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Salmonella enterica serovar Typhi genomic regions involved in low pH resistance and in invasion and replication in human macrophages

Blanca Dinora Mendoza-Mejía, Liliana Medina-Aparicio, Isela Serrano-Fujarte, Alejandra Vázquez, Edmundo Calva and Ismael Hernández-Lucas*

Abstract

Purpose: *Salmonella enterica* serovar Typhi, the etiological agent of typhoid fever, causes a systemic life-threatening disease. To carry out a successful infection process, this bacterium needs to survive alkaline and acid pH conditions presented in the mouth, stomach, small intestine, and gallbladder. Therefore, in this work, a genetic screening to identify *S.* Typhi genes involved in acid and circumneutral pH resistance was performed.

Methods: A collection of *S.* Typhi mutants deleted of fragments ranging from 6 to 80 kb were obtained by the Datsenko and Wanner method. Bacterial growth rate assays of each mutant were performed to identify *S.* Typhi genes involved in circumneutral and acid pH resistance. *S.* Typhi mutants deficient to growth at specific pH were evaluated in their capacity to invade and replicate in phagocytic cells.

Results: In this work, it is reported that *S.* Typhi ΔF4 (pH 4.5), *S.* Typhi ΔF44 (pH 4.5, 5.5, and 6.5), and *S.* Typhi ΔF73 (pH 4.5, 5.5, 6.5, and 7.5) were deficient to grow in the pH indicated. These three mutant strains were also affected in their ability to invade and replicate in human macrophages.

Conclusions: *S.* Typhi contains defined genomic regions that influence the survival at specific pH values, as well as the invasion and replication inside human cells. Thus, this genetic information probably allows the bacteria to survive in different human compartments for an efficient infection cycle.

Keywords: *S.* Typhi, pH, Macrophages

Introduction

Escherichia coli and *Salmonella* use specific genetic strategies to sense, respond, and survive at diverse pH values present in its human host.

E. coli contains five acid-resistance (AR) systems: AR1 (oxidative or glucose-repressed acid resistance system), which is positively regulated by RpoS and CRP (Bak et al. 2014), whereas AR2, AR3, AR4, and AR5 (De Biase and Lund 2015; Kanjee and Houry 2013) work as follows: glutamate decarboxylase for AR2, arginine decarboxylase for AR3, lysine decarboxylase for AR4, and ornithine decarboxylase for AR5, perform cytoplasmic decarboxylations of glutamate, arginine, lysine, and ornithine substrates, respectively. The products resulting from the decarboxylation reaction, γ-aminobutyric acid from AR2, agmatine from AR3, cadaverine from AR4, and putrescine from AR5, are exported to the periplasm by a specific antiporter for each (poly) amine-cognate amino acid pair. Thus, glutamate, arginine, lysine, or ornithine is imported from the periplasm to maintain

* Correspondence: ismael.hernandez@ibt.unam.mx
Departamento de Microbiología Molecular, Instituto de Biotecnología, Universidad Nacional Autónoma de México, 62210 Cuernavaca, Morelos, Mexico

the continuous functioning of the decarboxylases. These enzymes consume protons through their decarboxylation reactions, maintaining the intracellular pH homeostasis and avoiding cellular damage in the pH range of 2.0–2.5 (Audia et al. 2001; De Biase and Lund 2015; Foster 2004; Kanjee and Houry 2013). In addition, an acid resistance system in *E. coli*, the glutamine-dependent AR system (Lu et al. 2013), is widespread in many bacterial species, including those that are part of the human gut microbiome (Pennacchietti et al. 2018).

