Stabilization of the Virulence Plasmid pSLT of *Salmonella* Typhimurium by Three Maintenance Systems and Its Evaluation by Using a New Stability Test

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Certain *Salmonella enterica* serovars belonging to subspecies I carry low-copy-number virulence plasmids of variable size (50–90 kb). All of these plasmids share the *spv* operon, which is important for systemic infection. Virulence plasmids are present at low copy numbers. Few copies reduce metabolic burden but suppose a risk of plasmid loss during bacterial division. This drawback is counterbalanced by maintenance modules that ensure plasmid stability, including partition systems and toxin-antitoxin (TA) loci. The low-copy number virulence pSLT plasmid of *Salmonella enterica* serovar Typhimurium encodes three auxiliary maintenance systems: one partition system (*parAB*) and two TA systems (*ccdAB*ST and *vapBC2*ST). The TA module *ccdAB*ST has previously been shown to contribute to pSLT plasmid stability and *vapBC2*ST to bacterial virulence. Here we describe a novel assay to measure plasmid stability based on the selection of plasmid-free cells following elimination of plasmid-containing cells by ParE toxin, a DNA gyrase inhibitor. Using this new maintenance assay we confirmed a crucial role of *parAB* in pSLT maintenance. We also showed that *vapBC2*ST, in addition to contribute to bacterial virulence, is important for plasmid stability. We have previously shown that *ccdAB*ST encodes an inactive CcdB*ST* toxin. Using our new stability assay we monitored the contribution to plasmid stability of a *ccdAB*ST variant containing a single mutation (R99W) that restores the toxicity of CcdB*ST*. The “activation” of CcdB*ST* (R99W) did not increase pSLT stability by *ccdAB*ST. In contrast, *ccdAB*ST behaves as a canonical type II TA system in terms of transcriptional regulation. Of interest, *ccdAB*ST was shown to control the expression of a polycistronic operon in the pSLT plasmid. Collectively, these results show that the contribution of the CcdB*ST* toxin to pSLT plasmid stability may depend on its role as a co-repressor in coordination with CcdA*ST* antitoxin more than on its toxic activity.

**Keywords:** virulence plasmid, toxin-antitoxin, plasmid stability, transcriptional regulation, *Salmonella Typhimurium*
INTRODUCTION

During evolution bacterial pathogens acquire new genes dedicated to manipulate host processes. Many of these pathogen functions are encoded by chromosomal genes. Others, however, can be encoded by genes present in mobile genetic elements such as virulence plasmids. Horizontal transfer of these mobile genetic components has shaped the host adaptation strategies in several bacterial pathogens (Jackson et al., 2011). The presence of a virulence gene in a mobile element also facilitates its rapid acquisition or loss under distinct selective pressures. Enteric bacteria such as Escherichia coli, Shigella spp. and Salmonella enterica, frequently carry virulence genes in large transmissible low-copy-number plasmids (Sasakawa et al., 1986; Makino et al., 1988; Gulig et al., 1993). The S. enterica species are facultative intracellular bacteria that cause disease ranging from self-limiting gastroenteritis to more severe systemic infections (Rivera-Chávez and Bäumler, 2015). S. enterica subdivides into seven subspecies (I, II, IIIa, IIIb, IV, VI, and VII) (Tindall et al., 2005; Grimont and Weill, 2007) and subspecies I includes more than 2500 serovars (Grimont and Weill, 2007). Most of these serovars have adapted to infect warm-blooded hosts. One of the most extensively studied serovars of subspecies I is Typhimurium, which infects both humans and livestock. Serovar Typhimurium, together with a few other serovars of subspecies I, possesses a virulence plasmid (Jones et al., 1982). These plasmids have a variable size of 50–90 kb and share common features such as low copy number (1–2 plasmids per chromosome), a similar repFIB replicon (similar to the repFIB family) and a conserved set of virulence genes encoding toxins and fimbrial proteins (including spv and pef operons) (Bäumler et al., 1998; Rotger and Casadesús, 1999). The low copy number of the S. Typhimurium virulence plasmid (also called pSLT) could theoretically compromise its heritability to daughter cells during cell division. Despite this, pSLT is extremely stable in the host with ~10^7 segregants per cell generation, in a similar rate to that observed for other low-copy-number plasmids such as F and P1 (Austin and Abeles, 1983; Kline, 1985; Tinge and Curtiss, 1990a). Low-copy-number plasmids carry maintenance modules such as partition systems and toxin-antitoxin (TA) systems that ensure their proper segregation to nascent cells (Million-Weaver and Camps, 2014). Partition systems significantly increase the stability of plasmids by ensuring segregation of one copy of the plasmid to each sibling cell (Ebersbach and Gerdes, 2005). On the other hand, TA modules are typically bicistronic operons that encode an unstable antitoxin and a stable toxin (Chan et al., 2016; Lobato-Márquez et al., 2016). As a consequence of their different stabilities, antitoxin must be continuously produced to efficiently neutralize its cognate toxin (Gerdes et al., 1986). However, if the TA-encoding plasmid is lost, the antitoxin cannot be replenished and the free toxin eliminates or reduces the growth of daughter cells thus diluting plasmid-free cells in the population (Yamaguchi and Inouye, 2011). This phenomenon is called post-segregational killing (Gerdes et al., 1986).

