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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 28ºC 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 Δ*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 Δ*mlrB* strain could be masked by the presence of MlrA. However, the low and similar *csgD* expression observed in both Δ*mlrA* and Δ*mlrA* Δ*mlrB* mutants (Fig 1B), along with the lack of adhesion of the Δ*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; Walthers *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 IgaA 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 Δ*mlrB* Δ*mlrA* double mutant showed an *orf319* transcription level similar to that displayed by the Δ*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 Δ*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 Δ*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 Δ*mlrA* Δ*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 Δ*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 Δ*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 Δ*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

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

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

253 On the other hand, and while only MlrA activated *csgD* expression in laboratory
254 conditions (Fig. 1B), we showed an increased expression of *csgD* inside
255 macrophages in the absence of MlrB (Fig. 6) indicating that in this environment MlrB
256 acts as a repressor of the biofilm master regulator. In this sense, we propose a
257 counteracting role between MlrB and MlrA inside macrophages. This is further
258 supported by the increased survival of the Δ*mlrA* Δ*mlrB* double mutant strain
259 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

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

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

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

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

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

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

324

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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 μg 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 ELχ808).

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 37ºC for 30 min and then fresh D-MEM 10% FBS medium supplemented with gentamicin 100 µg/ml was added. After 1 h at 37ºC, 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 cm^2 plates as described previously (Núñez-Hernández et al., 2013). Briefly, cells were seeded in BioDish-XL 500-cm^2 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

411 times with PBS and then were incubated with fresh medium containing 100 µg/ml of
412 gentamicin. After 1 h at 37 ºC, infected cells were incubated with medium containing
413 gentamicin at a concentration of 30 µg/ml until 18 h post infection. At indicated time
414 points, infected macrophages were washed with PBS and lysed in a solution
415 containing 4% SDS, 1% acidic phenol, and 19% ethanol in water. After 30 min of
416 incubation at 4°C, intracellular bacteria were collected by centrifugation (27,500 g,
417 4°C, 30 min) and washed three times with 1 ml of a, 19% ethanol RNAse free
418 solution. For western blot analyses, intracellular bacteria were processed as
419 described previously (Pontel and Soncini, 2009; Pérez Audero *et al.*, 2010).
420 Immunodetection was carried out using mouse anti-FLAG monoclonal (Sigma-
421 Aldrich) or rabbit polyclonal anti-IgaA antibodies (Lobato-Márquez *et al.*, 2015).

422 **_csgD_ intracellular expression**

423 Measurement of *csgD* transcription from intracellular bacteria was performed using
424 WT/pPromcsgD-gfp or ΔmlrB/pPromcsgD-gfp strains. A gentamicin protection assay
425 was performed as described above, with modifications, as follows. RAW 264.7
426 macrophages were cultured in 6-well plates until they reached confluence.
427 WT/pPromcsgD-gfp or ΔmlrB/pPromcsgD-gfp strains were grown ON at 37ºC. At the
428 indicated time point, invaded cells were washed four times with PBS and lysed with
429 0,1% PBS-Triton X-100. Lysates were collected, centrifuged 5 min at 6500 rpm and
430 resuspended in PBS. Two hundred microliters of each sample were used to measure
431 GFP fluorescence in a Synergy 2 Microplate Reader (Biotek) spectrophotometer
432 (λ_{exc}=485 nm, λ_{em}=528 nm). One hundred microliters of each sample were used to
433 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.