Another mechanism developed by *Enterobacteriaceae*, including *E. coli*, for protection against low pH values is known as the acid-tolerance response (ATR) (Blattner et al. 1997), defined as the capacity to undergo an adaptive response to moderately acidic pH (4.5–5.8) that enhances the subsequent survival to extreme low pH (3.0) (Álvarez-Ordóñez et al. 2011; Audia et al. 2001; Foster and Hall 1990). Moreover, a recent report showed that the two-component system CpxRA directly senses acidification through protonation of the CpxA periplasmic histidine residues. Therefore, it activates transcription of the *fabA* and *fabB* genes that are essential in biosynthesis of unsaturated fatty acids to enhance the UFA content in membrane lipid, allowing *E. coli* to grow at acid pH (Xu et al. 2020). On the other hand, it has also been reported in *E. coli* that the glycolytic enzymes Glk, PykF, and Pgk are necessary for the rise in ATP under weakly acidic conditions and for survival in markedly acidic conditions (Zhang et al. 2020).

Thus, the response to acid pH has been well studied in *Enterobacteriaceae*, and the knowledge of the molecular mechanisms adopted by a range of Gram-positive and Gram-negative bacteria, mostly those affecting human health, for coping with acid stress are described in excellent reviews (Lund et al. 2014; Zhang et al. 2020).

The genetic elements implicated in the ATR have been widely characterized also in the pathogen *Salmonella enterica* serovar Typhimurium and includes the arginine decarboxylase and lysine decarboxylase systems, which are homologous to the AR3 and AR4 systems from *E. coli.* In *Salmonella,* however, these enzymes are induced at pH 4.5–6.4 (Alvarez-Ordóñez et al. 2010). Additionally, the ATR transcriptional regulators RpoS, Fur, PhoP/PhoQ, and OmpR induce the genetic expression of acid shock proteins (ASPs), such as GroEL, DnaK, HtpG, and HtpM, which prevent or repair the macromolecular damage caused by acid stress (Bang et al. 2000; Bearson et al. 1998; Foster 1991; Foster and Hall 1992; Lee et al. 1995). Furthermore, OmpR-regulated genes indicate that it drives a major reprogramming in bacteria in response to acid and osmotic stress (Chakraborty and Kenney 2018).

Interestingly, it has also been observed that other *Salmonella* serovars, such as Agona, Anatum, Enteritidis, Gaminara, Heidelberg, Javiana, Mbandaka, Michigan, Montevideo, Poona, Reading, Saintpaul, or Seftenberg, that are exposed to mild acid pH (4.3–5.8) displayed an increased resistance to extreme acid pH (3.0) (Alvarez-Ordóñez et al. 2009; Bacon et al. 2003; Leyer and Johnson 1992; Yuk and Schneider 2006). However, it is unknown whether these *Salmonella* serovars utilize the same genetic elements as *S.* Typhimurium for ATR response.

In *Salmonella*, acid pH regulates SPI-2 (*Salmonella* Pathogenicity Island 2) by controlling the SsrAB two-component system (Liew et al. 2019). Thus, this bacterium uses acid pH as a signal to drive a pathogenic infection process (Kenney 2019).

In *Salmonella enterica* serovar Typhi, an intracellular pathogen that causes typhoid fever in humans, the genetic elements involved in the resistance to acid or alkaline pH have not been characterized. As for any enteric pathogen, it is relevant to develop an efficient pH response, since it needs to survive to diverse pH values present in the saliva (6.3–7.3), stomach (1.5–4.0), macrophages (4.5), small intestine (7.5), and gallbladder (7.8) (Booijink et al. 2007; Evans et al. 1988; Steele-Mortimer 2008; Sutor and Wilkie 1976). Therefore, we describe herein the analysis of three *S.* Typhi genomic regions involved in resistance to acid and circumneutral pH, which are also fundamental for the invasion and replication of the bacteria in human macrophages.

Materials and methods

Bacterial strains and culture conditions

The bacterial strains used are listed in Table 1. *S.* Typhi IMSS-1 strains were grown aerobically at 37°C in Luria-Bertani (LB) [10 g tryptone, 5 g yeast extract, and 10 g NaCl per liter]. For growth rate assays in acid pH (4.5, 5.5, and 6.5), the medium was buffered with 80 mM MES [2-(N-morpholino)ethanesulfonic acid] (Castanheira et al. 2017). In the case of growth rate assays in neutral and alkaline pH (7.0, 7.5, and 8.0), the medium was buffered with 200 mM Tris-HCl (Medina-Aparicio et al. 2017). When required, kanamycin (Km) 30 μg ml^{-1} or ampicillin (Ap) 100 μg ml^{-1} was added to the growth media.

