow, S. (1998) Macrophage-dependent induction
473 of the *Salmonella* pathogenicity island 2 type III secretion system and its role in intracellular survival.
474 **30**: 175–188.

475 Coombes, B.K., Brown, N.F., Valdez, Y., Brumell, J.H., and Finlay, B.B. (2004) Expression and
476 Secretion of *Salmonella* Pathogenicity Island-2 Virulence Genes in Response to Acidification Exhibit
477 Differential Requirements of a Functional Type III Secretion Apparatus and SsaL *. **279**: 49804–

478 49815.

479 Crawford, R.W., Rosales-Reyes, R., Ramírez-Aguilar, M. de la L., Chapa-Azuola, O., Alpuche-
480 Aranda, C., and Gunn, J.S. (2010) Gallstones play a significant role in *Salmonella* spp. gallbladder
481 colonization and carriage. *Proc Natl Acad Sci U S A* **107**: 4353–8
482 <http://www.ncbi.nlm.nih.gov/pubmed/20176950>
483 <http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=PMC2840110>.

484 Datsenko, K.A., and Wanner, B.L. (2000) One-step inactivation of chromosomal genes in *Escherichia*
485 *coli* K-12 using PCR products. *Proc Natl Acad Sci U S A* **97**: 6640–6645
486 <https://www.ncbi.nlm.nih.gov/pubmed/10829079>.

487 Deiwick, J., Nikolaus, T., Erdogan, S., and Hensel, M. (1999) Environmental regulation of *Salmonella*
488 pathogenicity island 2 gene expression. *Mol Microbiol* **31**: 1759–1773 [https://doi.org/10.1046/j.1365-](https://doi.org/10.1046/j.1365-2958.1999.01312.x)
489 [2958.1999.01312.x](https://doi.org/10.1046/j.1365-2958.1999.01312.x).

490 Deiwick, J., Nikolaus, T., Shea, J.E., Gleeson, C., Holden, D.W., and Hensel, M. (1998) Mutations in
491 *Salmonella* pathogenicity island 2 (SPI2) genes affecting transcription of SPI1 genes and resistance
492 to antimicrobial agents. *J Bacteriol* **180**: 4775–4780 <https://pubmed.ncbi.nlm.nih.gov/9733677>.

493 Desai, S.K., and Kenney, L.J. (2019) Switching Lifestyles Is an in vivo Adaptive Strategy of Bacterial
494 Pathogens. **9**: 1–9.

495 Ellermeier, J.R., and Slauch, J.M. (2007) Adaptation to the host environment : regulation of the SPI1
496 type III secretion system in *Salmonella enterica* serovar Typhimurium. .

497 Gerstel, U., and Römling, U. (2003) The *csgD* promoter, a control unit for biofilm formation in
498 *Salmonella typhimurium*. *Res Microbiol* **154**: 659–667.

499 Gonzalez-Escobedo, G., and Gunn, J.S. (2013) Gallbladder epithelium as a niche for chronic
500 *Salmonella* carriage. *Infect Immun* **81**: 2920–2930 <https://www.ncbi.nlm.nih.gov/pubmed/23732169>.

501 Grant, C.E., Bailey, T.L., and Noble, W.S. (2011) FIMO : scanning for occurrences of a given motif.

502 **27**: 1017–1018.

503 Hensel, M. (2000) Salmonella Pathogenicity Island 2. *Mol Microbiol* **36**: 1015–1023

504 <https://doi.org/10.1046/j.1365-2958.2000.01935.x>.

505 Hensel, M., Hinsley, A.P., Nikolaus, T., Sawers, G., and Berks, B.C. (1999) The genetic basis of

506 tetrathionate respiration in Salmonella typhimurium. *Mol Microbiol* **32**: 275–287

507 <https://doi.org/10.1046/j.1365-2958.1999.01345.x>.