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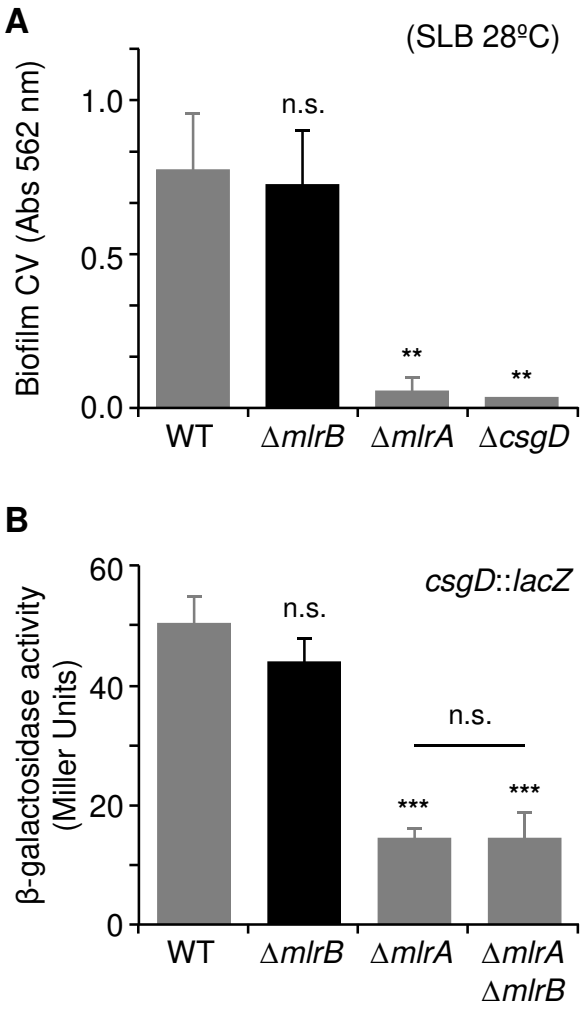
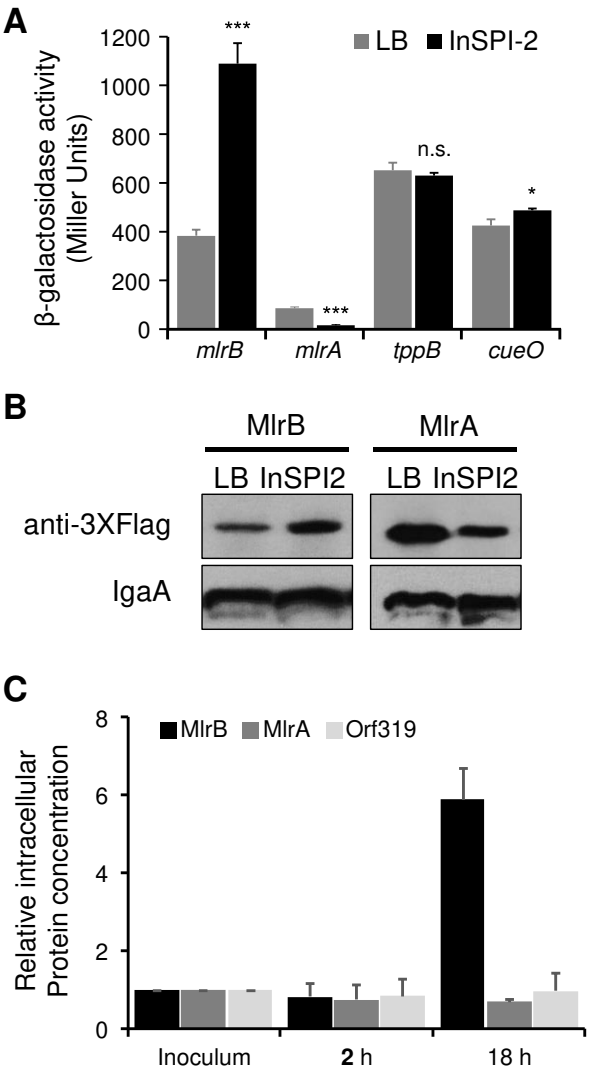


593

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

596 **A.** Adherence to polystyrene. 1:100 dilutions of ON SLB cultures of the indicated
597 strains were incubated in SLB medium, at 28 °C for 48 hours in polystyrene
598 microplates without shaking. After successive washes, staining was performed with
599 1% crystal violet and 562 nm Absorbance was determined. The data correspond to
600 average values of three independent experiments carried out by quadruplicate. The
601 error bars correspond to the SD. **B.** β-galactosidase activity from a *csgD*::*lacZ*

602 transcriptional fusion determined for wild-type (WT), Δ*mlrB*, Δ*mlrA* or Δ*mlrA* Δ*mlrB*
603 cells, grown overnight in LB medium, at 28 °C. The data correspond to mean values
604 of three independent experiments performed in duplicate. Error bars represent SDs.
605 Symbols above bars denote statistical differences between means, with respect to
606 WT. n.s., not significant; *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.
607



608

609 **Figure 2. MlrB expression increases inside macrophages.**

610 **A.** β-galactosidase activity determined for the indicated reporter strains, grown until
611 stationary phase in LB medium (gray bars) or InSPI-2 medium (black bars); values
612 reported are means and SD of three independent experiments. Symbols above bars
613 denote statistical difference between means from LB *versus* InSPI-2-grown cells.
614 n.s., not significant; *, $P < 0.05$; ***, $P < 0.01$; ***, $P < 0.001$. **B.** Western blot analysis
615 of MlrB and MlrA 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.