Construction of *S.* Typhi mutants

Forty deletions, ranging from 6 to 80 kb, of the *S.* Typhi genome were independently obtained using the mutagenesis procedure described by Datsenko and Wanner (2000). The target DNA fragment was replaced with selectable antibiotic resistance gene markers. Then, the resistance cassette was removed using the pCP20 plasmid. Each deletion was further characterized by sequencing to verify the authenticity. Individual gene deletions were also performed with the methodology mentioned before (Datsenko and Wanner 2000).

Table 1 Bacterial strains used in this study

Bacterial strains	Relevant characteristics	Reference
S. Typhi IMSS-1	*Salmonella enterica* serovar Typhi Vi serotype. Mexican clinical reference strain.	Puente et al. (1987)
S. Typhi IMSS-1 Δ*leuO*::km^r	*S.* Typhi containing a deletion of the LysR-type regulator LeuO	Hernández-Lucas et al. (2008)
S. Typhi IMSS-1 ΔSTY0159	*S.* Typhi containing a deletion of the LysR-type regulator STY0159	This study
S. Typhi IMSS-1 ΔSTY0277	*S.* Typhi containing a deletion of the LysR-type regulator STY0277	This study
S. Typhi IMSS-1 ΔSTY0341	*S.* Typhi containing a deletion of the LysR-type regulator STY0341	This study
S. Typhi IMSS-1 ΔSTY0651	*S.* Typhi containing a deletion of the LysR-type regulator STY0651	This study
S. Typhi IMSS-1 ΔSTY0730	*S.* Typhi containing a deletion of the LysR-type regulator STY0730	This study
S. Typhi IMSS-1 ΔSTY1693	*S.* Typhi containing a deletion of the LysR-type regulator STY1693	This study
S. Typhi IMSS-1 ΔSTY2510	*S.* Typhi containing a deletion of the LysR-type regulator STY2510	This study
S. Typhi IMSS-1 ΔSTY2660	*S.* Typhi containing a deletion of the LysR-type regulator STY2660	This study
S. Typhi IMSS-1 ΔSTY2821	*S.* Typhi containing a deletion of the LysR-type regulator STY2821	This study
S. Typhi IMSS-1 ΔSTY3158	*S.* Typhi containing a deletion of the LysR-type regulator STY3158	This study
S. Typhi IMSS-1 ΔSTY3293	*S.* Typhi containing a deletion of the LysR-type regulator STY3293	This study
S. Typhi IMSS-1 ΔSTY3415	*S.* Typhi containing a deletion of the LysR-type regulator STY3415	This study
S. Typhi IMSS-1 ΔSTY3547	*S.* Typhi containing a deletion of the LysR-type regulator STY3547	This study
S. Typhi IMSS-1 ΔSTY4196	*S.* Typhi containing a deletion of the LysR-type regulator STY4196	This study
S. Typhi IMSS-1 ΔSTY4468	*S.* Typhi containing a deletion of the LysR-type regulator STY4468	This study
S. Typhi IMSS-1 Δ*ltrR*	*S.* Typhi containing a deletion of the LysR-type regulator LtrR	Villareal et al. (2014)
S. Typhi IMSS-1 Δ*ltrR*/Δ*ompA*::km^r	*S.* Typhi containing two deletions, one of them correspond to the LysR-type regulator LtrR and the second deletion correspond to the OmpA outer membrane protein	This study