Classically, plasmid stability has been measured using antibiotic-resistance plasmid derivatives. Cells harboring the studied plasmid are positively selected in the presence of the selection antibiotic and those that have lost the plasmid are killed (Gerdes et al., 1985; del Solar et al., 1987). The main drawback of this technique is its sensitivity. Highly stable plasmids such as S. Typhimurium pSLT are below the sensitivity range of these assays. To solve this problem other methods relying in the direct selection of plasmid-free cells have been developed; for instance, the one based on the tetAR-chlorotetracycline system (Bochner et al., 1980; Maloy and Nunn, 1981). The tetA gene encodes a protein which resides in the cytoplasmic membrane and prevents cellular accumulation of tetracycline, thereby conferring resistance (Reyrat et al., 1998). However, TetA location in the bacterial membrane also causes the cell to become hypersensitive to lipophilic chelators such as fusaric or quininal acids (Bochner et al., 1980). Therefore, it is possible to select those cells that have lost the tetAR cassette. Inserted in a plasmid, the tetAR cassette can be used to select plasmid-free cells in special agar plates (Bochner-Maloy) containing fusaric acid (García-Quintanilla et al., 2006). Limitations of this method include poor reproducibility and the frequent occurrence of false positives (Li et al., 2013). Here, we have developed a novel, highly sensitive stability assay based on the negative selection of plasmid-containing cells. This assay is based on a cassette containing the ParE toxin-encoding gene of the parDE TA system and a kanamycin resistance gene (aph). ParE toxin targets DNA gyrase, blocks DNA replication and induces DNA breaks leading to cell death (Jiang et al., 2002). In our system ParE synthesis is controlled by a rhamnose-inducible promoter (PparE) (Maisononneuve et al., 2011). Once the aph-parE cassette has been inserted in the plasmid of interest and upon induction of PparE, only plasmid-free cells survive. Using this new tool we studied the contribution of the three main maintenance modules of the pSLT virulence plasmid of S. Typhimurium: the parAB partition system (Tinge and Curtiss, 1990b) and the ccdABST and vapBC2ST TA loci (Lobato-Márquez et al., 2015). We show that vapBC2ST TA module, which we recently demonstrated to be important to S. Typhimurium survival during non-phagocytic cells infection (Lobato-Márquez et al., 2015, 2016), also stabilizes pSLT plasmid. We show that the ccdABST TA system, known to impact pSLT heritability and encoding an inactive toxin (García-Quintanilla et al., 2006; Lobato-Márquez et al., 2015), conserves its TA transcriptional regulatory activity. Of interest, the ccdABST operon extends beyond the toxin gene including four additional open reading frames. Moreover, CcdABST TA complexes influence expression of downstream genes. We also demonstrate that stability of pSLT plasmid is not affected by a mutation (R99W) that restores CcdBST toxicity. We propose that the contribution of ccdABST to pSLT stability could be related to the regulatory activity of CcdA-ST:CcdB-ST complexes rather than to a post-segregational killing effect mediated only by CcdBST toxicity.