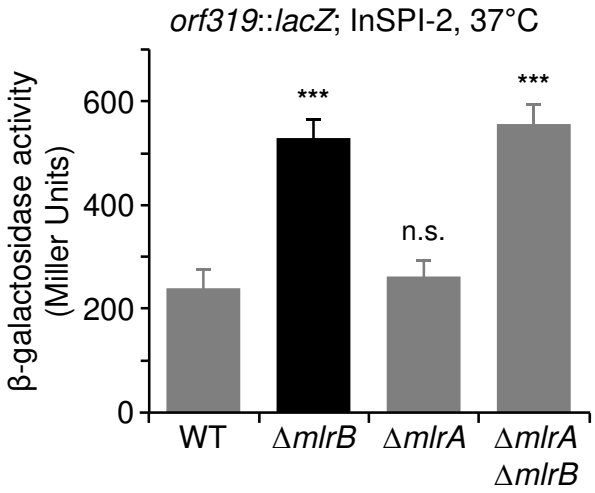
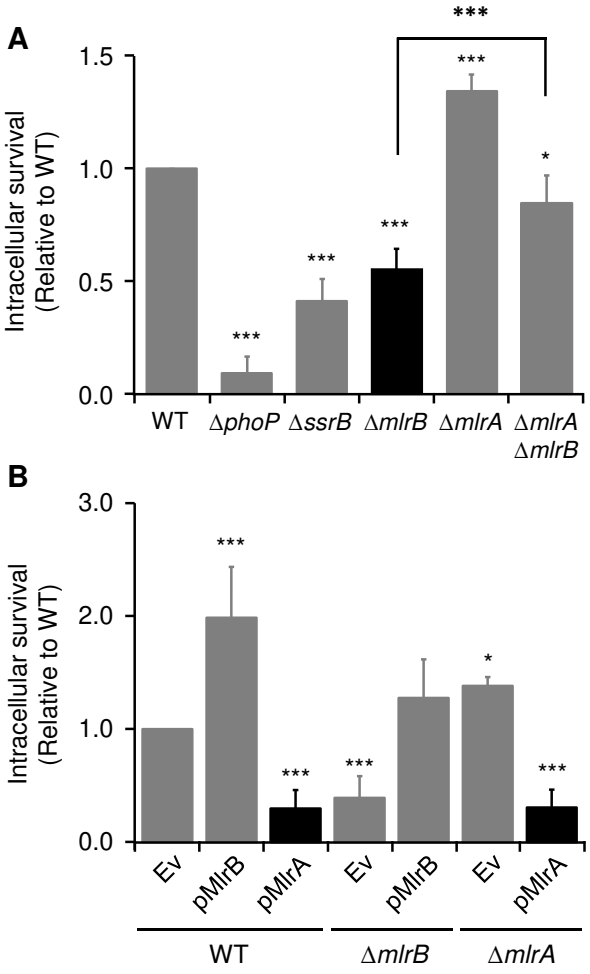


Figure 3. MlrB regulates *orf319* transcription under SPI-2-inducing conditions.

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 tree 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; *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.



633

634 **Figure 4. MlrB is required for intramacrophage survival.**

635 **A.** Survival of wild-type (WT), Δ*mlrB*, Δ*mlrA*, and Δ*mlrA* Δ*mlrB* *S.* Typhimurium
636 strains in RAW 264.7 macrophages at 18 h after infection. The Δ*phoP* and Δ*ssrB*
637 strains were included as controls. The values correspond to the average of at least
638 three independent experiments carried out in duplicate and the error bars represent
639 SD. **B.** Intramacrophage survival of wild-type (WT), Δ*mlrB*, and Δ*mlrA* strains
640 ectopically expressing MlrB or MlrA from a multicopy plasmid was determined as in
641 (A). (Ev) indicates the empty vector. The values correspond to the average of at least
642 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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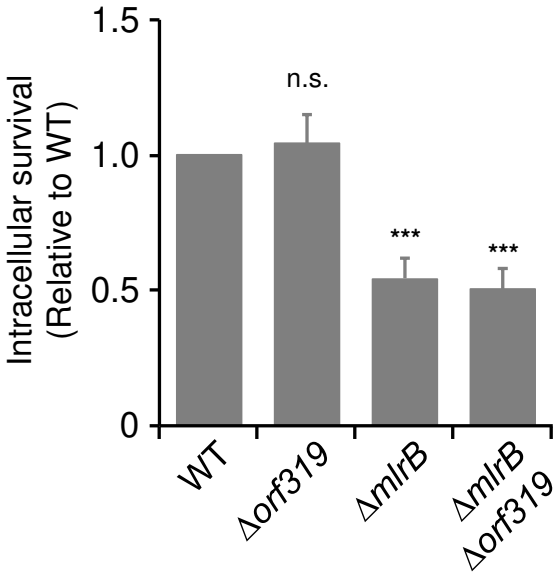
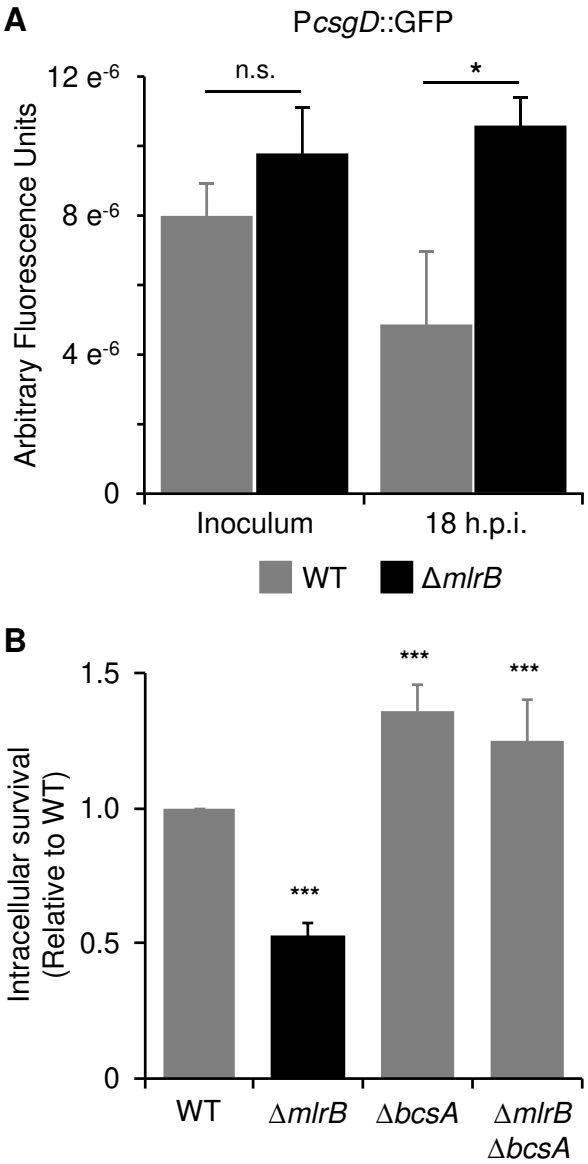


