 doi:10.1111/mmi.12610

The *Salmonella enterica* serovar Typhi *ltrR-ompR-ompC-ompF* genes are involved in resistance to the bile salt sodium deoxycholate and in bacterial transformation

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Summary

A characterization of the LtrR regulator, an *S.* Typhi protein belonging to the LysR family is presented. Proteomics, outer membrane protein profiles and transcriptional analyses demonstrated that LtrR is required for the synthesis of OmpR, OmpC and OmpF. DNA–protein interaction analysis showed that LtrR binds to the regulatory region of *ompR* and then OmpR interacts with the *ompC* and *ompF* promoters inducing porin synthesis. LtrR-dependent and independent *ompR* promoters were identified, and both promoters are involved in the synthesis of OmpR for OmpC and OmpF production. To define the functional role of the *ltrR-ompR-ompC-ompF* genetic network, mutants in each gene were obtained. We found that *ltrR*, *ompR*, *ompC* and *ompF* were involved in the control of bacterial transformation, while the two regulators and *ompC* are necessary for the optimal growth of *S.* Typhi in the presence of one of the major bile salts found in the gut, sodium deoxycholate. The data presented establish the pivotal role of LtrR in the regulatory network of porin synthesis and reveal new genetic strategies of survival and cellular adaptation to the environment used by *Salmonella*.

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Introduction

Salmonella enterica serovar Typhi is a Gram-negative bacterium, facultative, intracellular pathogen that causes typhoid fever in humans. This pathogen contains multiple families of transcriptional regulators including those of the LysR family. The LysR-type transcriptional regulators (LTTRs) are the largest family of transcriptional factors in prokaryotes (Pareja *et al.*, 2006). Currently, nearly four thousand members of this family have been identified in the genomes of proteobacteria, archaea and Gram-positive organisms. The structural organization of these proteins consists of an N-terminal DNA-binding helix–turn–helix motif and a C-terminal co-inducer-binding domain. In general, the LysR-type DNA-binding proteins can positively or negatively regulate target gene expression and repress their own transcription (Schell, 1993; Maddocks and Oyston, 2008). In *Salmonella*, LTTRs are involved in multiple biological processes including metabolism (Borum and Monty, 1976; Hansen *et al.*, 2006; Hernández-Lucas *et al.*, 2008; Kim *et al.*, 2008; Lewis *et al.*, 2009; Baños *et al.*, 2011; Turnbull *et al.*, 2012), porin synthesis (Fernández-Mora *et al.*, 2004; De la Cruz *et al.*, 2007; Hernández-Lucas *et al.*, 2008), detoxification (Hernández-Lucas *et al.*, 2008; Gallego-Hernández *et al.*, 2012), regulation of CRISPR/Cas system (Hernández-Lucas *et al.*, 2008; Medina-Aparicio *et al.*, 2011), virulence (Coynault *et al.*, 1992; Tenor *et al.*, 2004; Lawley *et al.*, 2006; Rodríguez-Morales *et al.*, 2006; Jakomin *et al.*, 2008; Lahiri *et al.*, 2008; Dillon *et al.*, 2012) and resistance to stress conditions (Christman *et al.*, 1989; Fang *et al.*, 2000; Lahiri *et al.*, 2008; Jennings *et al.*, 2011). Although these transcriptional factors have a global role in pathogenic bacteria, in *S.* Typhi only the function of the LeuO regulator has been explored (Fernández-Mora *et al.*, 2004; Hernández-Lucas *et al.*, 2008; Medina-Aparicio *et al.*, 2011; Gallego-Hernández *et al.*, 2012) and the performance of a large number of these proteins remain to be

established. Hence, the putative LysR-type protein STY0036 was selected for study since it is only found in the *Salmonella* genus and genome-wide screenings suggest a role of its orthologous (STM0030) in the pathogenicity of *Salmonella* Typhimurium (Eriksson *et al*., 2003; Lawley *et al*., 2006).

