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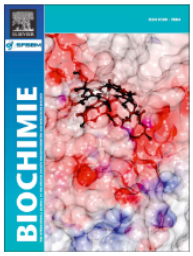

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Abstract

Bacterial survive and respond to adverse changes in the environment by regulating gene transcription through two-component regulatory systems. In *Salmonella* Typhimurium the STM1485 gene expression is induced under low pH (4.5) during

replication inside the epithelial host cell, but it is not involved in sensing or resisting to this condition. Since the RcsCDB system is activated under acidic conditions, we investigated whether this system is able to modulate STM1485 expression. We demonstrated that acid-induced activation of the RcsB represses STM1485 transcription by directly binding to the promoter. Under the same condition, the RstA regulator activates the expression of this gene. Physiologically, we observed that RcsB-dependent repression is required for the survival of bacteria when they are exposed to pancreatic fluids. We hypothesized that STM1485 plays an important role in *Salmonella* adaptation to pH changes, during transition in the gastrointestinal tract. We suggest that bacteria surviving the gastrointestinal environment invade the epithelial cells, where they can remain in vacuoles. In this new environment, acidity and magnesium starvation activate the expression of the RstA regulator in a PhoPQ-dependent manner, which in turn induces STM1485 expression. These levels of STM1485 allow increased bacterial replication within vacuoles to continue the course of infection.

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replication inside the epithelial host cell, but it is not involved in sensing or resisting to this condition. Since the RcsCDB system is activated under acidic conditions, we investigated whether this system is able to modulate STM1485 expression. We demonstrated that acid-induced activation of the RcsB represses STM1485 transcription by directly binding to the promoter. Under the same condition, the RstA regulator activates the expression of this gene. Physiologically, we observed that RcsB-dependent repression is required for the survival of bacteria when they are exposed to pancreatic fluids. We hypothesized that STM1485 plays an important role in *Salmonella* adaptation to pH changes, during transition in the gastrointestinal tract. We suggest that bacteria surviving the gastrointestinal environment invade the epithelial cells, where they can remain in vacuoles. In this new environment, acidity and magnesium starvation activate the expression of the RstA regulator in a PhoPQ-dependent manner, which in turn induces STM1485 expression. These levels of STM1485 allow increased bacterial replication within vacuoles to continue the course of infection.

Highlights

In *Salmonella* Typhimurium, STM1485 gene is negatively modulated by RcsB regulator.

The STM1485 gene expression is induced by RstA response regulator.

Under acidity environments, RstA and RcsB compete to control STM1485 gene expression.

The expression of STM1485 is directly repressed by RcsB into the host digestive tract.

Keywords

RcsCDB, RstAB, STM1485, *Salmonella*

1. Introduction

Salmonella spp. is one of the most frequent intracellular pathogens responsible for food-borne diseases in humans, causing several clinical cases ranging from self-limiting gastroenteritis to systemic typhoid fever. *Salmonella enterica* serovar Typhimurium (*S.* Typhimurium) is a human pathogen that can survive outside the host through biofilm formation or inside eukaryotic cells by expression of specific genes that allow bacteria to persist and replicate in extreme conditions such as acidic pH, anaerobiosis or limited carbon sources [1, 2]. Bacteria can respond to these adverse conditions, modulating the expression of those genes which products are required for environmental adaptation via the so-called two-component systems (TCS) [1].

The RcsC/RcsD/RcsB (RcsCDB) system is an unusual TCS because it consists of three proteins: the RcsC sensor that resembles a hybrid sensor domain, the RcsB response regulator, and the RcsD protein, which has been postulated as an intermediary in phosphate transference from RcsC to RcsB [3, 4] but, according to our results, it can also act as a sensor [5, 6, 7]. This system is able to control a wide variety of cellular functions such as *Salmonella* virulence, among others [8, 9, 10, 11, 12, 13, 14, 15, 16]. Although numerous factors that induce the RcsCDB system have been reported, the signal leading to this activation remains unknown. In spite of this, it has been shown that most of these factors affect the bacterial cell envelope [17, 18, 19, 20]. It was reported in *Escherichia coli* (*E. coli*) and *S.* Typhimurium, that low pH activates the RcsCDB system, conferring an acid resistance phenotype [21, 22].

In *S.* Typhimurium virulence, acid resistance mechanisms are crucial for bacteria survival to gastric acidity and the low pH in the intestinal and phagolysosomal environments [1, 23, 24]. In this sense, the RcsCDB system, with the concerted action of other regulatory factors, is essential to define the survival strategy and the bacterial potential to resist low pH [21]. Castanie-Cornet *et al.* (2007) reported that basal activity of RcsB and GadE regulators are absolutely necessary to activate *gadAB* expression

which results in the glutamate-dependent acid resistance. According to this, we investigated if other RcsB-modulated genes were involved in the acid resistance phenotype.

In *E. coli* it has been reported that the *asr* gene, encoding an acid-inducible protein, is required to acid tolerance and to colonize the intestine [25, 26]. It was demonstrated that expression of this gene is induced at low pH (<5.0) and low Mg^{2+} by the RstA response regulator, and under phosphate starvation by the PhoBR system [27, 28]. This *asr* gene is not present in *Salmonella*, but the STM1485 gene has been described as a homolog displaying 77% similarity at the nucleotide level and a lower identity in the amino acid sequences (~68%) [28]. It has been reported that the STM1485 gene is involved in *Salmonella* virulence, enabling intracellular replication within the host cell [29, 32]. However, little is known about the modulation of its expression. Accordingly, we investigated if RcsB is able to control STM1485 expression when the bacterium is exposed to low pH.

In this work, we demonstrated that the RcsCDB system negatively controls STM1485 gene transcription and exerts its effect by directly binding to a conserved sequence located in its promoter. In this context, we observed that the RstA regulator is also able to modulate STM1485 expression but in a positive manner, as it does on its homologous gene from *E. coli*, *asr*. Taken together, our results highlight the importance of TCS networks in the ability of *Salmonella* to trigger a rapid response after sensing environmental cues during host infection.

2. Materials and Methods

2.1 *Bacterial strains, growth conditions and molecular genetics techniques*

The bacterial strains used in this work where derived from the *S.* Typhimurium 14028s strain, and are detailed in Table 1, together with the plasmids. After construction

and sequencing analysis, the STM1485::*lacZY* transcriptional fusion was transferred to different background by Phage P22-mediated transductions as described [44, 55]. Bacteria were cultured in Luria-Bertani (LB) [45] or in N-minimal medium (0.5 M Trizma base, 0.5 M Bis-Tris, 50 mM KCl, 75 mM $(NH_4)_2SO_4$, 5 mM K_2SO_4, and 10 mM KH_2PO_4, pH 7.4) [46], at 37°C. PhoPQ activation was reached by growing the bacteria in N-minimal medium containing low-Mg^{2+} (10 μM $MgCl_2$) related to high Mg^{2+} (10 mM $MgCl_2$) [46]. RcsCDB acid activation was achieved by growing the bacteria in LB medium previously adjusted to pH 5 with HCl 0.1M. When necessary, 25 ∝g ml^{-1} chloramphenicol, 50 ∝g ml^{-1} kanamycin, and 50 ∝g ml^{-1} ampicillin were added to the medium.

Table 1: Bacterial strains and plasmids used in this study.

Strain or plasmid	Description[a]	Reference or source
Escherichia coli		
MC4100	F⁻*araD139 Δ(argF-lac)205 λ⁻ rpsL150* (Sm^r) *flbB5301 relA1 deoC1 pstF25*	CGSC
S. enterica serovar Typhimurium		
14028s	wild type	[51]
MDs1262	STM1485::*lacZY*	This work
MDs1284	STM1485::*lacZY rcsB::*Cm	This work
MDs1650	STM1485::*lacZY rcsC11*	This work
MDs1651	STM1485::*lacZY tolB::*Cm	This work
MDs1652	STM1485::*lacZY rcsB tolB::*Cm	This work
MDs1263	STM1485::*lacZY rstA*::Cm	This work
MDs1765	STM1485::*lacZY* PhoP*	This work
EG12711	*rcsB::*Cm	[6]
PB3145	*rstA*::Cm	[52]
Plasmids		
pUHE2-21	rep$_{pMB1}$ Ap *lacI*^{q}	[53]
p*rcsB*	pUHE2-21*lacI*^{q} harboring the *rcsB* gene	[7]
pT7-7-*rcsB*-His6	rep$_{PMB1}$ Ap^r pT7-*rcsB*-His6	[12]
pT7-7-*rstA*-His6	rep$_{PMB1}$ Ap^r pT7- *rstA* -His6	This work
p*rstA*	pUHE2-21*lacI*^{q} harboring the *rstA* gene	[52]

[a] Gene designations are summarized by Sanderson [54]

2.2 *β-galactosidase assays*

β-galactosidase activity was determined from stationary phase bacterial cultures in LB medium at pH 5 or pH 7, as indicated. The activity levels of this enzyme were measured as previously described [47]. For the *rcsB* or *rstA* genes overexpression, bacterial cultures harboring the p*rcsB* or the p*rstA* plasmids, respectively, were supplemented with IPTG to a 0.35 mM final concentration. Activity levels are presented as Miller Units.