RESULTS

Development of a New Assay to Measure Plasmid Stability

Due to the recognized problems of the tetAR-chlorotetracycline method to measure plasmid stability, we decided to develop a
novel negative selection method to measure the contribution to stability of the different maintenance modules encoded in pSLT plasmid (Figure 1). We took advantage of an aph-parE cassette of the pKD267 plasmid (Maisonneuve et al., 2011). This cassette carries a kanamycin resistance gene (aph) and the parE gene, which encodes the toxin of the parDE TA system. ParE toxin interacts with and blocks the DNA gyrase, causing inhibition of DNA synthesis, induction of breaks and nicks in the DNA and finally cell death (Jiang et al., 2002). In the aph-parE cassette, previously used for chromosomal scarless deletions (Maisonneuve et al., 2011; Lobato-Márquez et al., 2015), the toxin-encoding parE gene is controlled by a rhamnose-inducible promoter. Thus, when rhamnose is present as the only carbon source in the medium, ParE is synthesized and the cell is killed (Figure 1). Using aph-parE cassette to disrupt the maintenance modules of pSLT plasmid we could select plasmid-free bacteria. To distinguish plasmid curing from other events causing rhamnose resistance (e.g., mutations in PparE promoter or parE gene), we took advantage of the kanamycin resistance gene also present in the aph-parE cassette. The resulting pSLT plasmid derivates were thus tagged with two different markers. 

parAB Partition System and vapBC2ST Promote Stability of S. Typhimurium pSLT Plasmid

Previous studies proposed two important regions involved in S. Typhimurium pSLT plasmid stability: the parAB partition system and the TA module ccdABST (Tinge and Curtiss, 1990b; García-Quintanilla et al., 2006). Additionally, we identified another TA system, called vapBC2ST, encoded within the trbH gene (Lobato-Márquez et al., 2015) and homologous to the mvpAT locus encoded in the virulence plasmid of Shigella flexneri (Sayeed et al., 2000). We reported that vapBC2ST promotes Salmonella survival inside infected host cells. We now evaluated if similarly to ccdABST, and to other plasmidic TA loci, vapBC2ST may play a role in pSLT stability. Additionally, to test the sensitivity and the reproducibility of our method, we reevaluated the contribution of parAB and ccdABST using the new stability assay. We compared pSLT plasmids derivates lacking parAB, ccdABST or vapBC2ST with an isogenic strain in which aph-parE cassette was inserted in the gene spvA, which was previously shown to be innocuous for the stability of pSLT (García-Quintanilla et al., 2006). Stability assays demonstrated that disruption of vapBC2ST TA system resulted in a 5.5 ± 0.1 fold increase in the fraction of segregants after ∼10 generations of growth without selection pressure (Figure 2). This increase was more important than in the case of the pSLT derivate lacking ccdABST (4 ± 0.2) under the same growth conditions (Figure 2). In accordance to previous studies, disruption of parAB or ccdABST decreased pSLT stability (Tinge and Curtiss, 1990a; García-Quintanilla et al., 2006). The parAB partition system stabilizes pSLT plasmid 119 ± 3 and 163 ± 9 fold more efficiently than the vapBC2ST or ccdABST TA systems, respectively (Figure 2). Moreover, the pSLT wild type plasmid was 650.1 ± 190.2 fold more stable than pSLT lacking parAB (Figure 2). These