S. Typhi IMSS-1 Δ*ltrR*/Δ*ompR*::km^r	*S.* Typhi containing two deletions, one of them correspond to the LysR-type regulator LtrR and the second deletion correspond to the regulator OmpR	This study
S. Typhi IMSS-1 Δ*pmrAB*::km^r	*S.* Typhi containing a deletion in the two component system PmrAB	Medina-Aparicio et al. (2017)
S. Typhi IMSS-1 Δ*phoP*::km^r	*S.* Typhi containing a deletion in the two component system PhoP	Medina-Aparicio et al. (2017)
S. Typhi IMSS-1 Δ*narP*::km^r	*S.* Typhi containing a deletion in the two component system NarP	This study
S. Typhi IMSS-1 Δ*soxRS*::km^r	*S.* Typhi containing a deletion in the two component system SoxRS	This study
S. Typhi IMSS-1 Δ*arcA*	*S.* Typhi containing a deletion in the two component system ArcA	This study
S. Typhi IMSS-1 Δ*cpxRA*::km^r	*S.* Typhi containing a deletion in the two component system CpxRA	Medina-Aparicio et al. (2017)
S. Typhi IMSS-1 Δ*ssrB*::km^r	*S.* Typhi containing a deletion in the two component system SsrB	Medina-Aparicio et al. (2017)
S. Typhi IMSS-1 Δ*fis*::km^r	*S.* Typhi containing a deletion in the nucleoid-associated protein FIS	This study
S. Typhi IMSS-1 ΔSTY*hns*99::km^r	*S.* Typhi containing a deletion in the nucleoid-associated protein HNS	Flores-Valdez et al. (2003)
S. Typhi IMSS-1 Δ*lrp*::km^r	*S.* Typhi containing a deletion in the nucleoid-associated protein LRP	Medina-Aparicio et al. (2011)
S. Typhi IMSS-1 Δ*lrp*/Δ*hns*::km^r	*S.* Typhi containing two deletions, one of them correspond to the nucleoid-associated protein LRP and the second deletion correspond to the nucleoid-associated protein HNS	Medina-Aparicio et al. (2011)
S. Typhi IMSS-1 Δ*ihfA*::km^r	*S.* Typhi containing a deletion in the nucleoid-associated protein IHFA	Medina-Aparicio et al. (2017)
S. Typhi IMSS-1 Δ*malT*	*S.* Typhi containing a deletion in the LuxR-like MalT protein	This study
S. Typhi IMSS-1 Δ*sdiA*	*S.* Typhi containing a deletion in the LuxR-like SdiA protein	This study
S. Typhi IMSS-1 Δ*luxS*::km^r	*S.* Typhi containing a deletion in the LuxR-like LuxS protein	This study
S. Typhi IMSS-1 Δ*fur*::km^r	*S.* Typhi containing a deletion in the FuR-like FUR protein	This study
S. Typhi IMSS-1 Δ*crp*	*S.* Typhi containing a deletion in the CRP-like CRP protein	Medina-Aparicio et al. (2017)
S. Typhi IMSS-1 Δ*fnr*::km^r	*S.* Typhi containing a deletion in the CRP-FNR-like FNR protein	Medina-Aparicio et al. (2017)
S. Typhi IMSS-1 Δ*ompR*	*S.* Typhi containing a deletion in the OmpR regulator	Villareal et al. (2014)
S. Typhi IMSS-1 ΔSTY0123-STY0125::km^r	*S.* Typhi containing a deletion in the *thiQP-tpbA* genes	This study