Because the synthesis of the global regulator OmpR as well as of the major porins OmpC and OmpF were found to be dependent on STY0036, we designated it as LtrR for LysR-type OmpR regulator. DNA–protein interaction analyses showed that LtrR directly regulates *ompR*, inducing porin synthesis. 5′ RACE experiments and gene fusions showed the presence of an LtrR-dependent (*ompR*-P1) and LtrR-independent (*ompR*-P2) *ompR* promoters. Chromosomal substitutions demonstrated that both *ompR* promoters were involved in the synthesis of OmpR for production of OmpC and OmpF. Mutant analyses and complementation experiments revealed that *ltrR*, *ompR*, *ompC* and *ompF* have a role in bacterial transformation, a finding of interest since this phenomenon has not been widely studied in *Salmonella*. An additional phenotype for the *ltrR*, *ompR* and *ompC* genes was found, as they were required for optimal growth of *S*. Typhi in the presence of one of the major bile salts found in the gut, sodium deoxycholate. This is particularly interesting since this bacterium is able to survive and cause chronic infection in the bile storage organ, the gallbladder, and an association between *S*. Typhi and gallbladder cancer has been reported (Kumar *et al*., 2006; Tewari *et al*., 2010). Furthermore, genetic studies relating to the survival of this pathogen in the gallbladder and in the presence of bile is limited (van Velkinburgh and Gunn, 1999; Langridge *et al*., 2009; Lahiri *et al*., 2011). In conclusion, the data presented here establish the role of the LtrR protein in the regulation of porin synthesis, and provide evidence for a role of *ltrR*, *ompR*, *ompC* and *ompF* in the genetic strategies that *Salmonella* use to survive and adapt to its environment.

Results

The LtrR regulator is required for the synthesis of OmpR, OmpC and OmpF in S. *Typhi*

LysR-type proteins regulate a diverse set of genes, including those involved in virulence, metabolism, quorum sensing and motility (Schell, 1993). Expression analysis as well as microarray-based negative selection studies suggest that LtrR, a regulator found only in the *Salmonella* genus, is involved in the pathogenicity of *S.* Typhimurium (Eriksson *et al*., 2003; Lawley *et al*., 2006). In an effort to elucidate the role of LtrR in *S*. Typhi, a transcriptional fusion of the *ltrR* regulatory region was constructed and its expression evaluated in a rich medium A (MA) (Kawaji *et al*., 1979) and in N-minimal medium (N-MM). The latter is deficient in magnesium and phosphate, and several *Salmonella* Pathogenicity Island-2 genes are induced in this condition (Deiwick *et al*., 1999). *ltrR* was expressed in both media, although higher activity was found in N-MM than in MA (Fig. 1A) suggesting that this regulator may operate in specific host environments. To identify LtrR-regulated proteins, a proteomic analysis was performed. For these experiments, the *S.* Typhi wild-type strain IMSS-1 as well as an isogenic IMSS-1 Δ*ltrR* were grown in N-MM to OD_{595} of 0.6, and cellular proteins were separated by two-dimensional gel electrophoresis (2-DGE). About 600 proteins were resolved in each gel. Comparison of the proteomic profiles showed that one protein with a M_r of 25–30 kDa and a pI of 6.5–7.5 (spot 1), as well as a conglomerate of proteins with M_rs of 39–41 kDa and pIs of 4–5 (spot 2) were downregulated in the Δ*ltrR* strain (Fig. 1B). To identify these LtrR-regulated proteins, spots were excised, trypsin-digested and analysed by MALDI-TOF MS (Washburn *et al*., 2001). The results showed that the LtrR-dependent proteins were OmpR (spot 1), OmpC and OmpF (spot 2). The sequence coverage of the LtrR-regulated proteins were 48% for OmpR, 37% for OmpC, and 34% for OmpF, and the Mascot database search algorithm revealed that the proteins identified were identical to the corresponding proteins of several *S.* Typhi strains.

To further confirm the proteomic data, outer membrane protein profiles of the wild-type IMSS-1 and IMSS-1 Δ*ltrR* grown in N-MM to OD_{595} of 0.6 were determined, and this showed the presence of OmpC, OmpF and OmpA in the wild-type strain, while downregulation of OmpC and OmpF in the absence of *ltrR* was evident (Fig. 1C).

To determine that the lack of LtrR affects porin synthesis, we complemented the Δ*ltrR* with the pWSK29 (Wang and Kushner, 1991) plasmid containing a wild-type fragment of 1510 bp corresponding to *ltrR* and flanking sequences. As shown in Fig. 1C, the outer membrane profile of the complemented Δ*ltrR*/pWSK*ltrR* strain showed the reestablishment of the presence of the OmpF and OmpC porins, confirming the role of *ltrR* in their expression. Therefore, the LtrR protein is a positive regulator involved in the regulation of porin synthesis in *S*. Typhi.