2.3 *Quantitative real-time PCR (qRT-PCR)*

The qRT-PCR assay was carried out as previously described [12]. To this end, specific primers STM1485-QRT-Fwd (CTGCAATGGGTCTCTCTTC) and STM1485-QRT-Rev (GGTGGTCTGGGTTGTTT) were designed from the STM1485 coding sequence. cDNA was obtained by reverse transcription of mRNA harvested from wild type (14028s) strain, after 8 hours of growth in LB medium at pH 7 or 5. The mRNA levels of the STM1485 gene was normalized to the *gyrB* transcript levels at each pH essayed, amplified using primers *gyrB*-QRT-Fwd (CTCCTCACAGACCAAAGAT AAG) and *gyrB*-QRT-Rev (GCTCAGCAGTTCGTTCAT).

2.3 *Electrophoretic mobility shift assays (EMSA)*

The EMSA assay was carried out as described [48]. For this purpose, the STM1485 regulatory sequence containing both RcsB and RstA putative binding sites was amplified using primer #8024 (CGGATTCATATACGGTGTGCC) and #8023 (GCGGCAACAACCAGAGC) designed from wild type 14028s strain sequence. Approximately 2 pmol of PCR product were incubated with increasing RcsB-His6 protein concentrations, at room temperature, as previously described [12]. The RcsB-

His6 and RstA-His6 proteins were purified as described [12, 49]. Samples were run on 6% non-denaturing polyacrylamide gels, using Tris-borate-EDTA buffer and maintained at 4°C. After electrophoresis, the DNA fragments were stained with ethidium bromide and visualized under UV irradiation.

2.4 In vitro *gastrointestinal digestion*

The effect of salivary, gastric and intestinal fluids on wild type *S.* Typhimurium 14028s strain and the STM1485, *rcsB*, *rstA*, STM1485 *rcsB,* and STM1485 *rstA* mutants was performed as described [50]. Briefly, overnight cell cultures were washed twice with physiological solution (CaCl2, 0.22 g l^{-1}; NaCl, 6.2 g l^{-1}; KCl, 2.2 g l^{-1}; NaHCO3, 1.2 g l^{-1}, pH 7) and suspended in an equal volume of the same solution. Then, a dilution of 1:20 was mixed with saliva solution (lysozyme, 20 mg ml^{-1} and α-amylase, 2 mg ml^{-1}, pH 6,5), and incubated for 10 min at 37°C. An aliquot was used to determine the number of cell forming colonies (CFU). A second aliquot was diluted 1:10 in physiological solution, and then incubated at 1:2 rate with gastric fluids solution (NaCl, 125 mM; KCl, 7 mM; $NaHCO_3$, 45 mM; pepsin, 3 g l^{-1}; pH 3 adjusted with HCl, 80 to 160 mM) for 90 min, at 37°C. Then, an aliquot of this new sample was used to determine CFU and the remaining sample was incubated with pancreatic fluids solution (trypsin, 1 mg ml^{-1} and chymotrypsin, 1 mg ml^{-1}; pH 8 adjusted with NaOH 5N, pH 8) at the same conditions. After this treatment, CFU was determined by serial dilutions plated on LB agar and incubated at 37°C.

3. Results

3.1 *RcsB modulates the STM1485 expression in a pH-dependent pathway*

The STM1485 gene is induced when bacteria grow in acidic LB medium [31] and it is essential for intracellular replication and *Salmonella* virulence [32]. Since RcsB is involved in acid stress response, we decided to study the effect of RcsB in the pH-dependent activation of *S*. Typhimurium STM1485 gene. To this end, we quantified the levels of β-galactosidase activity produced by the chromosomal *lacZY* transcriptional fusion to the STM1485 promoter gene. We first determined the expression pattern of the STM1485::*lacZY* fusion in the wild type strain during growth in LB medium at pH 7. As shown in Fig. 1A, the promoter start to be active at early stage of the exponential growth (4 hours), achieving a maximal peak after 8 hours of incubation.

Considering that induction of STM1485 expression occurs in the eukaryotic cell when bacteria sense low pH and that RcsB is also activated in acid medium conditions (after a meal consumption gastrointestinal pH reaches 5 [33]), we decided to determine if STM1485 transcription could also be modulated at pH 5. Thus, β-galactosidase activity from the STM1485::*lacZY* fusion was determined in wild type genetic background, after 6 and 8 hours of growth in LB medium at pH 7 or 5, since they were the times points where maximum expression was observed. As shown in Fig. 1B (left panel), we observed that β-galactosidase activity decreased almost 5-fold when bacteria were grown at pH 5 compared to the levels observed at pH 7. However, no differences in bacterial growth rate were observed between the wild type and the STM1485 mutant cultured at pH 7 or pH 5 (Fig. S1). To confirm these results, the STM1485 transcription was analyzed by qRT-PCR, measuring the levels of STM1485 mRNA produced related to the mRNA level from the housekeeping gene *gyrB*. To this end, we used total RNA harvested from the wild type strain, growing 8 hours in LB medium at pH 7 or 5. In agreement with above results, we observed that the levels STM1485 mRNA were significantly lower when bacteria growth at pH 5 compared to the levels observed at pH

7 (Fig. 1B, right panel). Our results suggest that STM1485 gene expression is repressed under moderate acid conditions.

We hypothesized that the repression of STM1485 expression is due to the RcsCDB system pH-dependent activation. Accordingly, we suggest that other activation pathways of the RcsCDB system, such as *tolB* and *rcsC11* mutants, could produce the same repression of the gene under study. To test this hypothesis, the transcriptional activity of STM1485::*lacZY* fusion was determined in wild type strain and in *rcsB* and *rcsB* harboring p*rcsB* growing in LB medium at pH 7 or pH 5, as well as in *rcsC11*, *tolB* and *tolB rcsB* mutants, after 6 and 8 hours of growth. As shown in Fig. 1C, the decrease of β-galactosidase activity in the wild type strain at pH 5 was not observed in the absence of the *rcsB* gene. In addition, the STM1485 expression levels produced by the *rcsB* mutant growth at pH 7 or pH 5 displayed no significant differences. Moreover, we observed higher transcriptional levels in the *rcsB* mutant than those observed in the wild type strain at both times and pH assayed (Fig. 1C). When the *rcsB* chromosomal mutation was complemented with the p*rcsB* plasmid, at pH 7 these high levels of β-galactosidase were abolished (Fig. 1C). These results support the assumption that RcsB activation represses STM1485 transcription. To confirm this hypothesis, we analyzed STM1485 gene expression under another RcsCDB induction conditions such as the *rcsC11* and *tolB* mutations. While the *rcsC11* mutant constitutively activates the RcsCDB system [20, 34], *tolB* induces the system by altering outer membrane permeability [19]. The results obtained in LB at physiological pH demonstrated that, under both RcsCDB induction conditions, the STM1485 expression was repressed reaching similar levels than those observed in the wild type strain growing in LB at pH 5 (Fig. 1C). Moreover, when the *rcsB* gene was deleted from the chromosome of the *tolB* strain such repression was abolished (Fig. 1C). These data confirmed that activation of the RcsB regulator inhibits STM1485 gene expression.