Table 1 Bacterial strains used in this study *(Continued)*

Bacterial strains	Relevant characteristics	Reference
S. Typhi IMSS-1 ΔF1::km^r	Deleted from base 5114 to base 11,245 (6.131 kb, STY0005-STY0011).	This study
S. Typhi IMSS-1 ΔF2::km^r	Deleted from base 15,020 to 44,181 (29.161 kb, STY0014-STY0045).	This study
S. Typhi IMSS-1 ΔF3::km^r	Deleted from base 56,686 to base 104,274 (47.588 kb, STY0056-STY0106).	This study
S. Typhi IMSS-1 ΔF4::km^r	Deleted from base 115,571 to base 140,755 (25.184 kb, STY0114-STY0138).	This study
S. Typhi IMSS-1 ΔF14::km^r	Deleted from base 2,493,002 to base 2,499,616 (6.614 kb, STY2655-STY2662).	This study
S. Typhi IMSS-1 ΔF16::km^r	Deleted from base 2,445,015 to base 2,460,717 (15.702 kb, STY2610-STY2625).	This study
S. Typhi IMSS-1 ΔF17::km^r	Deleted from base 2,436,294 to base 2,443,543 (7.249 kb, STY2600-STY2608).	This study
S. Typhi IMSS-1 ΔF18::km^r	Deleted from base 2,400,919 to base 2,433,642 (32.723 kb, STY2564-STY2596).	This study
S. Typhi IMSS-1 ΔF19::km^r	Deleted from base 2,342,588 to base 2,400,843 (58.255 kb, STY2508-STY2563)	This study
S. Typhi IMSS-1 ΔF20::km^r	Deleted from base 2,311,811 to base 2,331,187 (19.376 kb, STY2483-STY2497)	This study
S. Typhi IMSS-1 ΔF22::km^r	Deleted from base 2,200,548 to base 2,215,156 (14.608 kb, STY2370-STY2383)	This study
S. Typhi IMSS-1 ΔF23::km^r	Deleted from base 2,150,560 to base 2 199,512 (48.952 kb, STY2321-STY2368)	This study
S. Typhi IMSS-1 ΔF24::km^r	Deleted from base 2,101,051 to base 2,150,557 (49.506 kb, STY2276-STY2320)	This study
S. Typhi IMSS-1 ΔF25::km^r	Deleted from base 2,052,806 to base 2,097,723 (44.917 kb STY2217-STY2271)	This study
S. Typhi IMSS-1 ΔF28::km^r	Deleted from base 1,936,029 to base 1,954,615 (18.586 kb, STY2083-STY2101)	This study
S. Typhi IMSS-1 ΔF29::km^r	Deleted from base 1,855,187 to base 1,935,559 (80.372 kb, STY1965-STY2081)	This study
S. Typhi IMSS-1 ΔF31::km^r	Deleted from base 1,803,420 to base 1,823,145 (19.725 kb, STY1910-STY1929)	This study
S. Typhi IMSS-1 ΔF36::km^r	Deleted from base 1,701,377 to base 1,712,613 (11.236 kb, STY1779-STY1791)	This study
S. Typhi IMSS-1 ΔF37::km^r	Deleted from base 1,674,560 to base 1,693,618 (19.058 kb, STY1755-STY1771)	This study
S. Typhi IMSS-1 ΔF38::km^r	Deleted from base 1,625,241 to base 1,674,133 (48.892 kb, STY1698-STY1754)	This study
S. Typhi IMSS-1 ΔF40::km^r	Deleted from base 1,581,741 to base 1,601,909 (20.168 kb, STY1653-STY1672)	This study
S. Typhi IMSS-1 ΔF41::km^r	Deleted from base 1,573,199 to base 1,580,739 (7.540 kb, STY1645-STY1651)	This study
S. Typhi IMSS-1 ΔF42::km^r	Deleted from base 1,551,982 to base 1,570,836 (18.854 kb, STY1616-STY1639)	This study
S. Typhi IMSS-1 ΔF43::km^r	Deleted from base 1,511,563 to base 1,551,982 (40.419 kb, STY1562-STY1615)	This study
S. Typhi IMSS-1 ΔF44::km^r	Deleted from base 1,453,776 to base 1,511,522 (57.746 kb, STY1496-STY1561)	This study
S. Typhi IMSS-1 ΔF45::km^r	Deleted from base 1,404,648 to base 1,451,382 (46.734 kb, STY1454-STY1493)	This study
S. Typhi IMSS-1 ΔF46::km^r	Deleted from base 1,359,382 to base 1,403,895 (44.513 kb, STY1409-STY1452)	This study
S. Typhi IMSS-1 ΔF47::km^r	Deleted from base 1,314,180 to base 1,359,236 (45.056 kb, STY1353-STY1408)	This study
S. Typhi IMSS-1 ΔF52::km^r	Deleted from base 1,085,337 to base 1,134,126 (48.789 kb, STY1114-STY1170)	This study
S. Typhi IMSS-1 ΔF54::km^r	Deleted from base 1,017,923 to base 1,058,825 (40.902 kb, STY1024-STY1081)	This study
S. Typhi IMSS-1 ΔF55::km^r	Deleted from base 1,003,596 to base 1,014,693 (11.097 kb, STY1005-STY1019)	This study
S. Typhi IMSS-1 ΔF59::km^r	Deleted from base 794,269 to base 849,155 (54.886 kb, STY0797-STY0854)	This study
S. Typhi IMSS-1 ΔF64::km^r	Deleted from base 606,760 to base 633,465 (26.705 kb, STY0605-STY0631)	This study
S. Typhi IMSS-1 ΔF73::km^r	Deleted from base 302,488 to base 366,847 (64.359 kb, STY0286-STY0357)	This study
S. Typhi IMSS-1 ΔF77::km^r	Deleted from base 2,876,422 to base 2,918,810 (42.388 kb, STY3004- STY3052)	This study
S. Typhi IMSS-1 ΔF78::km^r	Deleted from base 2,921,547 to base 2,960,653 (39.106 kb, STY3057- STY3094)	This study
S. Typhi IMSS-1 ΔF95::km^r	Deleted from base 3,526,926 to base 3,552,416 (25.490 kb, STY3674- STY3708)	This study
S. Typhi IMSS-1 ΔF107::km^r	Deleted from base 4,035,317 to base 4,105,650 (70.333 kb, STY4176- STY4239)	This study
S. Typhi IMSS-1 ΔF112::km^r	Deleted from base 4,367,185 to base 4,408,807 (41.622 kb, STY4481- STY4519)	This study
S. Typhi IMSS-1 ΔF114::km^r	Deleted from base 4,421,390 to base 4,472,347 (50.957 kb, STY4534- STY4596)	This study