Transcriptional analysis of ompR, ompC and ompF in S. *Typhi IMSS-1 and in the ltrR-deficient strain*

The proteomic and the outer membrane profiles showed that the LtrR protein is involved in the synthesis of the major porins. To define its role in the transcriptional regulation of *ompR*, *ompC* and *ompF*, transcriptional CAT fusions of the corresponding regulatory regions were obtained. Plasmids pKK8/*ompR*-383+169, pKK9/*ompC*-772+27 and pKK8/*ompF*-782+184 (Table S1) were transformed independently into *S.* Typhi IMSS-1 and *S.* Typhi

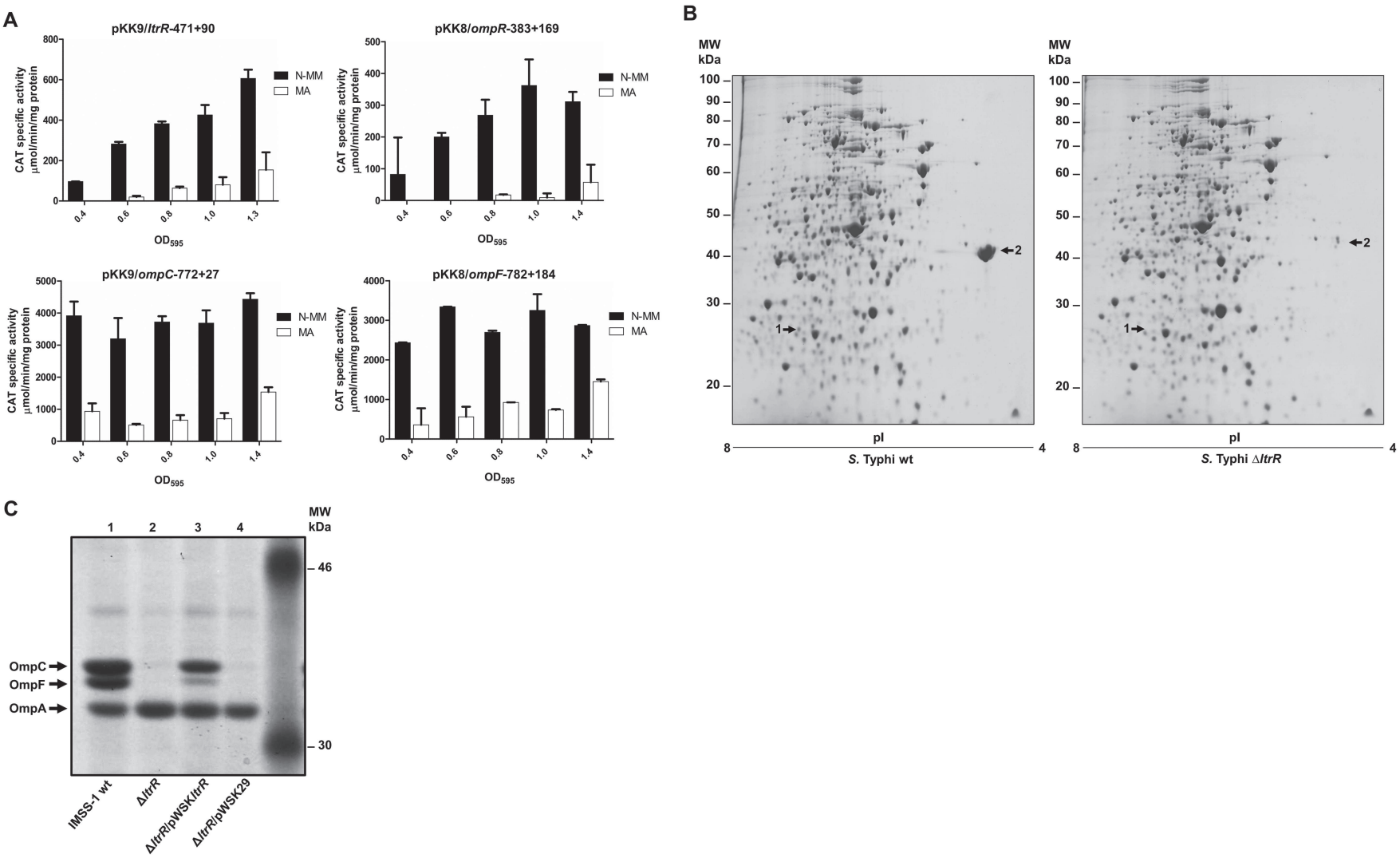


Fig. 1. Transcriptional expression of *ltrR, ompR, ompC, ompF* and the requirement of LtrR for porin synthesis.
A. Transcriptional profile of *S.* Typhi IMSS-1 harbouring plasmid pKK9/*ltrR*-471+90, pKK8/*ompR*-383+169, pKK9/*ompC*-772+27 or pKK8/*ompF*-782+184 grown in MA and N-MM. CAT-specific activities were measured at an OD_{595} of 0.4, 0.6, 0.8, 1.0 and 1.3 or 1.4. The values are the means ± standard deviations for two independent experiments performed in duplicate.
B. Proteome profiles of *S.* Typhi total protein extracts. *S.* Typhi wild type and *S.* Typhi Δ*ltrR* were grown in N-MM to an OD_{595} of 0.6. Spots 1 and 2 of the *S.* Typhi IMSS-1 wild-type strain were excised and identified by MALDI-TOF MS. Spot 1 corresponds to OmpR and spot 2 to OmpC and OmpF. Spots 1 and 2 were sequenced twice from independent 2-DGE gels. In the *S.* Typhi Δ*ltrR*, a diminished quantity of spot 1 and 2 is evident. At least three experiments were carried out, and representative 2-DGE gels are shown.
C. Electrophoretic pattern of Coomassie brilliant blue-stained outer membrane protein preparations, separated by 0.1% SDS-15% PAGE, from: *S.* Typhi IMSS-1 wild type (lane 1), Δ*ltrR* (lane 2), Δ*ltrR*/pWSK*ltrR* (lane 3), Δ*ltrR*/pWSK29 (lane 4), grown in N-MM to OD_{595} of 0.6. The major OMPs, OmpC, OmpF, and OmpA are indicated with an arrow.