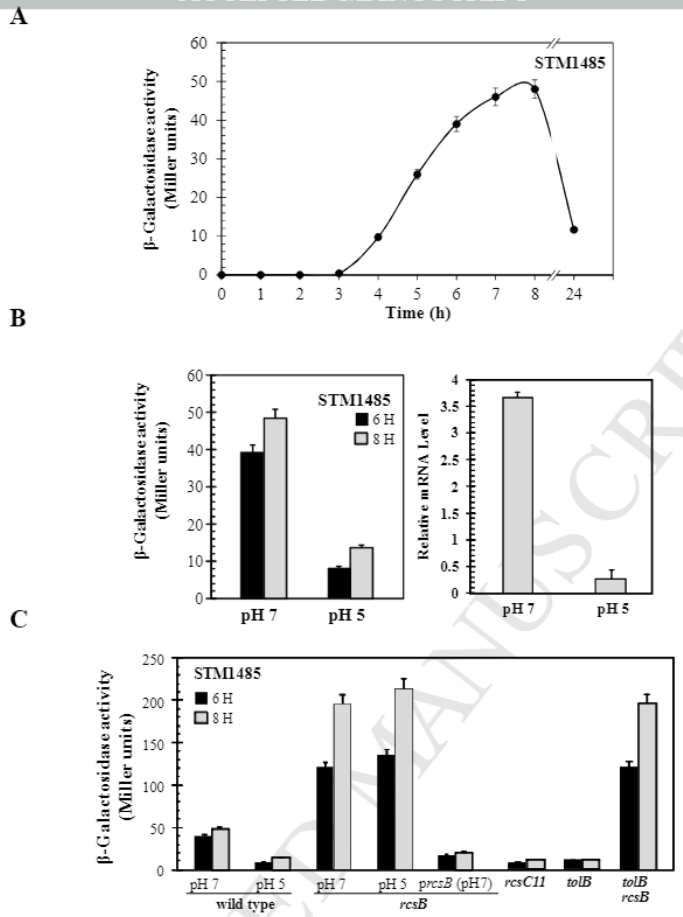

Fig. 1: **Regulation of STM1485 gene transcription.** A) β-galactosidase activity (Miller units) expressed by wild type strain harboring chromosomal STM1485::*lacZY* transcriptional fusion (MDs1262) was determined during bacterial growth in LB medium. B) Effect of the culture medium pH (7 or 5) on STM1485 gene expression, measured as β-galactosidase activity (Miller units, left panel) produced by wild type strain harboring the STM1485::*lacZY* fusion, after 6 and 8 hours of incubation (black and gray bars, respectively); and by qRT-PCR assays using mRNA from wild type strain harvested at 8 hours of growth in LB pH 7 and 5. C) β-galactosidase activity from STM1485*::lacZY* transcriptional fusion was determined in the wild type strain (MDs1262), *rcsB* (MDs1284), *rcsB* harboring p*rcsB*, *rcsC11*(MDs1650), *tolB*

(MDs1651) and *tolB rcsB* (MDs1652) mutants. The *rcsB* mutant complementation was assayed by 0.35 mM IPTG addition. These assays were performed in LB medium at pH 7 or pH 5 after 6 (black bars) or 8 hours (gray bars) of growth. The data correspond to mean values and standard deviation of three independent experiments carried out in duplicate.

3.2 *Bioinformatics search of a putative RcsB* cis*-acting element on STM1485 promoter*

According to the STM1485 gene expression data and using bioinformatic analysis, we looked in the STM1485 promoter region for a sequence that could act as a binding site for the RcsB regulator. For this purpose, the STM1485 promoter sequence was aligned with RcsB-conserved *cis*-acting elements found in other RcsB-dependent genes [35, 36]. Our bioinformatics analysis displayed a conserved sequence, located 12 bp upstream of the -35 box region and -52 nucleotides from the SMT1485 +1 transcription site, which displayed 75% identity to the binding site of other well characterized RcsB-regulated genes (Fig. 2A and 2B). We observed only a 7 bp difference between the sequences of RcsB box in the *ams* (*Erwinia amylovora*, Ea) and *ugd* (*Salmonella enterica*, Se) genes with the potential DNA binding site found in the STM1485 promoter, conserving specifics nucleotides required for binding (Fig. 2B). These data suggest that the phosphorylated active form of RcsB (RcsB-P) could be bound to the STM1485 promoter region and consequently repress STM1485 gene transcription.

In order to investigate whether RcsB exerts its negative effect on STM1485 gene expression by directly binding to the identified consensus sequence we conducted an electrophoretic mobility shift assay (EMSA). To this end, specific primers were used to amplify a 276 bp DNA fragment containing the STM1485 promoter elements as well as

the putative RcsB binding site and the start translation codon (Fig. 2A). As shown in Fig. 2C, when RcsB was added to the mixture containing the PCR product, a high molecular weight band (slower electrophoretic mobility) became apparent. In addition, we noted that the intensity of this retarded band was enhanced as the concentration of the regulator increased (50, 100, 200 or 300 µM), with the concomitant intensity reduction in the corresponding PCR product band (276 bp) (Fig. 2C). Based on these results, we suggest that the high molecular weight band corresponds to the complex formed by the RcsB protein bound to the DNA since these bands were not observed in the control (RcsB absence) (Fig. 2C). These results allowed us to confirm that the RcsB regulator binds to the specific sequence into the STM1485 promoter region, forming a DNA-RcsB complex to exert a direct repressor effect.