FIGURE 1 | Scheme of the method used to measure plasmid stability of S. Typhimurium pSLT derivates. A cassette containing a kanamycin resistance gene (aph) and the DNA gyrase inhibitor ParE-encoding gene was used to disrupt genes of interest (top). Grown cultures were plated in rhamnose-containing agar plates and synthesis of ParE toxin was induced. Cells that kept pSLT plasmid and therefore aph-parE cassette, were selectively killed (bottom).
We have recently demonstrated that Van Melderen et al., 1994 Lobato-Márquez et al., 1994). Cells that do not CcdB virulence, also mediates pSLT heritability. However, CcdBST CcdBST-dependent stability (Figure 3B). Due to the inability of CcdABST TA system to stabilize pSLT plasmid independently of CcdBST toxicity, we characterized the ccdABST operon in more detail. The Non-functional TA System ccdABST of S. Typhimurium Conserves Transcriptional Regulatory Activity We tested if the type II TA module ccdABST of S. Typhimurium behaves as a bona fide TA system in terms of transcriptional regulation. In the F plasmid the antitoxin CcdA of the ccdAB ortholog acts as a transcriptional repressor and the toxin enhances the repressor activity when TA complexes are formed in a proper stoichiometry (Tam and Kline, 1989; Salmon et al., 1994). Mutations in the last three amino acids of the CcdB toxin in the F plasmid eliminate its toxicity while maintaining its regulatory activity (Bahassi et al., 1995). To test the transcriptional activity of S. Typhimurium ccdABST, we fused the promoter of the TA system (PccdABST) to a promoter-less lacZ reporter gene. We measured β-galactosidase activity in the following genetic backgrounds: (i) pSLT wild type, (ii) pSLT plasmid cured, (iii) pSLT deficient for ccdBST gene, (iv) pSLT deficient for ccdABST operon, and (v) pSLT only lacking promoter PccdABST. β-galactosidase assays demonstrated that ccdABST TA module behaves as a classical type II TA system. When the whole system is present (wild type background), transcription of the operon is repressed. However, this repression is lost in the absence of CcdABST repressor complexes due to the loss of either ccdBST or ccdABST (Figure 4A). Interestingly, we did not observe differences in β-galactosidase activity when the system lacked only the toxin ccdBST or the whole operon arguing for an important role of CcdBST in transcriptional regulation. In many type II TA modules, transcriptional regulation relies on the toxin:antitoxin ratio. Thus, an excess of antitoxin results in TA complexes that are efficient repressors; however, when the number of toxin molecules increases, the stoichiometry of the complex changes and repression is relieved. This regulation feature is termed “conditional cooperativity” (Overgaard et al., 2008). Taking advantage of the inactive CcdBST toxin, we analyzed the conditional cooperativity phenomenon in the ccdABST TA module of pSLT plasmid by supplying in trans an extra dose of the inactive CcdBST toxin. We employed a plasmid that contains inactive ccdBST gene controlled by an arabinose-inducible promoter. To discard unspecific effects derived from protein over-production, the same experiment was carried out with the unrelated non-toxic VapCST toxin encoded in the new antitoxin, leading the toxin free to kill or reduce the growth of plasmid-free cells (Van Melderen et al., 1994). We asked whether in S. Typhimurium the toxicity of CcdBST could be important for pSLT stability. To test this hypothesis, we carried out stability assays using a pSLT plasmid in which de non-functional ccdBST was substituted by an activated ccdBST (R99W) variant. Stability assays showed no differences between the pSLT plasmid derivates containing wild type ccdBST or the toxic version ccdBST (R99W), suggesting that CcdBST toxicity is dispensable for ccdABST-dependent stability (Figure 3B). Due to the inability of CcdABST TA system to stabilize pSLT plasmid independently of CcdBST toxicity, we characterized the ccdABST operon in more detail.
FIGURE 3 | The toxic activity of CcdB<sub>ST</sub> is dispensable for the CcdAB<sub>ST</sub>-mediated pSLT stability. (A) Growth curves of S. Typhimurium strains expressing wild type non-active or CcdB<sub>ST</sub> (R99W) toxic proteins. Bacteria were grown at 37 °C with shaking in LB medium. The expression of the ccdB<sub>ST</sub> (R99W) toxin-encoding gene ceased bacterial growth. The arrow indicates the time point (90 min) at which CcdB<sub>ST</sub> synthesis was induced by arabinose addition. (B) Segregants fraction measurement of pSLT plasmid comparing pSLT wild type and a pSLT variant harboring the toxic version ccdB<sub>ST</sub> (R99W). Data represent the means and standard deviations from five independent experiments. Data were compared using Student’s T-test. ns, non significant.

FIGURE 4 | Transcriptional regulation of the ccdAB<sub>ST</sub> TA system. The 300 bp region upstream to ccdA<sub>ST</sub> was cloned as a transcriptional fusion with a promoter-less lacZ reporter gene in plasmid pMP220. (A) β-galactosidase was measured in different genetic backgrounds of S. Typhimurium SV5015 transformed with pPccdAB-lacZ: (i) pSLT wild type, (ii) a strain cured of pSLT, (iii) a pSLT plasmid deficient for ccdAB<sub>ST</sub> TA system (including the promoter of the system), (iv) a pSLT lacking the toxin ccdB<sub>ST</sub> gene, and (v) a pSLT in which the PccdAB<sub>ST</sub> promoter was eliminated. Absence of TA complexes releases the CcdAB<sub>ST</sub> transcriptional repression, resulting in increasing β-galactosidase activity (B) Wild type S. Typhimurium SV5015 was transformed with pPccdAB-lacZ and either a plasmid expressing the non-functional copies of CcdB<sub>ST</sub> or VapC<sub>ST</sub> to further measure β-galactosidase activity. Cultures were grown to OD<sub>600</sub> of 0.3 and then expression of ccdB<sub>ST</sub> or vapC<sub>ST</sub> genes was induced by adding 0.3% arabinose during 1 h. Excess of CcdB<sub>ST</sub> specifically shows conditional cooperativity effect as its overexpression derepresses transcription at PccdAB<sub>ST</sub> promoter of PSLT plasmid. Data represent the means and standard deviations from four independent experiments. ***P < 0.001 by one-way ANOVA.