Table 1. *ompR*, *ompC* and *ompF* expression profiles in *S.* Typhi IMSS-1 and in the isogenic *ltrR* mutant strain.

Plasmid	CAT-specific activity [μmol min^{-1} (mg protein)$^{-1}$]	
	S. Typhi IMSS-1	*S.* Typhi IMSS-1 Δ*ltrR*
pKK8/*ompR*-383+169	315 ± 12	145 ± 30
pKK9/*ompC*-772+27	4100 ± 276	< dl
pKK8/*ompF*-782+184	3180 ± 420	378 ± 25

Plasmids pKK8/*ompR*-383+169, pKK9/*ompC*-772+27 and pKK8/*ompF*-782+184 were transformed independently into *S.* Typhi IMSS-1 and *S.* Typhi IMSS-1 Δ*ltrR*. The CAT-specific activity of each strain was measured using samples grown in N-MM and collected at OD_{595} of 0.6. The standard deviation indicates the means of three independent experiments performed in duplicate. < dl (< detection limit) represent values between 0 and 5 CAT units.

IMSS-1 Δ*ltrR* and their transcriptional activity was evaluated. The CAT-specific activities at OD_{595} 0.6 in N-MM showed that *ompC* and *ompF* expression was reduced 100% and 88%, respectively, in the mutant (Table 1). For *ompR* a reduction of only 54% was observed in the Δ*ltrR* mutant (Table 1), suggesting the presence of both an LtrR-dependent and LtrR-independent *ompR* promoters (see below). The results obtained with the transcriptional fusions supported the proteomic and outer membrane profile data, showing the positive role of LtrR in the regulatory network of porin synthesis.

Since *ltrR* was more highly expressed in N-MM than in MA medium and this protein is involved in the transcriptional expression of *ompR*, *ompC* and *ompF,* we evaluated the transcriptional expression of these LtrR-dependent genes in both media. We found that the *ompR*, *ompC* and *ompF* regulatory regions were expressed at a higher in N-MM than in MA medium (Fig. 1A). Thus, the data demonstrated that this set of genes operate in N-MM.