A

```
-196  CGGATTCATATACGGTGTGCCTGTTATTCTGGATGGCGGATATT
-152  ACCGACTCTGCGACCAAAAATAACGGCCGGTAACGTAAAGCTAC
-108  CTATTATGGTGGGGGCTGACATCGCGTTACCGGGGGATTGTACA
 -64  TAACGTTACATGCCGAAACCAATCACTCACGGAAGCCTTTTGAT
                                  -35
 -20  AACAGCGATATAGTCATTTCACCGGCCCCGCAGTGGGGTTGAAT
          -10                 +1
 +24  GAAAACCAAATCGAGGGTATCAAAATGAAAAAAGTATTAGCTCT
 +68  GGTTGTTGCCGC
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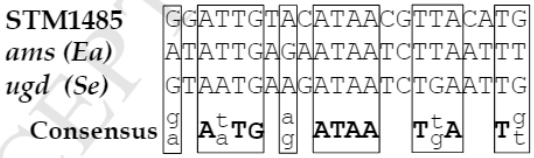
B



C

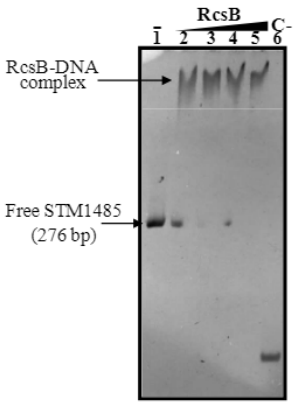


Fig. 2: **Identification of RcsB-binding site on the STM1485 promoter region.** A) The analysis was carried out using a 276 bp sequence containing the *cis*-elements that define the STM1485 promoter, where the -35 and -10 boxes are highlighted in bold, the transcription initiation site (+1) and the transcription start codon are underlined and the RcsB *cis*-acting element analyzed in this work is the boxed sequence. B) The putative RcsB binding site on STM1485 was identified by alignment of the conserved RcsB box identified in other RcsB-dependent genes: *ugd S. enterica* (Se) and *ams E. amylovora* (Ea) genes. The conserved nucleotide positions are highlighted in uppercase where the variable nucleotides are in lowercase in the consensus sequence. C) EMSA performed using a 276 bp PCR product harboring the *cis*-elements of the STM1485 promoter and the predicted RcsB-binding box (well 1, -), incubated with different RcsB-His6 protein concentrations (50, 100, 200 or 300 µM, wells 2 to 5). EMSA performed using a 150 bp PCR product of the STM1485 promoter (C-), lacking the conserved RcsB box, incubated with 300 µM RcsB-His6 protein (well 6).

3.3 *Effect of RstA on STM1485 gene transcription*

Since Ogasawara *et al.* (2007) previously reported that the RstA regulator induces the transcription of the *E. coli asr* gene under acid condition (pH 4.5) in a PhoPQ-dependent pathway, we investigated if the *Salmonella* STM1485 gene could be also regulated in same manner. To this end, we first analyzed the STM1485 promoter region to identify a putative RstA binding site. Our results demonstrated that the STM1485 promoter harbors a RstA *cis*-element of binding (Fig. 3A), located in the same position as the site identified in *E. coli*. The alignment of *asr* and STM1485 RstA-regulated sequences displays 92.89% identity (Fig. S2). However, this percentage decreased to 89.29% identity when the binding sequence identified in the STM1485 promoter region was aligned with the motif of other reported RstA-dependent genes

(Fig. 3B, left panel). This STM1485-RstA binding site was located 14 bp upstream of the -35 conserved box, overlapping the RcsB-binding site, which suggests that it could also negatively affect this gene expression. In addition, we found a second RstA putative binding site on the STM1485 promoter, located at -38 bp of the -35 box and displays 83.93% identity with the regulatory site of other RstA-dependent genes (Fig. 3B, right panel). As this new RstA binding box was located far from the -35 box, we can suggest that it could act as an RstA-positive regulation site, as in the *E. coli asr* gene.

In order to confirm the binding of RstA on the STM1485 promoter sequence we performed an EMSA. In this assay, we incubated the PCR product containing the STM1485 promoter sequence with different concentrations of the previously purified RstA regulator (100, 200 or 300 µM). As shown in Fig. 3C, RstA was able to form a complex with the PCR product of the regulatory sequence of STM1485, producing a slower migration band which intensity enhanced as the regulator protein concentration increases. Taken together, these results demonstrated that RstA could bind to the predicted regulatory sites on the STM1485 promoter region.

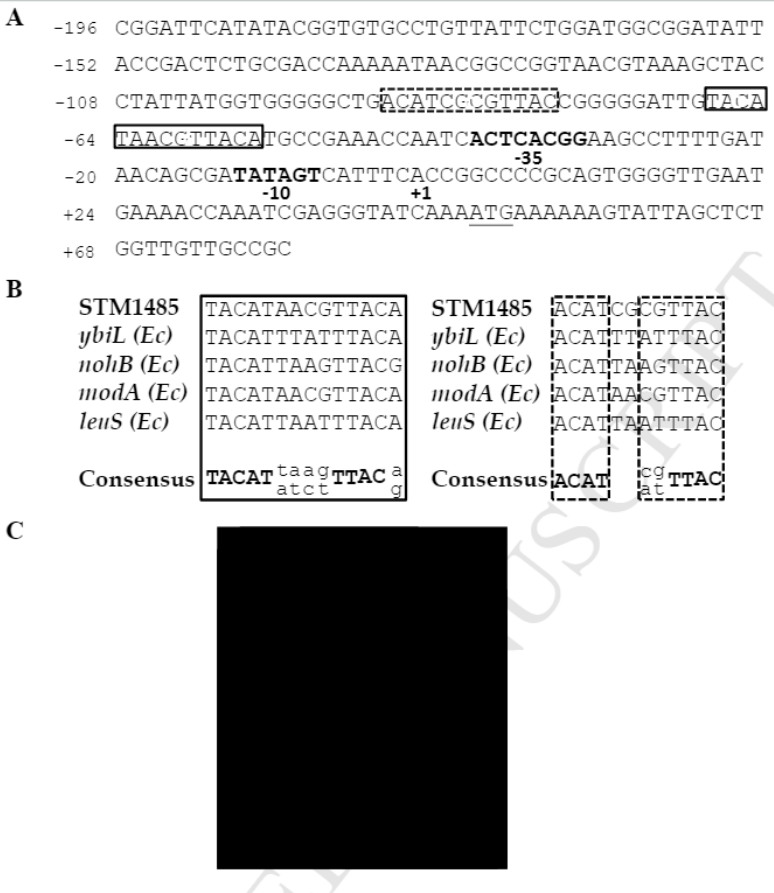


Fig. 3: **Localization of a putative RstA-binding site on the STM1485 promoter.** A) The 276 bp nucleotide sequence of the STM1485 promoter containing the -35 and -10 boxes is highlighted in bold; the transcription +1 site and the start codon are underlined, and both identified RstA binding sequences are boxed. B) Alignment of the RstA consensus motif identified in other RstA-dependent genes like *ybiL* (Ec), *nohB* (Ec), *modA* (Ec) and *leuS* (Ec) genes, with the STM1485 promoter sequences. The alignment with the first RstA identified motif is located on the left (continuous line box) and the second on the right (dash line box). The conserved nucleotides positions are highlighted in uppercase were the variable nucleotides are in lowercase in the consensus sequence. C) EMSA assay performed using the 276 bp STM1485-PCR product harboring the

RstA binding box (well 2), were incubated with different RstA-His6 protein concentrations (100, 200 or 300 µM, wells 3 to 5).

3.4 *RstA controls* in vivo *the STM1485 gene expression in* S. *Typhimurium*

In order to confirm the *in vivo* effect of RstA on STM1485 transcription, we measured the STM1485::*lacZY* fusion expression in wild type and *rstA* strains in physiological (pH 7) or acid (pH 5) medium. When β-galactosidase activity was determined, we found 2.6- and 1.5-fold decreased expression levels in *rstA* mutant compared to the wild type strain when both were grown at pH 7 for 6 or 8 hours, respectively (Fig. 4). However, non-significant expression differences between these strains were observed after growth at pH 5 (Fig. 4). These data suggest that, at physiological pH, RstA is required to induce STM1485 expression to reach wild type levels. Moreover, the results obtained in the acid medium could be explained assuming that in such condition the RcsCDB system is activated, repressing STM1485 gene transcription. We suggest that this repression occurs by competition with RstA for the binding site (preventing it from exert its inductive effect) or by an independent pathway where RstA binds to the other regulatory site identified further from the -35 box (Fig. 3B, right panel).

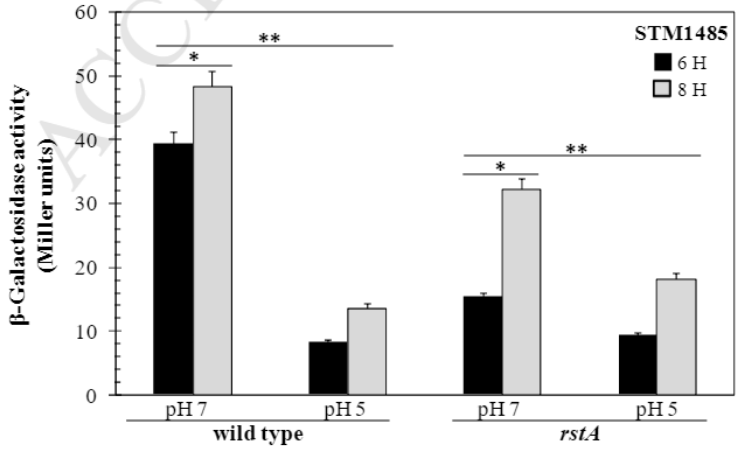


Fig. 4: **Effect of RstA on STM1485 gene transcription**. The promoter activity was determined using STM1485*::lacZY* transcriptional fusion were the β-galactosidase levels were quantified in wild type (MDs1262) and *rstA* (MDs1263) strains, growing in LB medium at pH 7 or 5, after 6 (black bars) or 8 hours (gray bars). The data correspond to mean values and standard deviation of three independent experiments carried out in duplicate. Asterisks represents statistically different β-galactosidase activity values (Tukey's test, P=0.05).

3.5 *Combined effects of RcsB and RstA regulators on STM1485 gene transcription*

In order to study the associated effect of RstA and RcsB on STM1485 promoter gene modulation, we determined its expression in the absence of one regulator but overexpressing the other one in bacteria growing in medium at physiological pH. On the one hand, we observed that *rcsB* overexpression in both, wild type and *rstA* strains, caused a decrease of 2- to 4-fold in β-galactosidase activity from the STM1485::*lacZY* fusion at 6 and 8 hours of assay, respectively (Fig. 5A). Moreover, we observed that the maximal repression occurred in the *rstA* mutant overexpressing *rcsB*. This data confirms that RcsB represses STM1485 transcription and suggests that such effect could occur by competing with RstA for the binding site. On the other hand, when we analyzed the effect of *rstA* overexpression (by IPTG addition) we observed about 4-fold induction of the STM1485 transcription in the wild type strain compared to the levels obtained in same background without IPTG (Fig. 5B). However, when the *rcsB* gene was eliminated from the chromosome and the RstA was overproduced, the STM1485 expression increased until it reached wild type levels (Fig. 5B). In addition, when the levels of STM1485 transcription produced by *rcsB* mutant under *rstA* overexpression were compared with those obtained in the absence of *rstA* induction, we only observed

weakly increased levels of ≅1.2 fold after 8 hours (Fig. 5B). These observations support the assumption that there is a competitive effect between these two regulators for bind the promoter region and control the expression of this gene; or that RstA could bound to the second conserved site on STM1485 promoter, acting only in the absence of RcsB.