Bacterial growth rate assays

S. Typhi IMSS-1, individual single-gene mutants, as well as strains with deletions of long DNA fragments was grown for 24 h on LB plates at 37°C. A bacterial colony was inoculated in liquid LB (5 ml) and grown aerobically at 37°C/16h. Then, 100 ml of LB medium at the corresponding pH value was inoculated with the pre-inoculum to give an initial optical density (OD) at 595 nm of 0.030.

The cultures were grown aerobically at 37°C for 12 h, with OD_{595} measurements being done every 2 h, using a spectrophotometer (Ultrospec 2100 *pro*, Amersham Bioscience).

Macrophage assays

The human monocyte cell line THP-1 was maintained in RPMI (Roswell Park Memorial Institute) (Sigma) medium supplemented with 2 mM L-glutamine, 10% fetal bovine serum (ByProducts), and 0.05 mM β-mercaptoethanol. The THP-1 monocyte cells were seeded at 1×10^5 cells per well in 24-well tissue-culture dishes and were differentiated to macrophages by addition of 50 ng ml^{-1} phorbol 12-myristate 13-acetate (PMA) for 24 h (Starr et al. 2018). Macrophage differentiation for 24 h is adequate, and several articles including Zeng et al. (2015), Park et al. (2007), Fitzgerald et al. (2000), and Madhvi et al. (2019) have proven it. The medium with PMA was removed, followed by the addition of 500 μl of fresh RPMI.