508 Hensel, M., Nikolaus, T., and Egelseer, C. (1999) Molecular and functional analysis indicates a

509 mosaic structure of Salmonella pathogenicity island 2. *Mol Microbiol* **31**: 489–498

510 <https://doi.org/10.1046/j.1365-2958.1999.01190.x>.

511 Humbert, M. V, Rasia, R.M., Checa, S.K., and Soncini, F.C. (2013) Protein Signatures That Promote

512 Operator Selectivity among Paralog MerR Monovalent Metal Ion Regulators *. **288**: 20510–20519.

513 Ibáñez, M.M., Cerminati, S., Checa, S.K., and Soncini, F.C. (2013) Dissecting the Metal Selectivity of

514 MerR Monovalent Metal Ion Sensors in Salmonella. **195**: 3084–3092.

515 Kader, A., Simm, R., Gerstel, U., Morr, M., and Römling, U. (2006) Hierarchical involvement of various

516 GGDEF domain proteins in rdar morphotype development of Salmonella enterica serovar

517 Typhimurium. **60**: 602–616.

518 Kröger, C., Colgan, A., Srikumar, S., Händler, K., Sivasankaran, S.K., Hammarlöf, D.L., *et al.* (2013)

519 An infection-relevant transcriptomic compendium for salmonella enterica serovar typhimurium. *Cell*

520 *Host Microbe* **14**: 683–695.

521 Lee, E.-J., Choi, J., and Groisman, E.A. (2014) Control of a Salmonella virulence operon by proline-

522 charged tRNA(Pro). *Proc Natl Acad Sci U S A* **111**: 3140–3145.

523 Lindenberg, S., Klauck, G., Pesavento, C., Klauck, E., and Hengge, R. (2013) The EAL domain

524 protein YciR acts as a trigger enzyme in a c-di-GMP signalling cascade in E . coli biofilm control.

525 *EMBO J* **32**: 2001–2014 <http://dx.doi.org/10.1038/emboj.2013.120>.

526 Lobato-Márquez, D., Moreno-Córdoba, I., Figueroa, V., Díaz-Orejas, R., and García-del Portillo, F.
527 (2015) Distinct type I and type II toxin-antitoxin modules control Salmonella lifestyle inside eukaryotic
528 cells. *Sci Rep* **5**: 9374.

529 López, C., Checa, S.K., and Soncini, F.C. (2018) CpxR/CpxA Controls scsABCD Transcription To
530 Counteract Copper and Oxidative Stress in Salmonella enterica Serovar Typhimurium. *J Bacteriol*
531 **200**.

532 MacKenzie, K.D., Palmer, M.B., Köster, W.L., and White, A.P. (2017) Examining the Link between
533 Biofilm Formation and the Ability of Pathogenic Salmonella Strains to Colonize Multiple Host Species.
534 *Front Vet Sci* **4**: 138 <https://www.ncbi.nlm.nih.gov/pubmed/29159172>.

535 Maruzani, R., Sutton, G., Nocerino, P., and Marvasi, M. (2019) Exopolymeric substances (EPS)
536 from Salmonella enterica : polymers , proteins and their interactions with plants and abiotic surfaces.
537 **57**: 1–8.

538 McGhie, E.J., Brawn, L.C., Hume, P.J., Humphreys, D., and Koronakis, V. (2009) Salmonella takes
539 control: effector-driven manipulation of the host. *Curr Opin Microbiol* **12**: 117–124
540 <https://pubmed.ncbi.nlm.nih.gov/19157959>.

541 Miller, J.H.H. (1972) *Experiments in Molecular Genetics*. Cold Spring Harbor Laboratory, Cold Spring
542 Harbor, New York.

543 Miller, W.G., Leveau, J.H., and Lindow, S.E. (2000) Improved gfp and inaZ broad-host-range
544 promoter-probe vectors. *Mol Plant Microbe Interact* **13**: 1243–1250.