S. Typhimurium chromosome (Lobato-Márquez et al., 2015). Upon arabinose addition, we specifically observed an increased transcriptional activity of the PccdAB<sub>ST</sub> promoter following CcdB<sub>ST</sub> but not VapC<sub>ST</sub> production (Figure 4B). These data demonstrate that the ccdAB<sub>ST</sub> TA system responds to conditional cooperativity.

ccdAB<sub>ST</sub> of S. Typhimurium pSLT Plasmid Conforms a Six-Gene Polycistronic Operon

In the E. coli F plasmid, ccdAB maps upstream of the resolvase-encoding gene resD. However, analysis of the regions flanking ccdAB<sub>ST</sub> TA system of pSLT showed that this locus could be genetically linked to four other downstream genes (Figure 5A).
The ccdB<sub>ST</sub> gene is separated by only one single nucleotide from the downstream gene SL1344_P1_0078 (PSLT029), which itself overlaps 4 bp with SL1344_P1_0077 (PSLT030). The next downstream gene is SL1344_P1_0076 (PSLT031 or rsdB). PSLT031 maps 33 bp downstream from the 3'-end of SL1344_P1_0077 (PSLT030) and 8 bp upstream from the 5'-end of SL1344_P1_0075 (PSLT032) (Figure 5A). These short intergenic regions led us to hypothesize that the TA system ccdB<sub>ST</sub> of pSLT plasmid could be encoded within a six-gene polycistronic operon. RT-PCR assays confirmed a polycistronic operon encompassing from ccdB<sub>ST</sub> to PSLT032 (Figure 5B).

**ccdA<sub>ST</sub>B<sub>ST</sub> Transcriptional Regulation Is Important to Control the Polycistronic Operon**

To further analyze the role of ccdA<sub>ST</sub>B<sub>ST</sub> in the polycistronic operon transcriptional control we asked if placed at the beginning of the operon, CcdA<sub>ST</sub>-CcdB<sub>ST</sub> TA complexes could modulate transcriptional expression of the operon in a TA system “classic” manner. PSLT031 or rsdB, placed at the penultimate position of the polycistronic operon, is annotated as a putative resolvase that could be important in multimer resolution during pSLT plasmid replication (Krause and Guiney, 1991). Thus, we used rsdB as a reporter to monitor the operon transcriptional regulation exerted by ccdA<sub>ST</sub>B<sub>ST</sub>. We tagged the rsdB gene with a 3xFLAG epitope at the 3'-end, and measured its protein levels in strains carrying: (i) wild type pSLT, (ii) engineered pSLT lacking the whole ccdA<sub>ST</sub>B<sub>ST</sub>, and (iii) pSLT lacking the 300 bp containing the ccdA<sub>ST</sub>B<sub>ST</sub> promoter. RsdB levels significantly decreased when the ccdA<sub>ST</sub>B<sub>ST</sub> TA system was altered, thus indicating that ccdA<sub>ST</sub>B<sub>ST</sub> acts as transcriptional repressor for the polycistronic operon (Figures 6A,B). As described above for the ccdA<sub>ST</sub>B<sub>ST</sub> TA system, we tested if the polycistronic operon could also respond to conditional cooperativity. We expressed the non-toxic CcdB<sub>ST</sub> variant and measured RsdB levels. Complementary, we used as a negative control the production of the unrelated toxin VapC<sub>ST</sub>. When CcdB<sub>ST</sub> was provided in trans (Supplementary Figure 1), RsdB levels increased accordingly to conditional cooperativity (Figure 6C). Altogether, these data demonstrate that CcdA<sub>ST</sub>B<sub>ST</sub> TA complexes influence the transcription of the polycistronic operon.
**DISCUSSION**