Wild-type *S.* Typhi and mutant strains were grown aerobically at 37°C in LB, collected at OD_{595} of 1.0 and pelleted at 1200*g* for 2 min. The cells were re-suspended in 1X phosphate-buffered saline (PBS). 2×10^5 THP-1 differentiated cells in each well were used for infection. Several authors including Park et al. (2007), Fitzgerald et al. (2000), and Madhvi et al. (2019) have used this amount of cell for infection. Macrophages were infected in duplicate with the bacterial strains at a multiplicity of infection (MOI) of 1:10, and the plates were incubated at 37°C in a 5% CO_2 atmosphere. After incubation for 20 min at 37°C to allow adhesion, extracellular bacteria were removed by washing cells twice with 500 μl 1X PBS. Then, 500 μl of RPMI, supplemented with 100 μg ml^{-1} gentamicin, was added to the wells for 2 h to eliminate extracellular bacteria. The medium was removed, and each well was washed with 500 μl PBS 1X. Eukaryotic cells were lysed by addition of 100 μl lysis solution (0.1% SDS, 1% Triton X-100 dissolved in 1X PBS) for 5 min (37°C, 5% CO_2 atm). Four hundred microliters of PBS was added, and intracellular bacteria were collected. Serial dilutions of the bacterial suspensions were plated in duplicate on LB agar plates to determine the colony-forming units (CFUs). The CFUs obtained indicate those bacteria that were able to invade the eukaryotic cell.

Evaluation of the replicative capacity was performed with the protocol described above. After infecting and removing the RPMI-gentamicin 100 μg ml^{-1} for 2 h, the wells containing macrophages were washed twice with 500 μl PBS 1X, and immediately, 500 μl RPMI, supplemented with gentamicin 10 μg ml^{-1}, was added. The THP-1 macrophages were incubated for 16 h at 37°C, 5% CO_2 atmosphere. Then, eukaryotic cells were lysed and plated in LB agar. The resulting number of bacteria corresponded to the replication capacity of the strain evaluated. Uninfected macrophage controls were included in the assays.

Results

S. Typhi genomic regions involved in acid and circumneutral pH resistance

To survive in free life and in the human host, *S.* Typhi must overcome numerous complex extracellular and intracellular environments, and one selective factor in any ecological niche is the pH. To determine the pH range at which the *S.* Typhi IMSS-1 wild type can survive, it was grown in LB medium at different pH values. The results showed that it was able to proliferate in pH 4.5, 5.5, 6.5, and 7.5, whereas at pH 1.0, 2.0, 3.0, 4.0, or 8.0, it was unable to replicate (Fig. 1).

Thus, to identify genes involved in *S.* Typhi pH resistance, 37 single-gene isogenic mutants, including the global regulatory transcriptional factors: CRP, FNR, IHF, FIS, H-NS, Lrp, ArcA, FuR, OmpR, CpxR/CpxA, SoxR, PhoP; sixteen LysR-type regulators (STY0159, STY0277, STY0341, STY0651, STY0730, STY1693, STY2510, STY2660, STY2821, STY3158, STY3293, STY3415, STY3547, STY4196, STY4468, and LtrR); other two-component regulators (PmrA/PmrB, NarP, and SsrB); LuxR-like proteins (MalT, SdiA, and LuxS); and double Δ*lrp*/Δ*hns*, Δ*ltrR*/Δ*ompA*, and Δ*ltrR*/Δ*ompR* mutant strains (Table 1) were evaluated in LB at acidic and circumneutral pH (4.5 and 7.5). The results of growth rate experiments showed that none of these transcriptional factors evaluated on their own had a role in pH resistance, since the corresponding mutants grew as the wild-type strain (data not shown). It is relevant to mention that many of these regulatory proteins are global regulators, fundamental in free-living cells and in the pathogenesis of many enterobacterial species, since they modulate a large number of genes in different conditions. Therefore, it was surprising that they did not show a role in *S.* Typhi pH resistance when tested individually.

Based on this result, several deletions of the bacterial chromosome were generated. Forty mutant strains containing deletions from 6 to 80 kb were obtained (Table 1). Thus, a total of 1.5 MB of the genome, represented by various deletions, were analyzed for its role in bacterial growth at pH 4.5, 5.5, 6.5, and 7.5. The results showed that *S.* Typhi ΔF4 with a deletion of 25 kb, *S.* Typhi ΔF44 with a deletion of 58 kb, and *S.* Typhi ΔF73 lacking 64 kb presented a deficiency for growing at acid and circumneutral pH, as compared with the wild-type strain.