This latter assumption was investigated using a physiological RstA induction condition through a PhoPQ-dependent pathway, in a non-RcsB activation background. It has been shown that the PhoPQ system is involved in the response to at least three different signals: low-Mg^{2+}, acidic pH and certain antimicrobial peptides [37]. The expression of genes encoding the RstAB system are induced under PhoPQ activation, and consequently the RstAB system activates the *asr* gene expression [37]. On the other hand, it has also been demonstrated that the constitutive activation of PhoP by low magnesium is produced in the PhoP* mutant, which consequently modulates the PhoP-dependent genes [38]. Thus, in this approach we analyzed STM1485 gene expression from wild type, *rcsB* and PhoP* genetic backgrounds grown in high or low magnesium concentrations. As shown in Fig. 5C, STM1485 transcription was increased 1.6 fold in the wild type strain grown in low magnesium concentration related to high magnesium condition (white and black bars, respectively). The same profile was observed in the *rcsB* mutant, but with 1.9 fold higher levels than the values obtained with high magnesium concentration and 1.3 fold higher than in the wild type strain under same growth condition (Fig. 5C). It is important to note that the *rcsB* mutant presented maximal STM1485 expression, suggesting that only in such scenario RstA is able to bind to the promoter region and activate the gene expression. These results were confirmed by studying the expression displayed by the PhoP* strain, where this regulator is constitutively activated [38]. Here, we observed that in both magnesium conditions the β-galactosidase levels were similar and higher than those from the wild type strain (Fig. 5C). These results confirm that RstA induces the expression of

STM1485, when it is abundant or when *rcsB* is absent. In addition, they support our hypothesis that there is a signal-dependent competition between RcsB and RstA for binding to the promoter to regulate STM1485 gene.

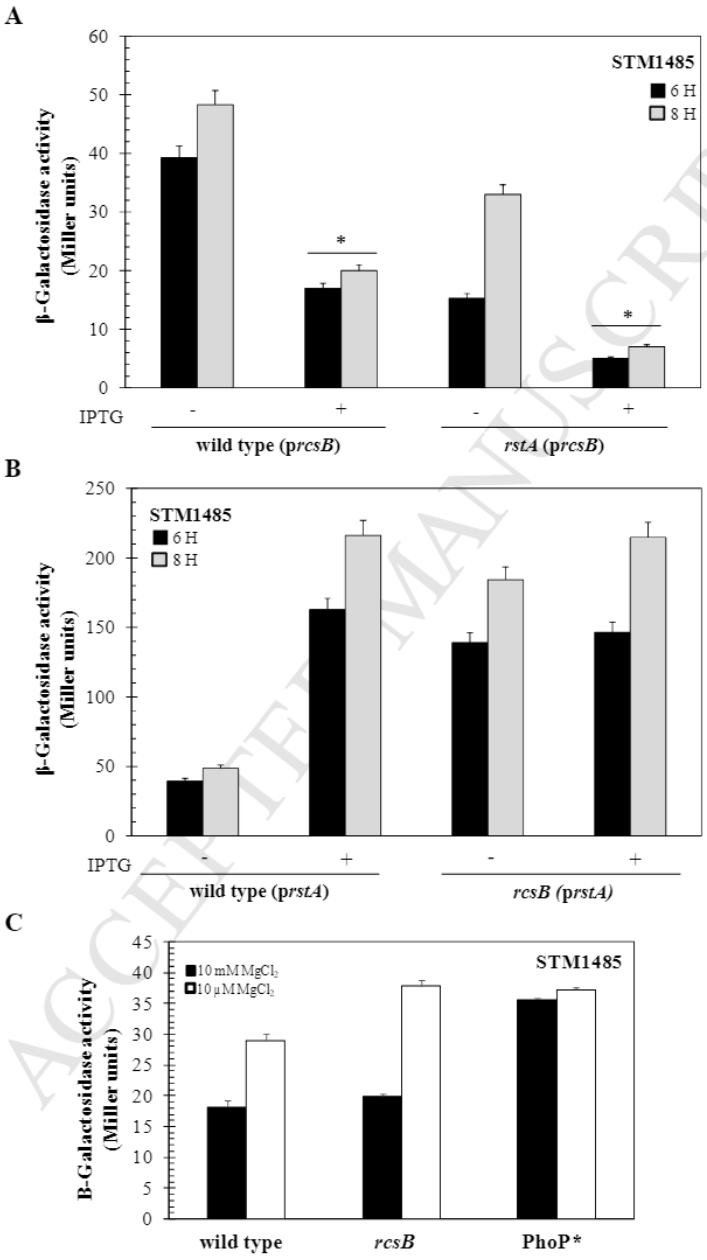


Fig. 5: **Effect of RcsB and RstA regulators on STM1485 gene transcription.** The β-galactosidase activities produced by the STM1485*::lacZY* transcriptional fusion were

determined (A) in wild type (MDs1262) and *rstA* (MDs1263) strains grown in LB medium at pH 7 and overproducing (+) or not (-) *rcsB* from p*rcsB*; and (B) in wild type (MDs1262) and *rcsB* (MDs1284) strains and overproducing (+) or not (-) *rstA* from p*rstA*. Gene expression was measured after 6 (black bars) or 8 hours (gray bars) of growth in LB medium. C) Effect of RstA/PhoPQ-dependent activation on STM1485 expression. The β-galactosidase activity produced by the STM1485*::lacZY* transcriptional fusion was determined in wild type, *rcsB* and PhoP* (MDs1765) strains grown in N-minimal medium containing high (10 mM) or low (10 ∝M) Mg^{2+} concentrations, black and white bars, respectively. All data correspond to mean values and standard deviation of three independent experiments, carried out in duplicate. Asterisk (*) represents statistically different β-galactosidase activity values (Tukey's test, P=0.05).

3.6 *Requirement of the STM1485 gene expression in the bacterial survival along gastrointestinal tract*

It has been previously demonstrated that STM1485 is essential for *Salmonella* survival during acid stress (pH 4) and replication within host cells [30, 32]. However, *Salmonella* behavior and gene regulation during the ingestion steps, where it undergoes gradual changes in the host's environmental pH, remains to be determined. Using the *in vitro* gastrointestinal digestion model we analyzed the role that different STM1485 expression levels play during the bacteria transition through the upper gastrointestinal tract. For this purpose, the wild type strain, STM1485, *rcsB* and *rstA* mutants, and STM1485 *rcsB* and STM1485 *rstA* double mutants were consecutively exposed to artificial salivary (pH 6.5), gastric (pH 3) and pancreatic (pH 8) solutions and the number of viable cells were determined after each treatment.

As shown in Fig. 6, no significant differences in bacterial viability were observed between the assayed strains treated with salivary or gastric solution. However, non-viable cells were detected when *rcsB* mutant was treated with artificial pancreatic fluid, while the *rstA* mutant displayed the same behavior as the wild type strain (Fig. 6). Interestingly, the STM1485 mutant was the strain that displayed the highest CFU numbers under the pancreatic fluid treatment, reaching higher values compared to the wild type strain (statistically significant differences of 4.2-fold). However, when the *rcsB* gene was deleted, the fitness of this mutant for pancreatic fluid decreased to levels even lower than wild type strain (Fig. 6). These results suggest that the expression of STM1485 is detrimental to the survival of the bacterium during its passage through the intestinal tract. According to the results presented above, we suggest that the RcsCDB system activation is fully required during this bacteria-host interaction step to inhibit STM1485 gene expression in order to allow bacteria survival.

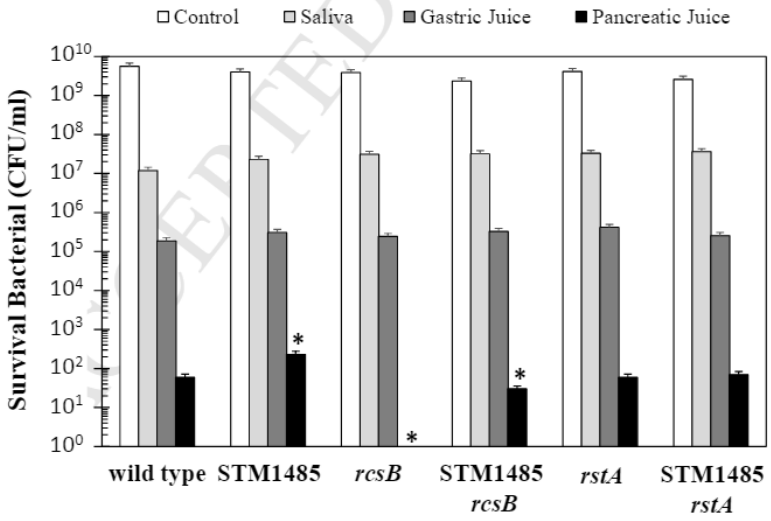


Fig. 6: Role of STM1485 gene expression in the gastrointestinal digestion model. The STM1485 requirement for bacterial survival was determined by viable-cell counting as log CFU/ml of wild type (14028s) strain and STM1485 (MDs1262), *rcsB*

(EG12711), STM1485 *rcsB* (MDs1284), *rstA* (PB3145) and STM1485 *rstA* (MDs1263) mutants exposed to physiologic solution (white bars, control), artificial salivary (light gray bars), gastric (dark gray bars) or pancreatic (black bars) fluids, as described in Materials and Methods. The CFU of each strains growing in physiological solution was determined as control (white bars). All assays were conducted at least three times and carried out by duplicate; the data correspond to mean values and standard deviation obtained from these assays. The asterisk (*) represents statistically different CFU values related to the wild type strain (Tukey test, P=0.05).

4. Discussion

Throughout the evolution process, enterobacteria have developed numerous strategies to adapt to surrounding changes. Many of these mechanisms are controlled at the transcriptional level by regulatory factors capable of binding to the promoter regions, modulating the expression of genes necessary for bacterium survival. One of the main barriers that these pathogens must overcome is the acid environment in the human digestive tract. It was previously demonstrated that enterobacteria modify the expression of specific acid shock genes (encoding for the acid shock proteins-ASPs) [27]. In *S.* Typhimurium, several acid shock genes are controlled by RpoS, Fur, PhoP, and OmpR transcriptional factors to produce the acid tolerance response (ATR) to extreme acidity (e. g. pH 3) [39, 40, 41, 42]. However, a large number of these ASPs remain uncharacterized, and their regulation pathway and function in bacterial survival and acid response are still unknown.

The RcsCDB system was also reported to be involved in the acid response. Castanie-Cornet *et al.* (2007) demonstrated that different levels of RcsB-P are able to modulate the glutamate-dependent acid resistance of *E. coli*. These authors reported that the basal levels of RcsB-P could act as transcriptional inductor of glutamate

decarboxylase genes *gadA* and *gadB* during the stationary growth phase, while an increase in RcsB-P leads to *gad* genes repression, with a concomitant decrease in acid resistance response. Moreover, this system is activated when the bacterium senses the medium acidity (pH≅5), leading to its adaptation by an independent mechanism of the *gad* gene products. More recently, it was demonstrated in *E. coli* that a moderate acidity of pH<5.0 strongly induces expression of the *asr* gene (*a*cid *s*hock *R*NA) [27]. The Asr protein is highly required to produce an acid tolerance response, allowing the bacterium to survive when is challenged by a more extreme acidity [27].