In this report we describe a novel method to measure plasmid stability in bacteria. This procedure is based on the use of an *aph-parE* cassette in which a rhamnose-inducible promoter controls synthesis of ParE toxin. When the *aph-parE* cassette is inserted in the plasmid of interest and rhamnose is present in the medium as the only carbon source, ParE is synthesized and plasmid-containing cells are selectively eliminated. This methodology allows direct selection of plasmid-free segregants in a reproducible and highly sensitive manner. As it has been described previously for many low-copy-number plasmids, the pSLT virulence plasmid of *S. Typhimurium* possesses at least three main mechanisms to ensure its stable maintenance in the cell: (i) a copy number control of replication mediated by *repB* and *repC* replicons; (ii) the *parAB* partition system; and (iii) the TA systems ccdAB<sub>ST</sub> and vapBC<sub>ST</sub>. In our study, we did not considered the influence of the conjugation machinery because although *S. Typhimurium* SV5015 pSLT is mobilizable, it is not self-transmissible (Ahmer et al., 1999). Using our novel stability assay, we reevaluated the contribution of ParAB and CcdAB<sub>ST</sub> to pSLT plasmid stability as a proof of concept for the reliability of our methodology. In accordance with the literature, we show that the ParAB partition system stabilizes the pSLT plasmid very efficiently. Moreover, as described for other plasmids, the partition system appeared more important for pSLT stability than the vapBC<sub>ST</sub> or ccdAB<sub>ST</sub> TA systems (Sia et al., 1995; Sengupta and Austin, 2011; Hernández-Arriaga et al., 2014). Several studies have demonstrated a moderately stabilizing effect of TA systems. Two examples are the *ccdAB* TA module of the fertility factor F (Ogura and Hiraga, 1983) and the *kis-kid* (also called *parD*) TA locus of the R1 plasmid (Bravo et al., 1987). These systems increase the stability of their host plasmids around 10-fold compared to mini-derivate plasmids (Hernández-Arriaga et al., 2014).
et al., 2014). However, there are exceptions to this rule. For instance, the parDE module of RK2 has a more important role in the stabilization of this plasmid than other TA systems (Roberts et al., 1994; Easter et al., 1997). Interestingly, the mvpTA TA system of the virulence plasmid pWR100 in S. flexneri is the principal contributor to plasmid stability, more than the partition system (Sayeed et al., 2005). This differs from the stability contribution of its ortholog in S. Typhimurium, vapBC2ST. Of note, MvpAT and VapBC2ST show more than 96% amino acid sequence identity. However, it has also been described that diverse experimental variables, including temperature, growth media or the strain analyzed in the assay can alter plasmid stability (Easter et al., 1997; Sayeed et al., 2005). The toxin MvpT is a specific endonuclease that cleaves the initiator tRNA (Winther and Gerdes, 2011), and the mvpTA TA system has been shown to stabilize the virulence plasmid of S. flexneri by post-segregational killing (Sayeed et al., 2000). On the other hand, the plasmidic toxin VapC2ST and its chromosomal paralog VapCST of S. Typhimurium conserve 82% amino acid sequence identity (Lobato-Márquez et al., 2015). Moreover, similar to MvpT toxin, the chromosomal VapCST toxin possesses tRNA endonuclease activity (Winther and Gerdes, 2011). These evidences imply that VapBC2ST may mediate pSLT plasmid stability by post-segregational killing.

The other TA system of pSLT is ccdABST. In this work we demonstrate that this TA module shows classic characteristics of type II TA loci, such as autorepression and conditional cooperativity. Moreover, ccdABST is highly conserved to its ortholog present in the F plasmid: 90 and 83% amino acid identity to CcdA and CcdB, respectively. One important amino acid substitution is the tryptophan 99 to arginine in CcdBST (Bahassi et al., 1995). Using a pSLT plasmid derive encoding a CcdBST (R99W) variant we demonstrate that CcdBST toxicity is not necessary for the contribution of this TA module to plasmid stability. Intriguingly, ccdABST forms part of a polycistronic operon with four other downstream genes. Moreover, CcdAST-CcdBST TA complexes contribute to the regulation of the expression of this operon. This result is surprising given that few exceptions escape the general rule of TA operons organization. These exceptions include TA modules with a third gene acting as the transcriptional repressor of the system (Zielenkiewicz and Cegłowski, 2005; Hallez et al., 2010) and a single case in which a chaperone, co-transcribed with a TA operon, facilitates the folding of the antitoxin and, therefore, its activity (Bordes et al., 2011). Although, RsdB levels decreased upon deletion of either the promoter of ccdABST TA module or the whole TA locus, we still detected RsdB by western blot. These results indicate that PccdABST does control the transcription of the operon but it may exist at least another additional promoter regulating the operon.

Future work should address how the unprecedented TA genomic organization of this novel polycistronic operon including ccdABST and its transcriptional regulation influence pSLT stability. pSLT is evolutionary related to F plasmid, yet in F ccdAB does not constitute such a polycistronic operon. The study of this particular TA system could shed light on the evolution and adaptation of TA modules to its bacterial host.