646

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

648 Survival of wild-type (WT), Δ*orf319*, Δ*mlrB*, and Δ*mlrB* Δ*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



655

656 **Figure 6. The defect in macrophage survival of the Δ*mlrB Salmonella* mutant**

657 **depends on CsgD expression.**

658 **A.** CsgD expression increases inside macrophages in the absence of MlrB. Arbitrary

659 fluorescence from a P*csgD*::*gfp* reporter plasmid expressed in wild-type (WT) or in

660 Δ*mlrB* strains inside RAW264.7 macrophages at 0 (inoculum) and 18 h post

661 infection. Values correspond to the ratio between fluorescence and the number of

662 intracellular surviving bacteria. The data correspond to the average of at least three
663 independent experiments carried out in duplicate and the error bars correspond to
664 the SD. **B.** MlrB effect on *Salmonella* survival within macrophages is *bcsA*
665 dependent. Relative survival of Δ*mlrB*, Δ*bcsA* and Δ*mlrB* Δ*bcsA* *strains* inside
666 RAW264.7 macrophages at 18 h after infection compared to the wild-type (WT)
667 strain. The values correspond to the average of at least three independent
668 experiments carried out in duplicate and the error bars represent SD. In **A** and **B**,
669 symbols above bars denote statistical significance between means, with respect to
670 WT. n.s., not significant; *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.
671

672 **Table S1. Bacterial strains and plasmids used in this study**

Strains *Salmonella* **Typhimurium**		
Strain or plasmid	**Relevant properties**	**Source**
14028s	Wild-Type	ATCC
PB13505	14028s Δ*mlrB*::*Cm*^{r} (-100)	This Study
PB11017	14028s Δ*orf319*::*Km*^{r}	This Study
PB10657	14028s Δ*mlrA*::*Km*^{r}	This Study
PB12982	14028s Δ*csgD*::*Km*^{r}	This Study
PB11273	14028s Δ*mlrB* Δ*mlrA*::*Km*^{r}	This Study
PB11019	14028s Δ*(orf319-mlrB)*::*Cm*^{r}	This Study
PB14054	14028s Δ*bcsA*::*Km*^{r}	This Study
PB14441	14028s Δ*mlrB* Δ*bcsA*::*Km*^{r}	This Study
PB10631	14028s *PcsgD*::*lacZ-Cm*^{r}	This Study
PB11286	14028s *PcsgD*::*lacZ-Cm*^{r} Δ*mlrB*	This Study
PB10915	14028s *PcsgD*::*lacZ-Cm*^{r} Δ*mlrA*::*Km*^{r}	This Study
PB11291	14028s *PcsgD*::*lacZ-Cm*^{r} Δ*mlrB* Δ*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 *et al.*, 1987)
PB7937	14028s Δ*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 Δ*ssrB*::*Cm*^{r} (-100)	This Study
PB12920	14028s *STM1389-lacZ*	This Study
PB13082	14028s *STM1389-lacZ* Δ*mlrB*::*Cm*^{r}	This Study
PB13291	14028s *STM1389-lacZ* Δ*mlrA*::*Km*^{r}	This Study
PB13286	14028s *STM1389-lacZ* Δ*mlrB*::*Cm*^{r} Δ*mlrA*::*Km*^{r}	This Study
MS7953s	14028s *phoP*::*Tn10*	(Fields *et al.*, 1986)
Plasmids		
pUH21-2lacIq	reppMB1, Amp^r, *lac*Iq	(Soncini et al., 1995)