Determination of the ltrR-ompR-ompC-ompF cascade for porin synthesis in S. *Typhi*

To evaluate whether LtrR interacts with *ompR*, *ompC* and *ompF*, electrophoretic mobility shift assays (EMSAs) were implemented with the purified LtrR protein and the *ompR*, *ompC* and *ompF* regulatory regions. LtrR was able to interact with a 5′ intergenic *ompR* fragment (383 bp) at 0.2 μM. However, at these concentrations it was unable to bind with the *ompC* (711 bp) and *ompF* (590 bp) promoter regions (Fig. 2A). This indicates that LtrR has a high affinity for the *ompR* regulatory region but not for those of *ompC* or *ompF*. EMSAs using the phosphorylated OmpR protein and the 5′ intergenic sequences of *ompC* and *ompF* showed that OmpR was able to interact with both regions at 0.4, 0.6 and 0.8 μM (Fig. 2B). These results support the model where the LtrR protein directly induces *ompR* expression, upon which OmpR binds to the regulatory region of *ompC* and *ompF* to induce their synthesis in *S.* Typhi IMSS-1.

Identification of the ompR sequences that mediate LtrR-dependent expression in S. *Typhi*

To define the *ompR* sequences involved in the LtrR-dependent expression, 5′-RACE experiments in N-MM at OD_{595} 0.6 were performed. In the wild-type *S.* Typhi IMSS-1 strain, two transcriptional initiation sites at 34 and 165 bp

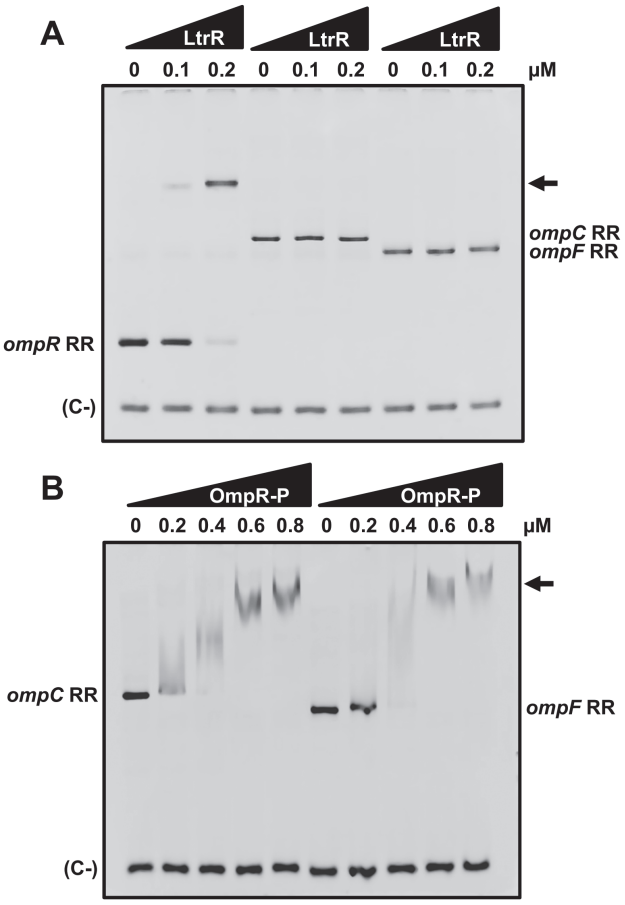


Fig. 2. DNA–protein interactions of the LtrR and OmpR proteins with their target genes.
A. Electrophoretic mobility shift assays (EMSAs) were performed with purified LtrR 6x-His and the 5' intergenic regulatory regions (RR) of *ompR* (383 bp), *ompC* (711 bp) and *ompF* (590 bp).
B. EMSAs of the phosphorylated OmpR 6x-His in the presence of the 5′ intergenic regions of *ompC* (711 bp) and *ompF* (590 bp) are presented. Increasing concentrations of purified LtrR 6x-His or phosphorylated OmpR 6x-His proteins were incubated with the corresponding DNA fragments. The EMSA experiments were resolved in 6% polyacrylamide gels, and stained with ethidium bromide. The −383 to −133 bp intergenic region of *ompR* that does not interact with LtrR or OmpR was used as a negative control (C–) for the EMSA. Arrows indicate DNA–protein complex.

upstream of the *ompR* ATG codon were found (Fig. 3A and B). To validate the presence of the –165 bp *ompR*-P2 promoter, fusions containing sequences from –383 to –133 and –383 to –173 were constructed (Fig. 3C) and their transcriptional expression was tested in the wild type and in the *ltrR* mutant. Activity values of 210 and 211 with plasmid pKK8/*ompR*-383-133 were detected in *S.* Typhi IMSS-1 and *S.* Typhi IMSS-1 Δ*ltrR*, respectively, while pKK8/*ompR*-383-173 showed null expression in both strains, indicating that there is an *ompR* promoter between –383 and –133 whose activity is independent of LtrR (*ompR*-P2, Fig. 3D). These data support the result obtained with the complete *ompR* regulatory region pKK8/*ompR*-383+169, where transcriptional expression independent of *ltrR* was also observed (Table 1, Fig. 3D).