545 Nuccio, S.-P., and Bäuml, A.J. (2014) Comparative analysis of Salmonella genomes identifies a
546 metabolic network for escalating growth in the inflamed gut. *MBio* **5**: e00929-14.

547 Núñez-Hernández, C., Tierrez, A., Ortega, A.D., Pucciarelli, M.G., Godoy, M., Eisman, B., et al.
548 (2013) Genome expression analysis of nonproliferating intracellular Salmonella enterica serovar
549 Typhimurium unravels an acid pH-dependent PhoP-PhoQ response essential for dormancy. *Infect*

550 *Immun* **81**: 154–165 <https://www.ncbi.nlm.nih.gov/pubmed/23090959>.

551 Ogasawara, H., Yamamoto, K., and Ishihama, A. (2010) Regulatory role of MlrA in transcription
552 activation of csgD, the master regulator of biofilm formation in *Escherichia coli*. *FEMS Microbiol Lett*
553 **312**: 160–168.

554 Ogasawara, H., Yamamoto, K., and Ishihama, A. (2011) Role of the biofilm master regulator CsgD in
555 cross-regulation between biofilm formation and flagellar synthesis. *J Bacteriol* **193**: 2587–2597.

556 Pérez Audero, M.E., Podoroska, B.M., Ibáñez, M.M., Cauerhff, A., Checa, S.K., and Soncini, F.C.
557 (2010) Target transcription binding sites differentiate two groups of MerR-monovalent metal ion
558 sensors. *Mol Microbiol* **78**: 853–865.

559 Petersen, E., Mills, E., and Miller, S.I. (2019) Cyclic-di-GMP regulation promotes survival of a slow-
560 replicating subpopulation of intracellular *Salmonella* Typhimurium. *Proc Natl Acad Sci U S A* **116**:
561 6335–6340.

562 Pezza, A., Pontel, L.B., López, C., and Soncini, F.C. (2016) Compartment and signal-specific
563 codependence in the transcriptional control of *Salmonella* periplasmic copper homeostasis. .

564 Pitts, B., Hamilton, M.A., Zelter, N., and Stewart, P.S. (2003) A microtiter-plate screening method for
565 biofilm disinfection and removal. **54**: 269–276.

566 Pontel, L.B., and Soncini, F.C. (2009) Alternative periplasmic copper-resistance mechanisms in Gram
567 negative bacteria. **73**: 212–225.

568 Pontes, M.H., Lee, E.-J., Choi, J., and Groisman, E.A. (2015) *Salmonella* promotes virulence by
569 repressing cellulose production. *Proc Natl Acad Sci U S A* **112**: 5183–5188
570 <http://www.ncbi.nlm.nih.gov/pmc/articles/PMC4413311/>.

571 Serra, D.O., Richter, A.M., and Hengge, R. (2013) Cellulose as an Architectural Element in Spatially
572 Structured *Escherichia coli* Biofilms. **195**: 5540–5554.

573 Simm, R., Ahmad, I., Rhen, M., Guyon, S. Le, and Römling, U. (2014) Regulation of biofilm formation
574 in *Salmonella enterica* serovar Typhimurium. **9**: 1261–1282.

575 Steele-Mortimer, O., Brumell, J.H., Knodler, L.A., Méresse, S., Lopez, A., and Finlay, B.B. (2002) The
576 invasion-associated type III secretion system of *Salmonella enterica* serovar Typhimurium is
577 necessary for intracellular proliferation and vacuole biogenesis in epithelial cells. *Cell Microbiol* **4**: 43–
578 54 <https://doi.org/10.1046/j.1462-5822.2002.00170.x>.

579 Thiennimitr, P., Winter, S.E., and Bäuml, A.J. (2012) *Salmonella*, the host and its microbiota. *Curr*
580 *Opin Microbiol* **15**: 108–114 <https://www.ncbi.nlm.nih.gov/pubmed/22030447>.