pBAD30	Ori P15A, araC, Ampr	(Guzman et al., 1995)
pPROBE(NT)	Ori V, Km^r	(Miller *et al.*, 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	*flp*, Amp^r, Cm^r	(Datsenko y Wanner, 2000)
pFZY1	Amp^r, F'lac replicon, lacZ + Y + A +	(Koop *et al.*, 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
p*sseA::lacZ*	pFZY1::*sseA; sseA* promoter region cloned in pFZY1 Amp^r	(Zaharik *et al.*, 2002)
pBAD::*mlrA*	*mlrA* coding sequence cloned in pBAD30 Amp^r	This Study
p*csgD*::*gfp*	pPROBE(NT)::P*csgD*; *csgD* promoter region cloned in pPROBE(NT) Km^r	This Study

673
674

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	CCATTGGCGTCAGTCGTCGTACTTTCAATAAGGGGGA ATCGTGTAGGCTGGAGCTGCTTCG	This Study
1389 P2	TGGCGGGCGGGTTACCCGCCGTTAGCGATGCGCAG GGTTACATATGAATATCCTCCTTA	This Study
mlrA P1	CGTCTAAAGTTAAACCGGGACCTCGCGAGCAAGGGT GAAACGCTGTGTAGGCTGGAGCTGCTTCG	This Study
mlrA P2	GTTAATAAAAGGAGTATACATTAAAGCGAATTTGTTAG CTTCCATATGAATATCCTCCTTA	This Study
csgDP1(F)	CAGCTGTCAGATGTGCGATTAAAAAAAGTGGAGTTTC ATCGTGTAGGCTGGAGCTGCTTCG	This Study
csgDP2(R)	CTCTGCTGCTACAATCCAGGTCAGATAGCGTTTCATG GCCCATATGAATATCCTCCTTAG	This Study
bcsA P1	AACGTCCGCCGGGAGCCTGCGATGAGCGCCCTTTCC CGGTGTAGGCTGGAGCTGCTTCG	This Study
bcsA P2	TCATCGCATTATCATCATTGTTGAGCCTGAGCCATAA CCCCATATGAATATCCTCCTTA	This Study
1390 3xFLAG Fw	CGCAACGTGGTCGCTTCGCTGGAGGTTATCACACTA GACTACAAAGACCATGACGG	This Study
mlrA CTF FLAG	ATGGCGCGCCCAGGGACGCGACATTCATCCCCTTGG CGTTGACTACAAAGATGACGACGAT	This Study
ssrB P1 Fw	CATTACTTAATATTATCTTAATTTTCGCGAGGGCAGCA AAGTGTAGGCTGGAGCTGCTTCG	This Study
ssrB P2 Rv -100	CTCATCATATTCATCCGGTGTGTTTCGACGGTTTTTAT 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	CCCCCCGGGACTACTATGGACTTC	This Study
PCsgB rv XmaI	CCCCCCGGGCGCACCCAGTATTGT	This Study