Regarding the presence of an LtrR-dependent *ompR* promoter (*ompR*-P1) whose mRNA initiation site was located 34 bp upstream of the *ompR* ATG codon, a fusion encompassing the sequence from –134 to –1 (pKK8/*ompR*-134-1) was constructed (Fig. 3C) and its transcriptional activity was tested in the wild type and in the *ltrR* mutant. Values of 140 were observed in the wild type while in the *ltrR*-deficient strain it had no activity, demonstrating that the LtrR-dependent *ompR* promoter was located in the –134 to –1 region (Fig. 3D). Hence individual nucleotide substitutions were made in the putative –10 (TAAGAAT → CCCCCCC; pKK8/*ompR*-134-1/A) and –35 (TGGTGA → CCCCCC; pKK8/*ompR*-134-1/B) sequences of the transcriptional start site located at 34 bp upstream of the *ompR* ATG codon (Fig. 3A). The transcriptional activity of these constructs in the wild type and in the *ltrR* mutant were null (Fig. 3D), thus providing a more precise localization of the LtrR-dependent *ompR* promoter.

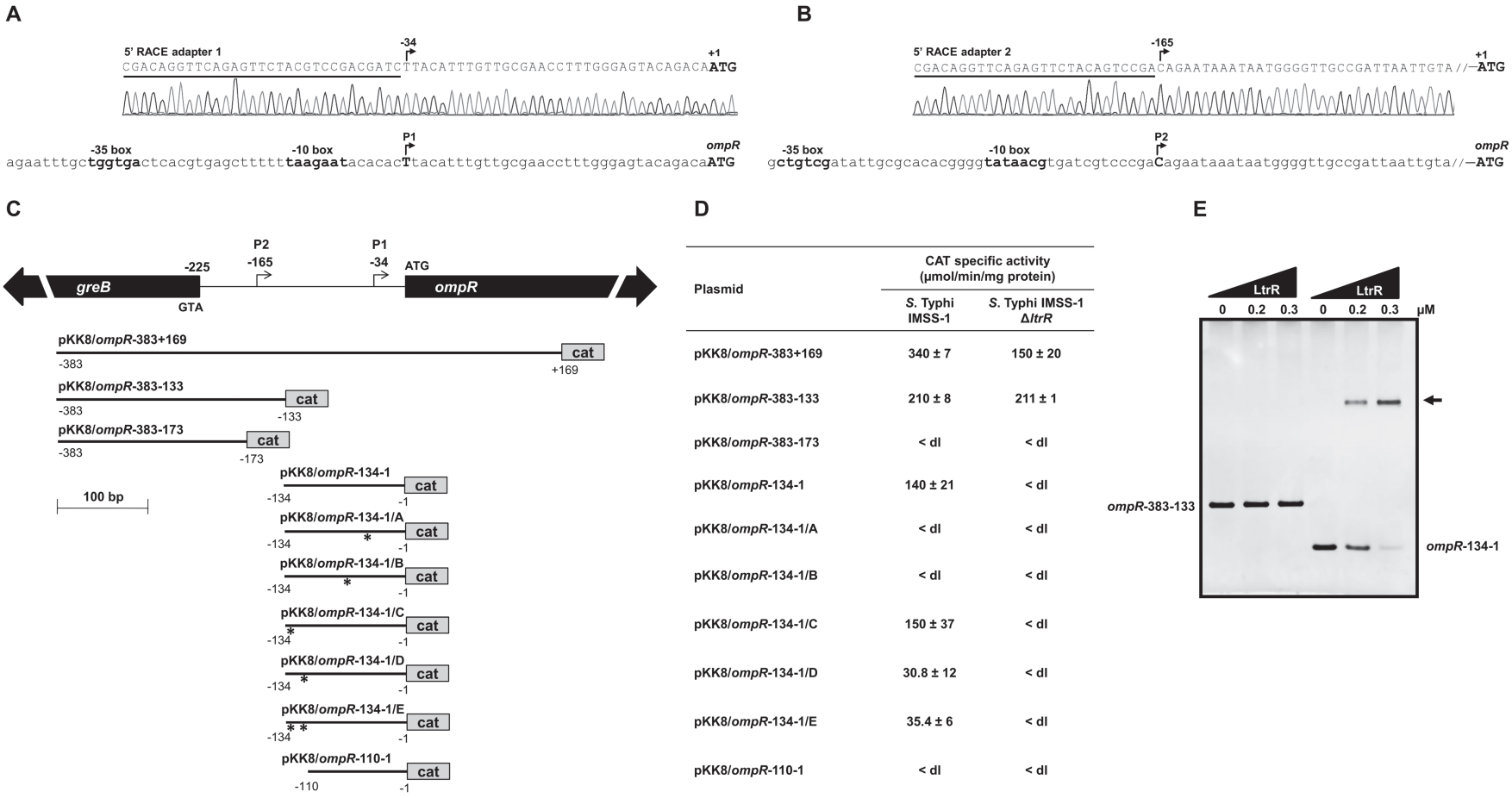
EMSAs were performed to establish whether LtrR binds to the 5′ intergenic –134 to –1 *ompR* region. LtrR was found to interact with this DNA fragment at 0.2 and 0.3 μM, and was unable to interact with *ompR* sequences –383 to –133 at the same concentrations (Fig. 3E). Therefore, the –134 to –1 region of the *ompR*-P1 promoter contains the LtrR-interacting sequences. To identify the specific region involved in *ompR* activation mediated by LtrR, an additional fusion encompassing regions from –1 to –110 was constructed (Fig. 3C). The lack of expression of this fusion in the wild-type strain and in the *ltrR* mutant indicates that nucleotides –134 to –110 are involved in LtrR-mediated induction of *ompR* (Fig. 3D).