Since the RcsCDB system is activated at moderate acidity (pH 5) and considering that this condition induces the *asr* gene expression in *E. coli*, we asked ourselves whether in *Salmonella* the STM1485 gene could be controlled by RcsB activation. In order to answer this question, we first analyzed STM1485 expression along bacterial growth. In accordance with other authors, its expression was detected earlier in the growth at physiological pH, reaching maximum levels during the stationary phase, probably regulated by RpoS [28]. In addition, we observed that STM1485 expression was inhibited in acid medium, which is in agreement with Seputiene *et al.* (2004) findings, who demonstrated that the STM1485 gene is not expressed at pH 5. However, we showed that this gene is expressed at pH 7, data not reported in the work mentioned above. These differences can be explained considering that expression levels in each study were determined at different points of the growth: 6 and 8 vs. 4 hours. Moreover, the non-concordant results can be attributed to the culture medium used in each work, LB vs LPM (low phosphate) medium. This assumption is supported by Suziedeliene *et al.* (1999), who reported that under acid pH the levels of *E. coli asr* gene were strongly reduced in LB medium respect to the values obtained in minimal medium or in phosphate starvation condition (LPM) [28]. It is worth mentioning that this phosphate starvation is the signal that induces the PhoBR two-

component system, which is one of the main positive regulation mechanisms that control *asr* gene expression. Thus, we decided to determine the effect of the RcsCDB system activation by moderate acid pH, using LB media to eliminate the PhoBR positive effect.

Taking into account the results discussed above, we suggest that the repression of the STM1485 gene expression at pH 5 could be produced by activation of RcsCDB-dependent acid conditions. We confirmed this assumption when the repression of the STM1485 transcription was observed in the absence of the *rcsB* gene. When RcsCDB was activated by conditions other than acid shock, such as the *rcsC11* or *tolB* mutants, repression of the STM1485 gene was maintained at levels similar to those observed at pH 5. Furthermore, no differences were observed in the rate of bacterial growth at pH 7 and pH 5, suggesting that the STM1485 gene product is not involved in bacterial survival at constant acid condition, at least under our *in vitro* assays.

Moreover, into the SMT1485 promoter region we found a sequence homologous to the RcsB-binding sites from other RcsB-regulated genes. The localization of this sequence suggests that it could serve as a negative regulation point, which was confirmed by EMSA. These results demonstrated that the RcsB exerts its repressive effect by direct binding to the specific sequence. Taken together, these results demonstrate that activation of the RcsCDB system leads to a direct and negative control of STM1485 gene expression during stationary bacterial growth.

Seputiene *et al.* (2004) reported that the alignment of the *asr* promoter sequence with those belonging to a wide range of enterobacteria species displayed high nucleotide sequence identity between the −70 to −30 position, relative to its transcriptional start site [31]. In this work, we identified the putative promoter sequence from STM1485, which presented 74% sequence identity with the corresponding sequence from the *E. coli asr* gene. (Fig. S2). However, we detected several gaps and non conserved

nucleotides in the −70 to −30 region, affecting the RcsB regulation site as well as other different factors that modulate *asr* gene transcription (Fig. S2) [31]. In spite of this, our results suggest that *asr* and STM1485 genes are controlled in the same way by the RcsB regulator.

In the present work, we also investigated if STM1485 gene acid-dependent expression could be modified by the RstA transcriptional factor, as reported for the *E. coli asr* gene [26]. Ogasawara *et al.* (2007) reported that expression of the *asr* gene is positively controlled by the RstBA system activation following a signal cascade that fully requires PhoPQ induction by low Mg^{2+} [37, 43]. The authors demonstrated that RstA binds to a conserved 23-nucleotide sequence located at -55 pb from the *asr* transcription start site, the region where we detected gaps and nucleotide changes. Considering these data, we searched for a putative RstA binding site in the STM1485 promoter region and surprisingly we identified two sequences displaying homology with the RstA promoter consensus. The first RstA regulatory sequence was located overlapping the predicted RcsB-binding site in same position as in *E. coli asr* gene and differ in only one nucleotide (Fig. S2). The second putative binding site was located farther from the -35 box, in the region where the greatest divergence between *E. coli* and *Salmonella* was observed (Fig. S2). However, this binding site displayed a large number of nucleotides position conserved in the alignment between STM1485 promoter and those from other RstA-dependent genes (Fig. 3B), suggesting that could acts as RstA regulatory site. These data and the levels of transcription demonstrate that RstA exerts a positive regulatory effect on the STM1485 gene by directly binding. In addition, we found that RstA could bind to this promoter sequence only when RcsB is absent or inactive, since an induction of STM1485 expression in the *rcsB* mutant or under magnesium starvation conditions (PhoP-dependent RstA activation) was observed. These results allow us to suggest that both RstA binding sites are required for

the regulator to exert its effect on the *Salmonella* gene. We also propose that the RstA induction is important at physiological pH conditions to maintain the expression of STM1485, where RcsB is inactive and unable to repress it.

Finally, in this work the physiological importance of the regulation exerted by RcsB and RstA was determined in an *in vitro* gastrointestinal model. Our results demonstrated that *Salmonella* is able to survive the pancreatic fluid possibly due to the RcsB repression of STM1485 gene. Thus, we conclude that the STM1485 gene product plays an important role in *Salmonella* survival, especially in relation to the exposition to extreme pH changes. Based on our results and those from other authors, we propose that in acid environments the RcsB and RstA transcriptional factors compete to regulate the STM1485 expression in an opposite manner.

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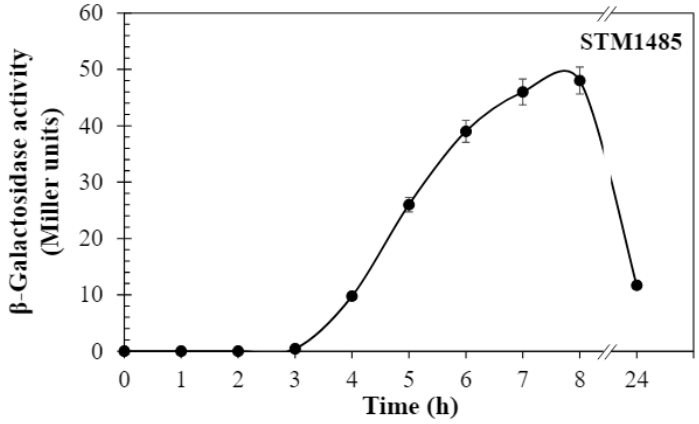
Table 1: Bacterial strains and plasmids used in this study.

Strain or plasmid	Description[a]	Reference or source
Escherichia coli		
MC4100	F⁻ *araD139 Δ(argF-lac)205 λ⁻ rpsL150* (Sm^r) *flbB5301 relA1 deoC1 pstF25*	CGSC
S. enterica serovar Typhimurium		
14028s	wild type	[51]
MDs1262	STM1485::*lacZY*	This work
MDs1284	STM1485::*lacZY rcsB::*Cm	This work
MDs1650	STM1485::*lacZY rcsC11*	This work
MDs1651	STM1485::*lacZY tolB::*Cm	This work
MDs1652	STM1485::*lacZY rcsB tolB::*Cm	This work
MDs1263	STM1485::*lacZY rstA*::Cm	This work
MDs1765	STM1485::*lacZY* PhoP*	This work
EG12711	*rcsB::*Cm	[6]
PB3145	*rstA*::Cm	[52]
Plasmids		
pUHE2-21	rep$_{pMB1}$ Ap *lacI*^{q}	[53]
p*rcsB*	pUHE2-21*lacI*^{q} harboring the *rcsB* gene	[7]
pT7-7-*rcsB*-His6	rep$_{PMB1}$ Ap^r pT7-*rcsB*-His6	[12]
pT7-7-*rstA*-His6	rep$_{PMB1}$ Ap^r pT7- *rstA* -His6	This work
p*rstA*	pUHE2-21*lacI*^{q} harboring the *rstA* gene	[52]

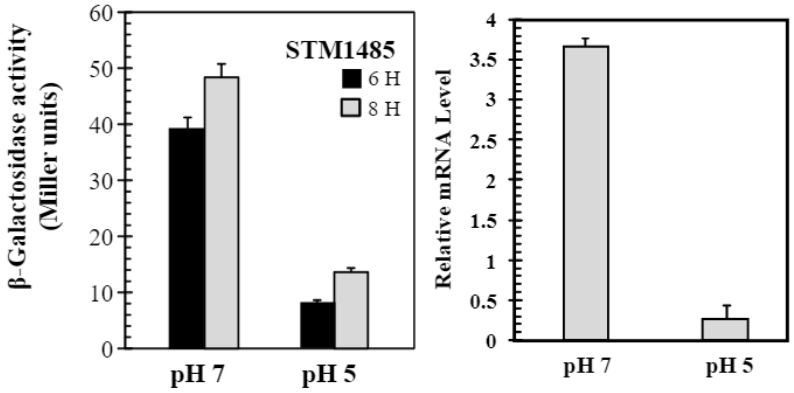
[a] Gene designations are summarized by Sanderson [54]

Figure 1

A


STM1485
β-Galactosidase activity (Miller units)
Time (h)
0
10
20
30
40
50
60
0 1 2 3 4 5 6 7 8 24

B


STM1485
6 H
8 H
β-Galactosidase activity (Miller units)
pH 7
pH 5
Relative mRNA Level
0
0.5
1
1.5
2
2.5
3
3.5
4

C

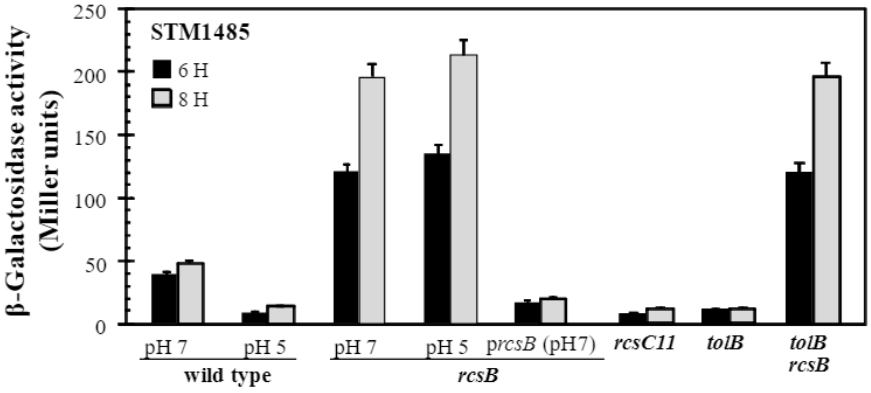
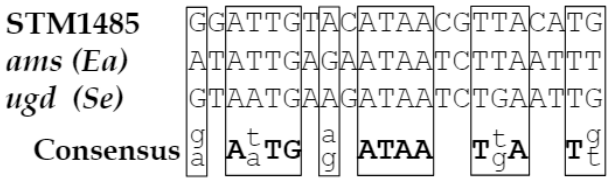

STM1485
6 H
8 H
β-Galactosidase activity (Miller units)
0
50
100
150
200
250
pH 7
pH 5
wild type
pH 7
pH 5
prcsB (pH7)
rcsB
rcsC11
tolB
tolB rcsB

Figure 2

A