676
677

A

```
                 _________HTH_________
MlrB    -MSYSIGEFARLCGINAATLRAWQRRYGLLKPQRTDGGHRLYSDDDIRQALSILDWVRKG
MlrA    MALYTIGEVALLCDINPVTLRAWQRRYGLLKPQRTDGGHRLFNDADIDRIREIKRWIDNG
           *:***.* **.**..**********************:.* ** .  .*  *: :*

MlrB    VPISQVNPLLSRPVIRLGDNWITIQETMLQHLHEGRIDALRQLIYDCGREYPRAELVTHL
MlrA    VQVSKVKVLLSSDSSEQPNGWREQQEILLHYLQSSNLHSLRLWVKERGQDYPAQTLTTNL
        * :*:*: ***       :.*   ** :*::*:...: :**  : : *.:**   *.*:*

MlrB    LRPLRSKVSAHLPAVMTLREILDGIIIAYTSFCLEGDRKAPGNNAFISGWNLSDHCEIWL
MlrA    FVPLRRRLQCQQPALQALLGILDGILINYIALCLASARKKQGKDALVIGWNIHDTTRLWL
        : *** .:..: **: :*  *****:* * ::** . **  *::*:: ***: *   :**

MlrB    EALTRTGQELRLNVLPSPPVVLAPELFAQRKWFLVTTGKLTAGQKKQLAQWRNVVASLEV
MlrA    EGWVASQQGWRIDVLAHSLSQFRPELFDGKTLLVWCGENQTLAQQQQLLAWRAQGRDIHP
        *. . : *  *::**. .   : ****  .. ::    : * .*::**  **    .:

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.

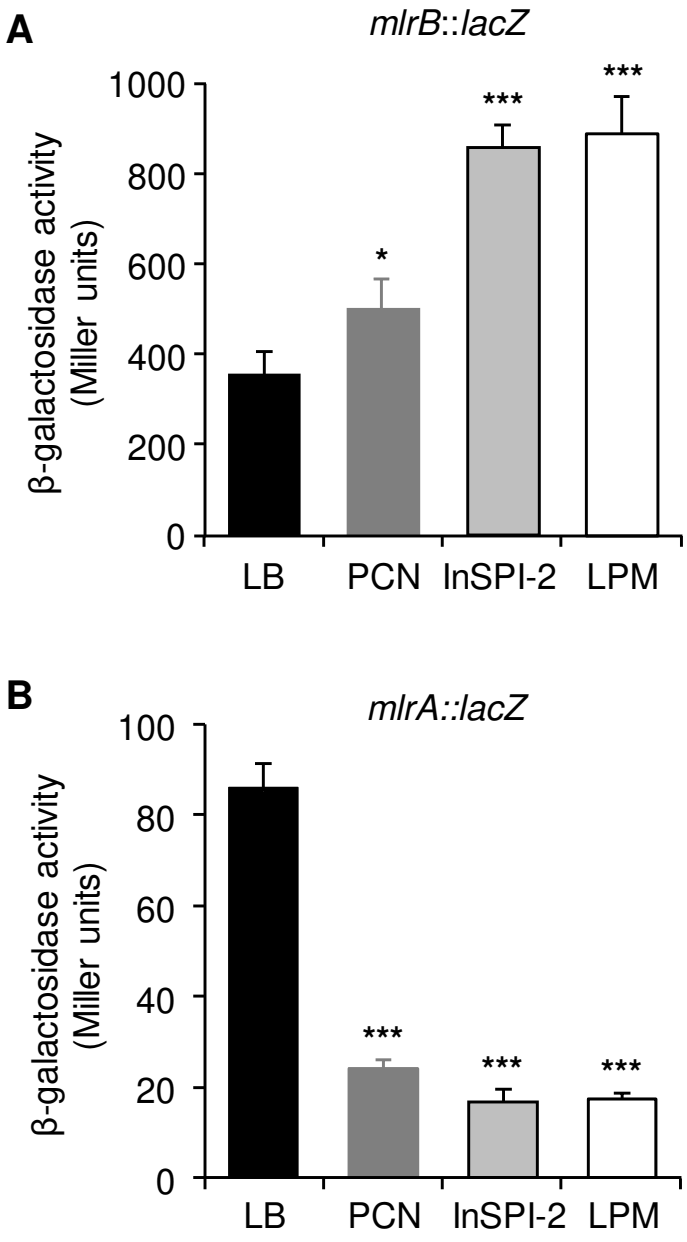
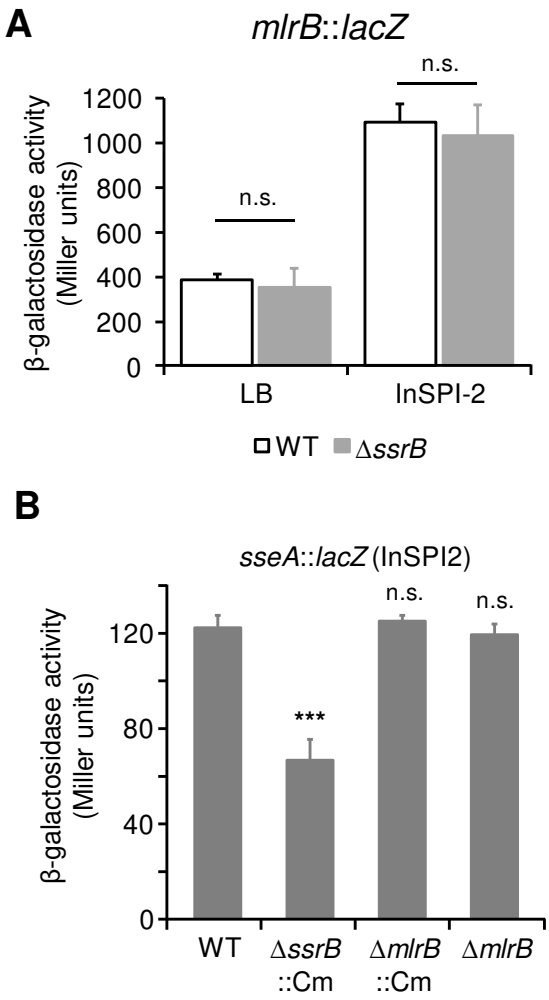


Figure S2. Comparative *mlrB* and *mlrA* expression in different growth media. β-galactosidase activity from **A**) *mlrB*::*lacZ*; or **B**) *mlrA*::*lacZ* transcriptional fusions, determined form cells grown to stationary phase in the indicated culture media, at 37°C. The values shown are the average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



695

696 **Figure S3. *mlrB* transcriptional induction under SPI-2 condition is not**
697 **SsrA/SsrB-dependent. A.** β-galactosidase activity of a *mlrB*::*lacZ* transcriptional
698 fusion from wild-type (WT) or Δ*ssrB*::*Cm* mutant cells grown overnight either in LB