The –134 to –110 region contains the palindromic sequence ATATTTAA-N_7-TTAAATAT at –133 to –111 bp upstream of the OmpR translational start site: this fragment contains the LysR-type consensus sequence T-N_{11}-A (Maddocks and Oyston, 2008). Specific substitutions at nucleotides –130 to –128 (TTT → CCC, pKK8/*ompR*-134-1/C), –116 to –114 (AAA → CCC, pKK8/*ompR*-134-1/D) and a double –130 to –128/–116 to –114 (TTT → CCC/AAA → CCC, pKK8/*ompR*-134-1/E) substitution were constructed (Fig. 3C). By their expression profiles, we observed that nucleotides –130 to –128 were not determinant in *ompR* expression mediated by LtrR, since the same activity was obtained with fusions pKK8/*ompR*-134-1 and pKK8/*ompR*-134-1/C (Fig. 3D). However, the AAA nucleotides, located at 116 to 114 bp upstream of the translational start-site of the OmpR regulatory protein, have a fundamental role in *ompR* activation mediated by LtrR, since a clear decrease (80%) of transcriptional expression was observed with substitution pKK8/*ompR*-134-1/D (Fig. 3D), and also with the double substitution pKK8/*ompR*-134-1/E (Fig. 3D). All these fusions were evaluated in the wild-type *S.* Typhi strain as well as in the Δ*ltrR*. These data are consistent with the positive role of LtrR in the expression of *ompR*, encoding the master regulator for porin synthesis.

LtrR-dependent and -independent ompR promoters are involved in OmpR synthesis for OmpC and OmpF production

5′ RACE experiments as well as transcriptional fusions support the presence of two *ompR* promoters: an LtrR-dependent *ompR* promoter (*ompR*-P1) and an LtrR-independent *ompR* promoter (*ompR*-P2). To determine the contribution of these *ompR* promoters in porin production, individual strains harbouring chromosomal substitutions in the –10 region of each promoter, as well as a double promoter mutant were obtained (Table S1). These strains, as well as the wild type, Δ*ltrR* and Δ*ompR* mutants were transformed with fusions containing the complete regulatory region of *ompC* (–772+27) and *ompF* (–782+184). The transcriptional activity data in the Δ*ltrR*, and Δ*ompR* mutants as well as in the individual substitutions of each *ompR* promoter showed similar residual *ompC* activity (Fig. 4A), indicating that the LtrR-dependent *ompR* promoter as well as the LtrR-independent *ompR* promoter are necessary for *ompC* induction. Null *ompC* expression was observed in the double *ompR* promoter mutant as expected. To support this result, porin electrophoretic profiles were performed and the results show the absence of OmpC in the Δ*ompR* and in each mutant of the *ompR* promoters, as well as in the double *ompR* promoter mutant (Fig. 4B). Therefore, the transcriptional data and the porin profiles support the notion that the two *ompR* promoters are required in OmpR synthesis for OmpC production.