MATERIALS AND METHODS

Bacterial Strains, Plasmids and Growth Conditions

*S. enterica* serovar Typhimurium SV5015 (a SL1344 His⁺ derivate strain Mariscotti and Garcia-del Portillo, 2009) was used as parental strain (S. Typhimurium SL1344 accession number: NC_016810.1). All strains and plasmids used in this study are listed in **Supplementary Table 1**. Bacteria were grown at 37°C with shaking at 150 rpm in Luria-Bertani (LB) medium. When necessary antibiotics were added at the following concentrations: kanamycin, 50 μg/ml; ampicillin, 50 μg/ml; cloramphenicol, 20 μg/ml.

A transcriptional fusion PccdABST-lacZ was designed to measure the transcriptional activity of PccdABST promoter. A 300 bp DNA sequence upstream of ccdAST containing the promoter of ccdAST (Tam and Kline, 1989; Madl et al., 2006) was PCR-amplified, digested with EcoRI-KpnI and ligated with the large EcoRI-KpnI fragment of plasmid pMP220 (Spaïnk et al., 1987). The resulting plasmid was confirmed by DNA sequencing.

Construction of *S. Typhimurium* Mutants

Oligonucleotide primers used in these procedures are listed in **Supplementary Table 2**. For disruption of pSLT plasmid maintenance modules, the deletion method described by Maisonneuve et al., was used (Maisonneuve et al., 2011). The strain used as control on stability assays was design inserting an aph-parE cassette in the spaV gene of pSLT. Disruption of this gene does not alter pSLT stability (Ahmer et al., 1999; Garcia-Quintanilla et al., 2006).

A similar protocol to that involving generation of deletion mutants was used to introduce the amino acid substitution R99W in CcdBST. Briefly, the aph-parE module was first introduced in ccdAST gene. Then the cassette was cleaned up with a PCR-amplified DNA fragment bearing the nucleotide change C→T in the position 73,232 of pSLT plasmid corresponding to the first nucleotide of the arginine 99 (R99) codon.

Construction of *S. Typhimurium* recombinant strain expressing tagged RsdB-3xFLAG was carried out as previously described (Uzzau et al., 2001). 3x-FLAG tagging was performed at the 3’-end of the PSLT031 gene.

All mutants were verified and confirmed by PCR.

Plasmid Stability Assays

Before starting stability assays, bacteria were grown in LB containing 50 μg/ml kanamycin. For plasmid stability assays all bacterial strains were grown in 10 ml LB medium (10:1 flask:medium volume ratio) without selection pressure for 16 h (~10 generations) at 37°C and 150 rpm. We did not observe alterations in the growth rate of the pSLT plasmid derivates lacking parAB, ccdABST or vapBC2ST compared to pSLT wild type plasmid. Aliquots of 1 ml of the culture were collected into 1.5 ml eppendorf tubes and bacteria were pelleted in a MiniSpin® Eppendorf centrifuge 1 min at 12,000 rpm at room temperature. Supernatants were discarded and bacterial pellets were washed twice with phosphate buffered saline (PBS) pH 7.4. This ensures proper elimination of LB medium traces that
otherwise could interfere with the growth in M9-rhamnose plates. Serial dilutions were done in PBS pH 7.4 and 100µl of the appropriate aliquots plated onto LB- or M9-rhamnose-agar plates. Typically a 1:10⁷ dilution was used to quantify total bacterial population in LB-agar plates, and dilutions in the range 1:1–10³ were used to determine the number of segregants in M9-rhamnose-agar plates. Plates were incubated for 24 h (LB-agar) or 48–72 h (M9-rhamnose-agar) at 37°C before counting of the colony forming units. Colony forming units grown in M9-rhamnose-agar were tested for their kanamycin resistance on antibiotic-containing LB plates. This is a sensitive assay that effectively eliminates plasmid-containing cells, thus allowing a direct selection of plasmid-free segregants.

β-Galactosidase Activity Measurements
Bacteria containing the plasmid with transcriptional fusion PcecAB-lacZ were grown to an optical density (OD₆₀₀) of 0.6 at 37°C and 150 rpm in LB. Then, β-galactosidase activity was measured as previously described (Miller, 1972).