699 or in InSPI-2 culture media at 37 °C. Values correspond to the average of three
700 independent experiments carried out in duplicate. Error bars correspond to the SD.
701 **B.** β-galactosidase activity of a *sseA*::*lacZ* transcriptional fusion from wild-type (WT),
702 Δ*ssrB*::Cm (polar effect), Δ*mlrB* or Δ*mlrB*::Cm (polar effect) strains. Cells were grown
703 overnight in InSPI-2 media at 37 °C. The values represent the average of three
704 independent experiments carried out in duplicate. Error bars correspond to the SD.
705 In both cases, symbols above bars denote statistical significance between means as
706 compared to the WT strain. n.s., not significant; *, $P < 0.05$; **, $P < 0.01$; ***, $P <$
707 0.001.

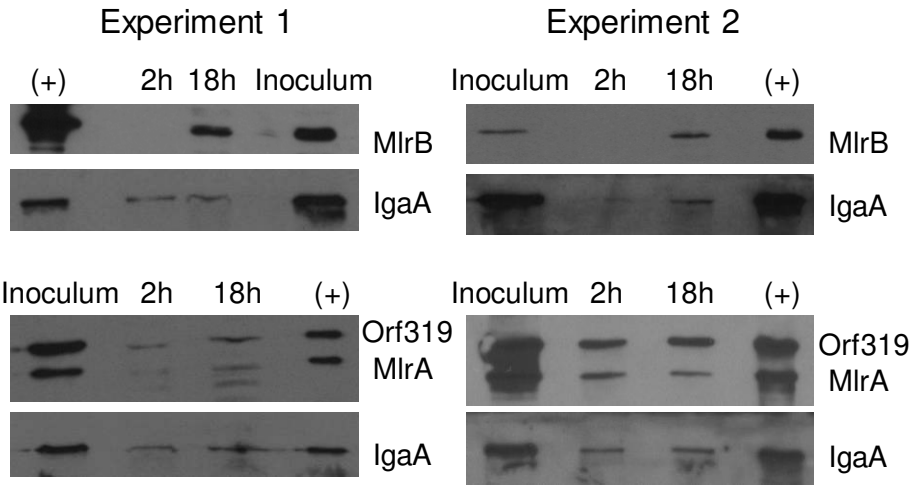


Figure S4. MlrB and MlrA protein levels inside macrophages. MlrB and MlrA protein levels detected by Western blots in total protein extracts obtained from *S.* Typhimurium *mlrB*::3×FLAG or *mlrA*::3×FLAG *orf319*::3×FLAG tagged strains grown inside RAW264.7 macrophages. The low-migrated bands in the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

A

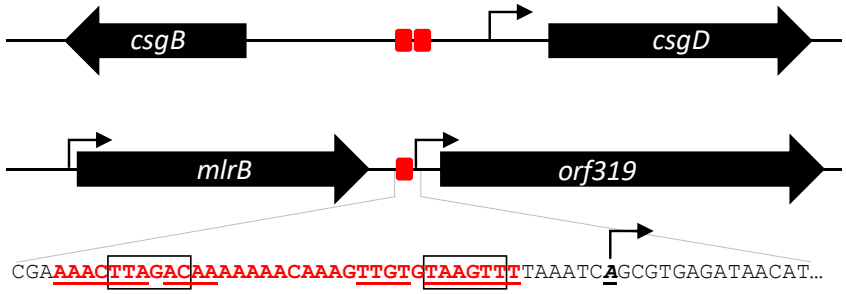
Escherichia coli

P_{csgD}	**AAAGTTGTACATTTGGTTTTTATTGCACAATTTT**
rplU	CAACTTGTACGGTAACGTTTATCTGTGCCATTTT
yrbA	CAAATTATACAATTTCGCCAGGGTATACACTTTG
bppB	AAAATCGTGCAAATTCAATACATTGCAGAATTTT
cadC	AAAAATGTTCAGAAAATAATCCATGGAAAATTGT

MEME (palindromes) → FIMO (*S.* Typhimurium LT2)

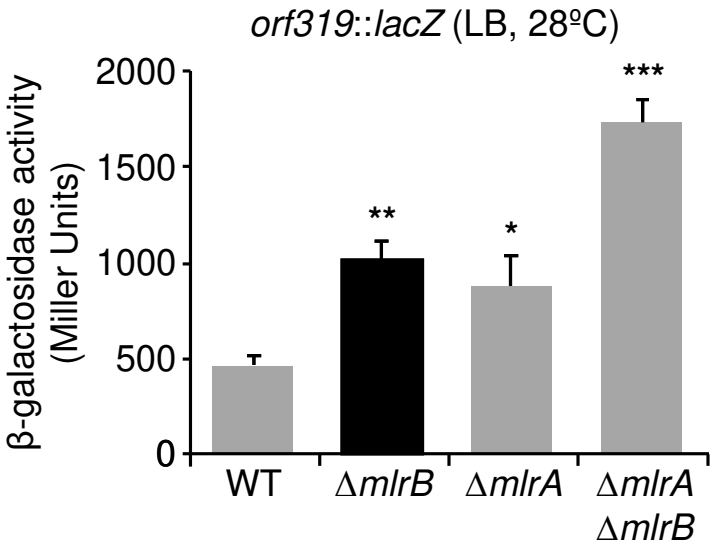
p-value	**Matched Sequence**	
6.98 e^{-08}	**AAACTTACACAACTTTGTTTTTTTGTCTAAGTTT**	**(P_{orf319})**
8.73 e^{-08}	ATAATTGTATATACATTTTTGTATGTACAATTAG	UTR *STM4528*