In the case of *ompF*, the expression results in the Δ*ltrR* and Δ*ompR* show CAT activity values around 418 (Fig. 4A); therefore, this gene showed OmpR-independent activity. A higher value was observed in each of the –10 substitutions of the *ompR* promoters and, as expected, the double promoter mutant displayed similar *ompF* induction as the Δ*ompR* (Fig. 4A). To further support these data,



Plasmid	CAT specific activity (μmol/min/mg protein)	
	S. Typhi IMSS-1	*S.* Typhi IMSS-1 Δ*ltrR*
pKK8/*ompR*-383+169	340 ± 7	150 ± 20
pKK8/*ompR*-383-133	210 ± 8	211 ± 1
pKK8/*ompR*-383-173	< dl	< dl
pKK8/*ompR*-134-1	140 ± 21	< dl
pKK8/*ompR*-134-1/A	< dl	< dl
pKK8/*ompR*-134-1/B	< dl	< dl
pKK8/*ompR*-134-1/C	150 ± 37	< dl
pKK8/*ompR*-134-1/D	30.8 ± 12	< dl
pKK8/*ompR*-134-1/E	35.4 ± 6	< dl
pKK8/*ompR*-110-1	< dl	< dl

Fig. 3. Genetic organization of the *ompR* regulatory region and determination of the LtrR-dependent *ompR* promoter sequences in *S.* Typhi IMSS-1.

A and B. Mapping of the *ompR* transcriptional start sites. 5'-RACE experiment were performed using total RNA from *S.* Typhi wild-type strain IMSS-1 grown in N-MM to an OD_{595} of 0.6. The nucleotide immediately after the adapter 1 or 2 (underlined) corresponds to the transcriptional start site (bent arrow), the LtrR-dependent *ompR* transcriptional start site (*ompR*-P1) was located at 34 bp upstream of the *ompR* ATG codon (A) and an LtrR-independent *ompR* transcriptional initiation site (*ompR*-P2) was identified at 165 bp upstream of the translational *ompR* start site (B).

C. The *ompR* 5' intergenic regulatory region is flanked by the *greB* gene and contains 225 bp. The bent arrows represent the transcriptional start sites located 34 and 165 bp upstream of the *ompR* translational start codon. Below the diagram of the *ompR* regulatory region, CAT transcriptional fusions of different length are shown. The fusions were named (bold) according to the translational initiation site; the numbers below each fusion represent the distance upstream and downstream of the ATG start site of *ompR*. Site-directed mutagenesis in the LtrR-dependent *ompR* promoter −10 (pKK8/*ompR*-134-1/A) and −35 (pKK8/*ompR*-134-1/B) sequences and substitutions in nucleotides $_{-133}$ATATTTAA-N_7-TTAAATAT$_{-111}$: TTT→CCC (pKK8/*ompR*-134-1/C); AAA→CCC (pKK8/*ompR*-134-1/D) and TTT→CCC, AAA→CCC (pKK8/*ompR*-134-1/E) are denoted by an asterisk in each fusion.

D. Determination of the LtrR-dependent *ompR* promoter sequences by transcriptional gene fusions. The columns represent the fusions and their expression in wild-type *S.* Typhi IMSS-1 and the *ltrR* mutant strain. CAT-specific activities were measured in N-MM at an OD_{595} of 0.6. The values are the means ± standard deviations for at least three independent experiments performed in duplicate, < dl (< detection limit) represent values between 0 and 5 CAT units.

E. LtrR interacts with the *ompR* regulatory region. EMSA was performed using increasing concentrations of the LtrR purified protein and the fragments of the regulatory region of *ompR*, −383 to −133 and −134 to −1. DNA–protein complex were only visualized with the −134 to −1 *ompR* fragment that contains the LtrR-dependent sequences. Arrows indicate DNA–protein complex.