581 Thompson, J.A., Liu, M., Helaine, S., Holden, D.W., Holden, D.W., and Helaine, S. (2011)
582 Contribution of the PhoP / Q regulon to survival and replication of *Salmonella enterica* serovar
583 Typhimurium in macrophages. 2084–2093.

584 Waldner, L.L., Mackenzie, K.D., Kö, W., and White, A.P. (2012) From Exit to Entry: Long-term
585 Survival and Transmission of *Salmonella*. 128–155.

586 Walther, D., Carroll, R.K., Navarre, W.W., Libby, S.J., Fang, F.C., and Kenney, L.J. (2007) The
587 response regulator SsrB activates expression of diverse *Salmonella* pathogenicity island 2 promoters
588 and counters silencing by the nucleoid-associated protein H-NS. **65**: 477–493.

589 Winter, S.E., Thiennimitr, P., Winter, M.G., Butler, B.P., Huseby, D.L., Crawford, R.W., *et al.* (2010)
590 Gut inflammation provides a respiratory electron acceptor for *Salmonella*. *Nature* **467**: 426–429.

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592

Figure 1. MlrB is not affecting CsgD expression or biofilm formation under laboratory conditions.

A. Adherence to polystyrene. 1:100 dilutions of ON SLB cultures of the indicated strains were incubated in SLB medium, at 28 °C for 48 hours in polystyrene microplates without shaking. After successive washes, staining was performed with 1% crystal violet and 562 nm Absorbance was determined. The data correspond to average values of three independent experiments carried out by quadruplicate. The error bars correspond to the SD. **B.** β -galactosidase activity from a $csgD::lacZ$

transcriptional fusion determined for wild-type (WT), $\Delta mlrB$, $\Delta mlrA$ or $\Delta mlrA \Delta mlrB$ cells, grown overnight in LB medium, at 28 °C. The data correspond to mean values of three independent experiments performed in duplicate. Error bars represent SDs. Symbols above bars denote statistical differences between means, with respect to WT. n.s., not significant; *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

Figure 2. MrB expression increases inside macrophages.

A. β -galactosidase activity determined for the indicated reporter strains, grown until stationary phase in LB medium (gray bars) or InSPI-2 medium (black bars); values reported are means and SD of three independent experiments. Symbols above bars denote statistical difference between means from LB *versus* InSPI-2-grown cells. n.s., not significant; *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$. **B.** Western blot analysis of MrB and MrA proteins labeled with the 3xFLAG epitope. Whole cell extracts were

prepared from stationary phase cultures grown in the indicated media. Prior to processing, OD_{600 nm} of each sample was adjusted to 1. Detection of IgaA with Anti-IgaA polyclonal antibodies was used as load control. **C.** Relative concentration of MlrB and MlrA determined from *Salmonella* cells grown inside macrophages at the indicated times. Concentrations were estimated from digitized autoradiographs by pixel densitometry. Original WB obtained from two independent experiments are included in Fig. S4.

Figure 3. MlrB regulates *orf319* transcription under SPI-2-inducing conditions.

β-galactosidase activity from an *orf319::lacZ* transcriptional fusion expressed on wild-type (WT), Δ*mlrB*, Δ*mlrA* and Δ*mlrA* Δ*mlrB* cells grown overnight in InSPI-2 medium at 37°C. The data correspond to mean values of three independent experiments performed out in duplicates. Error bars represent SD. Symbols above bars denote statistical differences between means, with respect to WT. n.s., not significant; *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

Figure 4. MlrB is required for intramacrophage survival.