```
-196 CGGATTCATATACGGTGTGCCTGTTATTCTGGATGGCGGATATT
-152 ACCGACTCTGCGACCAAAAATAACGGCCGGTAACGTAAAGCTAC
-108 CTATTATGGTGGGGGCTGACATCGCGTTACCGGGGGATTGTACA
 -64 TAACGTTACATGCCGAAACCAATCACTCACGGAAGCCTTTTGAT
                          -35
 -20 AACAGCGATATAGTCATTTCACCGGCCCCGCAGTGGGGTTGAAT
          -10          +1
 +24 GAAAACCAAATCGAGGGTATCAAAATGAAAAAAGTATTAGCTCT
 +68 GGTTGTTGCCGC
```

B



C

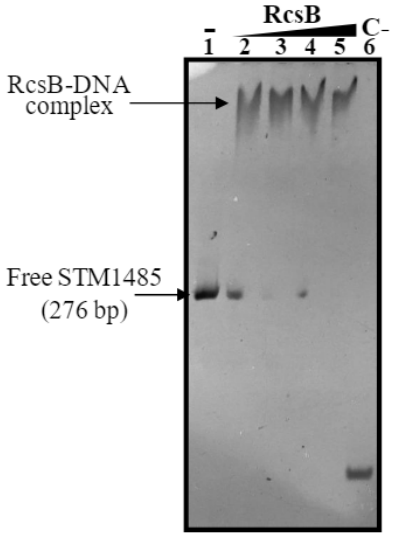


Figure 3

A