2.25 e^{-07}	ACAATAATGCAAATTATTTTATGTGTATAGCTTT	interg. reg.
2.26 e^{-07}	ACAGACGGGCAGTAAATATTCTGCGTATAATTTT	[int *STM2982*]
2.35 e^{-07}	CAATATTTACGGTTAAGTTTTAACGCAAAACTTG	[int *STM0982*]
2.45 e^{-07}	**AAAATCATACAAATTATAATAATTCATTGATTTT**	**(P_{csgD})**
Visual Insp.	**AAAGTTGTACATTTCGCTGTTATTGCATAGATTT**	**(P_{csgD})**

B



716

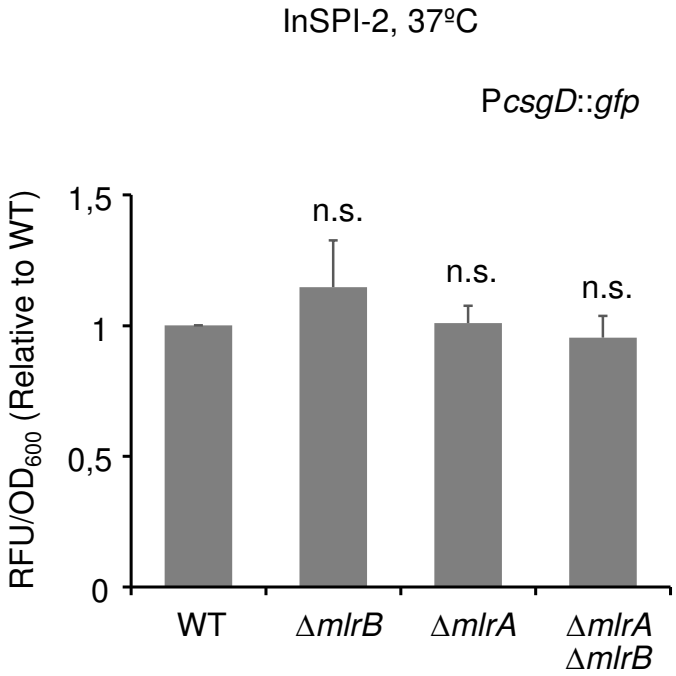
717 **Figure S5. *In silico* screening for MlrA/MlrB DNA binding sites in the *S.***
718 **Typhimurium genome. A.** A positional weight matrix was constructed using MlrA
719 binding sequences reported in *E. coli* (Ogasawara *et al.*, 2010) based on
720 MEME/MAST, and used to screen the *S.* Typhimurium LT2 genome. Among the
721 predicted MlrA-binding sites, the program detected one in the *csgD-csgB* intergenic
722 region, and we identified a second by visual inspection. The program also identified
723 a possible MlrA-binding site in the promoter region of *orf319*. **B.** Predicted MlrA/MlrB
724 binding sites (red boxes) and *csgD* and *orf319* transcription initiation sites (arrows)
725 (Kröger *et al.*, 2013) are depicted. The DNA sequence of *orf319* promoter region is
726 included. Boxes indicate the predicted -10 and -35 promoter elements. Underlined
727 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. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

736



737

738 **Figure S7. MlrB is not affecting CsgD expression under InSPI2 inducing**
739 **conditions.** Arbitrary fluorescence from a P*csgD*::*gfp* reporter plasmid expressed
740 from wild-type (WT), Δ*mlrB*, Δ*mlrA*, or the Δ*mlrA* Δ*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.