A. Survival of wild-type (WT), $\Delta mlrB$, $\Delta mlrA$, and $\Delta mlrA \Delta mlrB$ *S. Typhimurium* strains in RAW 264.7 macrophages at 18 h after infection. The $\Delta phoP$ and $\Delta ssrB$ strains were included as controls. The values correspond to the average of at least three independent experiments carried out in duplicate and the error bars represent SD. **B.** Intramacrophage survival of wild-type (WT), $\Delta mlrB$, and $\Delta mlrA$ strains ectopically expressing MlrB or MlrA from a multicopy plasmid was determined as in (A). (Ev) indicates the empty vector. The values correspond to the average of at least three independent experiments carried out in duplicate and the error bars represent

643 SD. Asterisks denote statistical significance between means, with respect to WT.

644 n.s., not significant; *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

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646

647 **Figure 5. *orf319* inactivation does not affect intramacrophage survival.**

648 Survival of wild-type (WT), $\Delta orf319$, $\Delta mlrB$, and $\Delta mlrB \Delta orf319$ *S. Typhimurium*
649 inside RAW264.7 macrophages at 18 h after infection. The values correspond to the
650 average of at least three independent experiments carried out in duplicate and the
651 error bars represent SD. Symbols above bars denote statistical significance between
652 means, with respect to WT. n.s., not significant; *, $P < 0.05$; **, $P < 0.01$; ***, $P <$
653 0.001.

654

Figure 6. The defect in macrophage survival of the $\Delta mlrB$ *Salmonella* mutant depends on CsgD expression.

A. CsgD expression increases inside macrophages in the absence of MlrB. Arbitrary fluorescence from a *PcsgD::gfp* reporter plasmid expressed in wild-type (WT) or in $\Delta mlrB$ strains inside RAW264.7 macrophages at 0 (inoculum) and 18 h post infection. Values correspond to the ratio between fluorescence and the number of

intracellular surviving bacteria. The data correspond to the average of at least three independent experiments carried out in duplicate and the error bars correspond to the SD. **B.** MlrB effect on *Salmonella* survival within macrophages is *bcsA* dependent. Relative survival of $\Delta mlrB$, $\Delta bcsA$ and $\Delta mlrB \Delta bcsA$ strains inside RAW264.7 macrophages at 18 h after infection compared to the wild-type (WT) strain. The values correspond to the average of at least three independent experiments carried out in duplicate and the error bars represent SD. In **A** and **B**, symbols above bars denote statistical significance between means, with respect to WT. n.s., not significant; *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

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Table S1. Bacterial strains and plasmids used in this study

Strains		
<i>Salmonella</i> Typhimurium		
Strain or plasmid	Relevant properties	Source
14028s	Wild-Type	ATCC
PB13505	14028s $\Delta mlrB::Cm^r$ (-100)	This Study
PB11017	14028s $\Delta orf319::Km^r$	This Study
PB10657	14028s $\Delta mlrA::Km^r$	This Study
PB12982	14028s $\Delta csgD::Km^r$	This Study
PB11273	14028s $\Delta mlrB \Delta mlrA::Km^r$	This Study
PB11019	14028s $\Delta (orf319-mlrB)::Cm^r$	This Study
PB14054	14028s $\Delta bcsA::Km^r$	This Study
PB14441	14028s $\Delta mlrB \Delta bcsA::Km^r$	This Study
PB10631	14028s $PcsgD::lacZ-Cm^r$	This Study
PB11286	14028s $PcsgD::lacZ-Cm^r \Delta mlrB$	This Study
PB10915	14028s $PcsgD::lacZ-Cm^r \Delta mlrA::Km^r$	This Study
PB11291	14028s $PcsgD::lacZ-Cm^r \Delta mlrB \Delta mlrA::Km^r$	This Study
PB11333	14028s $mlrA-lacZ-Km^r$	This Study
PB12016	14028s $mlrB-lacZ-Cm^r$	This Study
PB3062	14028s $tppB-lacZ-MudI$	(Gibson <i>et al.</i> , 1987)
PB7937	14028s $\Delta cueO::lacZ$	(Pontel and Soncini, 2009)
PB14016	14028s $mlrA::3xFLAG-Km^r$	This Study
PB11641	14028s $mlrB::3xFLAG-Km^r$	This Study
PB13572	14028s $orf319::3xFLAG mlrA::3xFLAG-Km^r$	This Study
PB11976	14028s $\Delta ssrB::Cm^r$ (-100)	This Study
PB12920	14028s $STM1389-lacZ$	This Study
PB13082	14028s $STM1389-lacZ \Delta mlrB::Cm^r$	This Study
PB13291	14028s $STM1389-lacZ \Delta mlrA::Km^r$	This Study
PB13286	14028s $STM1389-lacZ \Delta mlrB::Cm^r \Delta mlrA::Km^r$	This Study
MS7953s	14028s $phoP::Tn10$	(Fields <i>et al.</i> , 1986)
Plasmids		
pUH21-2lacIq	reppMB1, Amp ^r , lacIq	(Soncini <i>et al.</i> , 1995)