For the conditional cooperativity experiments, bacteria containing pCcdB or pVapC plasmids (Lobato-Márquez et al., 2015) were grown in LB to an OD₆₀₀ of 0.3 at 37°C and 150 rpm in the presence of 50 µg/ml kanamycin. Inactive CcdBₜₜ or VapCₜₜ toxins were synthesized upon induction with 0.3 ℧ (w/v) L-arabinose. β-galactosidase activity was assessed as in the rest of strains after 1 h of induction. The chromosomally-encoded S. Typhimurium VapCₜₜ was used as a control to discard unspecific effects of protein expression in β-galactosidase measurements.

Reverse Transcriptase PCR (RT-PCR)
To determine the presence of a polycistronic operon controlled by ccdABₜₜ total RNA was extracted from wild type S. Typhimurium SV5015 (Mariscotti and Garcia-del Portillo, 2009) grown in LB at 37°C until OD₆₀₀ ~ 0.3. Volume corresponding to 1 absorbance unit at OD₆₀₀ was lysed in 100 µl lysis buffer (lysozyme 50 mg/ml, 0.3% SDS). Cells extracts were processed using RNasey minit kit (#74104, Quiagen). cDNA was constructed employing ThermoScript RT-PCR (Promega) using 0.5 µl cDNA was amplified by PCR (Pfu DNA polymerase, #M774B, Promega) using 0.5 µM of primers annealing with ccdBₜₜ, SL1344_P1_0078 (PSLT032), SL1344_P1_0077 (PSLT030), rsdB, and SL1344_P1_0075 (PSLT032) (Supplementary Table 2). PCR amplification was carried out in duplicate using cDNA and RNA as a negative control. PCR products were visualized in 0.8% (w/v) agarose gels stained with ethidium bromide. Band densitometry was determined using Quantity One v.4.6.3 software (Bio-Rad, Berkeley, CA) as previously described (Molina-Garcia and Giraldo, 2014; Lopez-Villarejo et al., 2015).

Detection of RsdB Levels by Western Blotting and Protein Levels Quantification
Bacterial cultures were grown 16 h at 37°C and 150 rpm. Same amount of bacterial cells were collected (volumes were adjusted based on OD₆₀₀), centrifuged (1 min at 12,000 rpm) and resuspend in Laemmli buffer. (Laemmli, 1970). Bacterial protein extracts were resolved in SDS-PAGE using 15% polyacrylamide gels and processed for Western blot assays. Levels of the S. Typhimurium DnaK protein were used as loading control. RsdB or DnaK detection were performed using anti-FLAG antibody (#F3165, Sigma-Aldrich) 1:2000 (2 h) or anti-DNAP 1:10,000 (1 h), respectively, dissolved in TBS-TWEEN buffer (137 M M NaCl, 0.1% m/v Tween 20 and 20 mM Tris-HCl pH 7.5) containing 3% non-fat milk. RsdB expression levels were calculated by western blotting experiments using extracts prepared from at least four independent experiments and pSLT plasmid variants expressing 3xFLAG-tagged RsdB. Mean data were taken as the relative expression levels of the proteins. Band densitometry was determined using Quantity One v.4.6.3 software (Bio-Rad, Berkeley, CA) as previously described.

Statistical Analyses
Statistical significance was analyzed with GraphPad Prism v7 software (GraphPad Inc., La Jolla, CA) using one-way analysis of variance (ANOVA) with Dunnett’s multiple comparison post-test for Figures 2, 4, 6B. In the comparison test used for Figure 3B a Student’s T-test analysis was used. A P ≤ 0.05 was considered significant. Data are presented as mean ± standard deviation of the mean (SEM).

AUTHOR CONTRIBUTIONS
DL and RD: Conceived and designed the experiments; DL, LM, and IM: Performed the experiments; DL, LM, IM, FG, and RD: Analyzed the data; DL: Wrote the paper.

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SUPPLEMENTARY MATERIAL
The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fmolb.2016.00066

Supplementary Figure 1 | Control assays showing proper protein synthesis of CcdBₜₜ and VapCₜₜ in the experiments involving conditional cooperativity regulation of rsdB in Figure 6C (main text). Equal amounts of total protein extracts were loaded in each lane. Bacteria were grown in LB medium to OD₆₀₀ of 0.3, time at which CcdBₜₜ or VapCₜₜ expression was induced with 0.3% arabinose.

Supplementary Table 1 | Bacterial strains and plasmids used in this study.
Supplementary Table 2 | Oligonucleotides used in this study.
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The reviewer GDS declared a shared affiliation, though no other collaboration, with several of the authors IM, RD to the handling Editor, who ensured that the process nevertheless met the standards of a fair and objective review.