```
-196 CGGATTCATATACGGTGTGCCTGTTATTCTGGATGGCGGATATT
-152 ACCGACTCTGCGACCAAAAATAACGGCCGGTAACGTAAAGCTAC
-108 CTATTATGGTGGGGGCTGACATCCCGTTACCGGGGGATTGTACA
 -64 TAACGTTACATGCCGAAACCAATCACTCACGGAAGCCTTTTGAT
                          -35
 -20 AACAGCGATATAGTCATTTCACCGGCCCCGCAGTGGGGTTGAAT
        -10          +1
 +24 GAAAACCAAATCGAGGGTATCAAAATGAAAAAAGTATTAGCTCT
 +68 GGTTGTTGCCGC
```

B

STM1485	TACATAACGTTACA	**STM1485**	ACATCGCGTTAC
ybiL (Ec)	TACATTTATTTACA	*ybiL (Ec)*	ACATTTATTTAC
nohB (Ec)	TACATTAAGTTACG	*nohB (Ec)*	ACATTAAGTTAC
modA (Ec)	TACATAACGTTACA	*modA (Ec)*	ACATAACGTTAC
leuS (Ec)	TACATTAATTTACA	*leuS (Ec)*	ACATTAATTTAC
Consensus	**TACAT** taag atct **TTAC** a g	**Consensus**	**ACAT** cg at **TTAC**

C

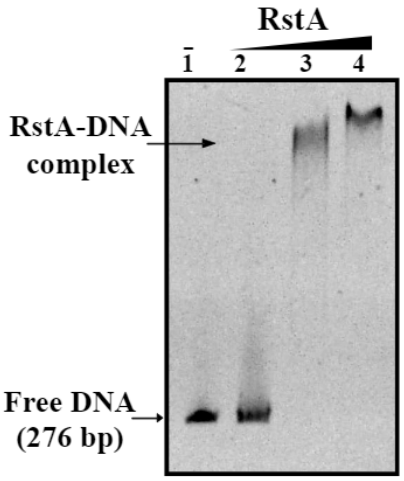


Figure 4

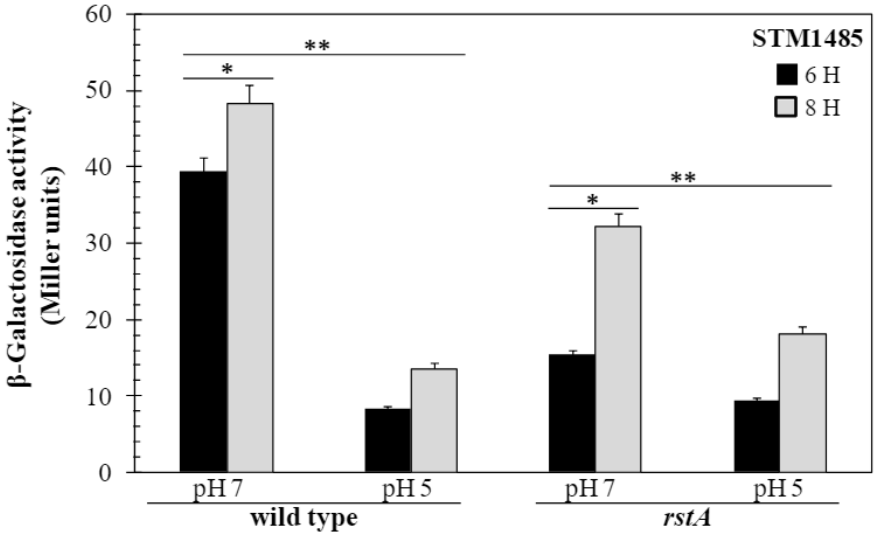
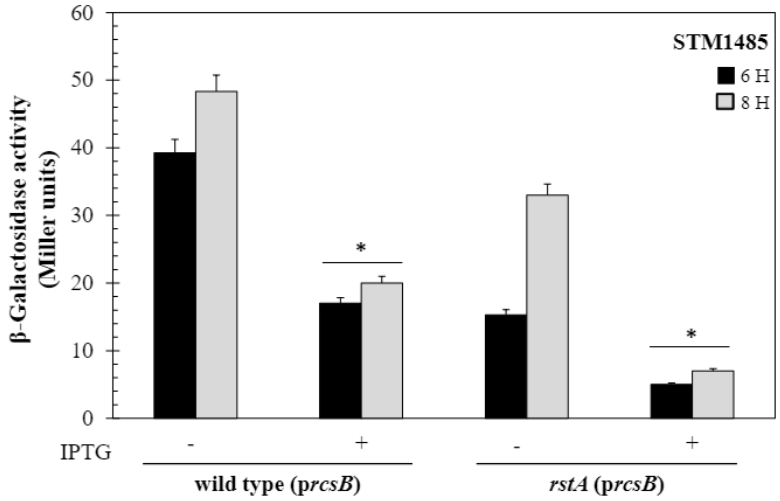
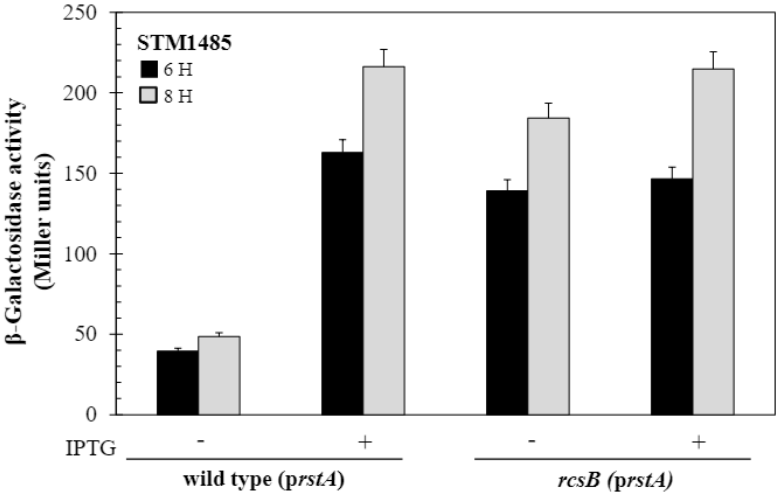

β-Galactosidase activity
(Miller units)
60
50
40
30
20
10
0
STM1485
6 H
8 H
*
**
*
**
pH 7
pH 5
pH 7
pH 5
wild type
rstA

Figure 5

A


STM1485
6 H
8 H
β-Galactosidase activity (Miller units)
60
50
40
30
20
10
0
*
*
IPTG
-
+
-
+
wild type (p*rcsB*)
rstA (p*rcsB*)

B


STM1485
6 H
8 H
β-Galactosidase activity (Miller units)
250
200
150
100
50
0
IPTG
-
+
-
+
wild type (p*rstA*)
rcsB (p*rstA*)

C

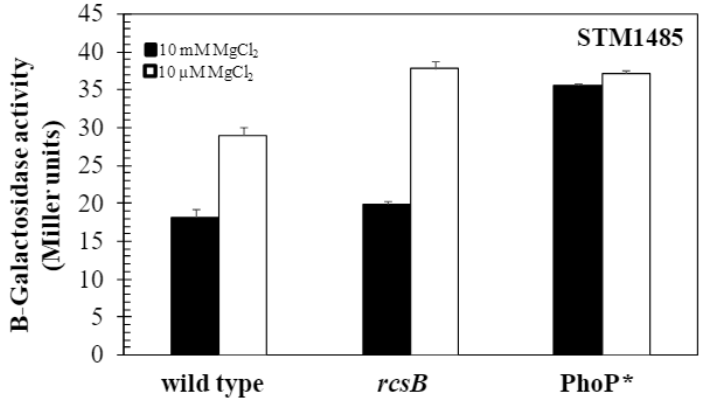
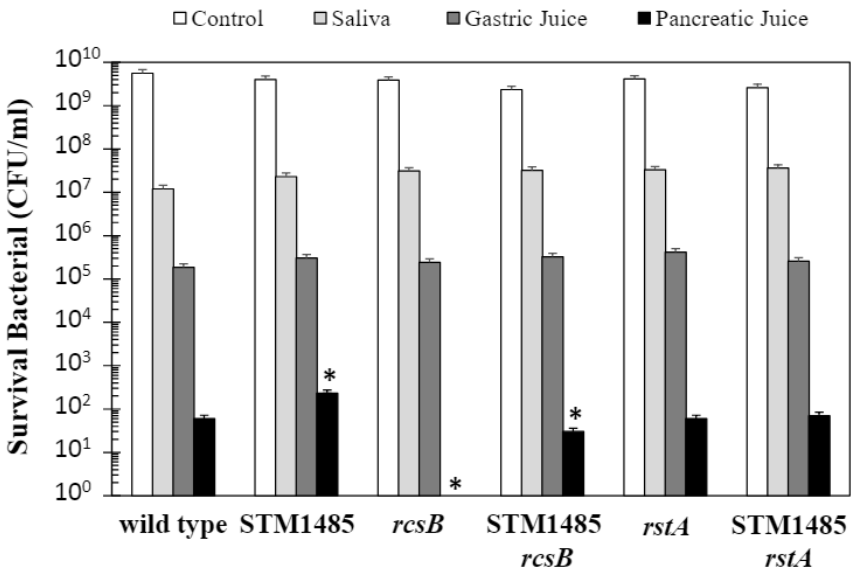

STM1485
10 mM MgCl2
10 µM MgCl2
B-Galactosidase activity (Miller units)
45
40
35
30
25
20
15
10
5
0
wild type
rcsB
PhoP*

Figure 6


Control
Saliva
Gastric Juice
Pancreatic Juice
Survival Bacterial (CFU/ml)
10^{10}
10^{9}
10^{8}
10^{7}
10^{6}
10^{5}
10^{4}
10^{3}
10^{2}
10^{1}
10^{0}
*
*
*
wild type
STM1485
rcsB
STM1485 rcsB
rstA
STM1485 rstA

Highlights

In *Salmonella* Typhimurium, STM1485 gene is negatively modulated by RcsB regulator.

The STM1485 gene expression is induced by RstA response regulator.

Under acidity environments, RstA and RcsB compete to control STM1485 gene expression.

The expression of STM1485 is directly repressed by RcsB into the host digestive tract.