pBAD30	Ori P15A, araC, Amp ^r	(Guzman et al., 1995)
pPROBE(NT)	Ori V, Km ^r	(Miller <i>et al.</i> , 2000)
pKD3	Ori R6K, Cm ^r , Amp ^r	(Datsenko y Wanner, 2000)
pKD4	Ori R6K, Km ^r , Amp ^r	(Datsenko y Wanner, 2000)
pKD46	ParaB-γ-β-exo, Ori R101, Amp ^r	(Datsenko y Wanner, 2000)
pCP20	<i>flp</i> , Amp ^r , Cm ^r	(Datsenko y Wanner, 2000)
pFZY1	Amp ^r , F'lac replicon, lacZ + Y + A +	(Koop <i>et al.</i> , 1987)
pBAD30	Ori P15A, araC, Amp ^r	(Guzman et al., 1995)
pPB1480	pUH::mlrB; mlrB coding sequence cloned in pUH21-2lacIq Amp ^r	This Study
pPsseA::lacZ	pFZY1::sseA; sseA promoter region cloned in pFZY1 Amp ^r	(Zaharik <i>et al.</i> , 2002)
pBAD::mlrA	mlrA coding sequence cloned in pBAD30 Amp ^r	This Study
pPcsgD::gfp	pPROBE(NT)::PcsgD; csgD promoter region cloned in pPROBE(NT) Km ^r	This Study

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675 **Table S2. Oligonucleotides used in this study**

Primer	5' → 3' Sequence	Reference
1390 P1	TCATGATATAGTAGAATCCCCTTATTTAACGGGCTTTA CCGTGTAGGCTGGAGCTGCTTCG	This Study
1390 P2-100	CCGGTGGTCACCAGGAACCATTTTCTCTGGGCGAAC CATATGAATATCCTCCTTA	This Study
1389 P1	CCATTGGCGTCAGTCGTCGACTTTCAATAAGGGGGA ATCGTGTAGGCTGGAGCTGCTTCG	This Study
1389 P2	TGGCGGGCGGGTTACCCGCCGTTAGCGATGCGCAG GGTTACATATGAATATCCTCCTTA	This Study
mlrA P1	CGTCTAAAGTTAAACCGGGACCTCGCGAGCAAGGGT GAAACGCTGTGTAGGCTGGAGCTGCTTCG	This Study
mlrA P2	GTTAATAAAAGGAGTATACATTAAAGCGAATTTGTTAG CTTCCATATGAATATCCTCCTTA	This Study
csgDP1(F)	CAGCTGTCAGATGTGCGATTAATAAAGTGGAGTTTC ATCGTGTAGGCTGGAGCTGCTTCG	This Study
csgDP2(R)	CTCTGCTGCTACAATCCAGGTCAGATAGCGTTTCATG GCCCATATGAATATCCTCCTTAG	This Study
bcsA P1	AACGTCCGCCCGGGAGCCTGCGATGAGCGCCCTTTCC CGGTGTAGGCTGGAGCTGCTTCG	This Study
bcsA P2	TCATCGCATTATCATCATTGTTGAGCCTGAGCCATAA CCCCATATGAATATCCTCCTTA	This Study
1390 3xFLAG Fw	CGCAACGTGGTCGCTTCGCTGGAGGTTATCACACTA GACTACAAAGACCATGACGG	This Study
mlrA CTF FLAG	ATGGCGCGCCCGAGGACGCGACATTCATCCCTTGG CGTTGACTACAAAGATGACGACGAT	This Study
ssrB P1 Fw	CATTACTTAATATTATCTTAATTTTCGCGAGGGCAGCA AAGTGTAGGCTGGAGCTGCTTCG	This Study
ssrB P2 Rv -100	CTCATCATATTCATCCGGTGTGTTTCGACGGTTTTAT ACCATATGAATATCCTCCTTA	This Study
1390 WT P1	GCGCAACGTGGTCGCTTCGCTGGAGGTTATCACACT ATAGGTGTAGGCTGGAGCTGCTTCG	This Study
1389 WT P1	GTACCCTGATACATTAGGGCTGCGCTTAGCCATCACC TGAGTGTAGGCTGGAGCTGCTTCG	This Study
1390 Fw	GAGGATCCATATGTCGTATTCTATC	This Study
1390 Rv	ACCCAAGCTTCTATAGTGTGATAAC	This Study
MlrAKpnI_SD fw	CGGGGTACCTATAAGGAGGAAAAACATATGGCGCTTT ACACAATTG	This Study
Mer 7 CTR	ACCCAAGCTTAAACGCCAAGGGGATG	This Study
PcsgD rv xmaI	CCCCCGGGACTACTATGGACTTC	This Study
PCsgB rv XmaI	CCCCCGGGGCGCACCCAGTATTGT	This Study

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                                HTH
MlrB  -MSYSIGEFARLCGINAATLRAWQRRYGLLPQRTDGGHRLYSDDDIRQALSILDWVRKG
MlrA  MALYTTIGEVALLCDINPVTLRAWQRRYGLLPQRTDGGHRLFNDAIDIRIREIKRWIDNG
      *.***.* **.*..*****.....*.* ** . .* *:.*

MlrB  VPISQVNPLLSRPVIRLGDNWITIQETMLQHLHEGRIDALRQLIYDCGREYPRAEVLVTHL
MlrA  VQVSKVKVLLSSDSSEQPNGWREQQEILLHYLQSSNLHSLRLWVKERGGDYPAQTLTTNL
      * :.*:.* **      :.* ** :*:*:...: ** : :*.:** *.:.*

MlrB  LRPLRSKVS AHLPAVMTLREILDGII IAYTSFCLEGRKAPGNNAFISGWNLSDHCEIWL
MlrA  FVPLRRRLQCQQPALQALLGILDGILINYIALCLASARKKQKDALVIGWNIHDTTRLWL
      : ** .:..: ** :.* ***** * * :.* . ** *:*: : ** : * :.*

MlrB  EALTRTQELRLNVLPSPVVLAPELFAQRKWFVLTGKLTAQKKQLAQWRNVVASLEV
MlrA  EGWVASQQGWRIDVLAHSLSQFRPELFDGKTLVWCGENQTLAQQQQLLAWRAQGRDIHP
      *. . : * *:*. . : **** . . : : * .*:.* ** .:

MlrB  ITL
MlrA  LGV
      : :

```

B

Figure S1. Amino acid alignment of MlrA and MlrB and *mlrB* genomic context.

A. Alignment of amino acid sequences of MlrA (access ACY89107.1) and MlrB (access ACY88162.1) from *S. Typhimurium* strain LT2 (McClelland et al., 2001). Asterisks (*) indicate identical residues while dots (.) and double dots (:) indicate similarity. The predicted DNA-interacting helix-turn-helix region (HTH) is indicated (Humbert *et al.*, 2013). **B.** Scheme of the SPI2 region containing *mlrB* and its neighbor genes.

687

688 **Figure S2. Comparative *mlrB* and *mlrA* expression in different growth media.**
689 β -galactosidase activity from **A**) *mlrB::lacZ*; or **B**) *mlrA::lacZ* transcriptional fusions,
690 determined from cells grown to stationary phase in the indicated culture media, at
691 37°C. The values shown are the average of three independent experiments carried
692 out in duplicate. Error bars correspond to the SD. Symbols above bars denote
693 statistical significance between means, with respect to LB. n.s., not significant; *, P
694 < 0.05; **, P < 0.01; ***, P < 0.001.

Figure S3. *mlrB* transcriptional induction under SPI-2 condition is not SsrA/SsrB-dependent. **A.** β-galactosidase activity of a *mlrB::lacZ* transcriptional fusion from wild-type (WT) or Δ*ssrB*::Cm mutant cells grown overnight either in LB or in InSPI-2 culture media at 37 °C. Values correspond to the average of three independent experiments carried out in duplicate. Error bars correspond to the SD. **B.** β-galactosidase activity of a *sseA::lacZ* transcriptional fusion from wild-type (WT), Δ*ssrB*::Cm (polar effect), Δ*mlrB* or Δ*mlrB*::Cm (polar effect) strains. Cells were grown overnight in InSPI-2 media at 37 °C. The values represent the average of three independent experiments carried out in duplicate. Error bars correspond to the SD. In both cases, symbols above bars denote statistical significance between means as compared to the WT strain. n.s., not significant; *, P < 0.05; **, P < 0.01; ***, P < 0.001.

Figure S4. MlrB and MlrA protein levels inside macrophages. MlrB and MlrA protein levels detected by Western blots in total protein extracts obtained from *S. Typhimurium* *mlrB*::3xFLAG or *mlrA*::3xFLAG *orf319*::3xFLAG tagged strains grown inside RAW264.7 macrophages. The low-migrated bands in the MlrA Western blots correspond to Orf319-3xFLAG. The figure shows two different experiments. Anti-IgaA was used as load control.

Figure S5. *In silico* screening for MlrA/MlrB DNA binding sites in the *S. Typhimurium* genome. A. A positional weight matrix was constructed using MlrA binding sequences reported in *E. coli* (Ogasawara *et al.*, 2010) based on MEME/MAST, and used to screen the *S. Typhimurium* LT2 genome. Among the predicted MlrA-binding sites, the program detected one in the *csgD-csgB* intergenic region, and we identified a second by visual inspection. The program also identified a possible MlrA-binding site in the promoter region of *orf319*. **B.** Predicted MlrA/MlrB binding sites (red boxes) and *csgD* and *orf319* transcription initiation sites (arrows) (Kröger *et al.*, 2013) are depicted. The DNA sequence of *orf319* promoter region is included. Boxes indicate the predicted -10 and -35 promoter elements. Underlined is the putative MlrB/MlrA recognized dyad.

728

729 **Figure S6. MlrB and MlrA co-dependent control of *orf319* transcription in LB.**

730 β-galactosidase activity of *orf319::lacZ* transcriptional fusion determined in wild-type
731 (WT), Δ*mlrB*, Δ*mlrA*, and Δ*mlrA* Δ*mlrB* *S. Typhimurium* strains grown overnight in
732 LB at 28°C. The values correspond to the average of three independent experiments
733 carried out in duplicate and the error bars correspond to the standard deviation.
734 Asterisks denote statistical significance between means, with respect to WT. *, P <
735 0.05; **, P < 0.01; ***, P < 0.001.

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737

738 **Figure S7. MlrB is not affecting CsgD expression under InSPI2 inducing**
739 **conditions.** Arbitrary fluorescence from a *PcsgD::gfp* reporter plasmid expressed
740 from wild-type (WT), $\Delta mlrB$, $\Delta mlrA$, or the $\Delta mlrA \Delta mlrB$ strains grown at 37°C in
741 InSPI-2. The data correspond to the average of at least three independent
742 experiments carried out in duplicate and the error bars correspond to the SD. n.s.,
743 not significant.