Notably, *S.* Typhi ΔF4 showed 48% of growth rate, measured by OD_{595}, and a 35% in the number of viable cells at pH 4.5 after 12 h of incubation, as compared

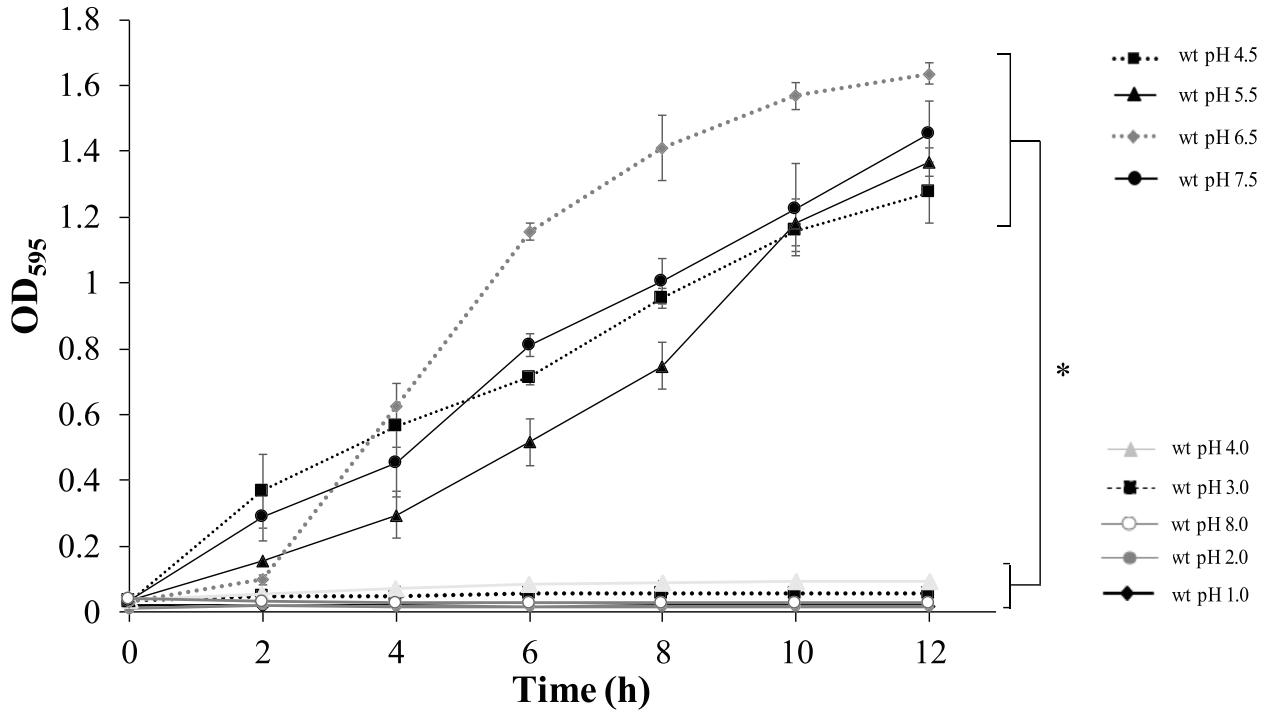


Fig. 1 Growth kinetics of *S.* Typhi IMSS-1 at different pH values. *S.* Typhi IMSS-1 wild-type strain was grown in LB medium at pH 1.0, 2.0, 3.0, 4.0, 4.5, 5.5, 6.5, 7.5, and 8.0. The cultures were incubated at 37°C, 200 rpm. Mean±standard deviation is presented. Statistical significance (*$P < 0.05$) was determined using 1-way ANOVA with Tukey post hoc test

with the values displayed by the wild-type strain. *S.* Typhi ΔF4 grew similar as the parental *S.* Typhi IMSS-1 in pH 5.5, 6.5, and 7.5 (Fig. 2).

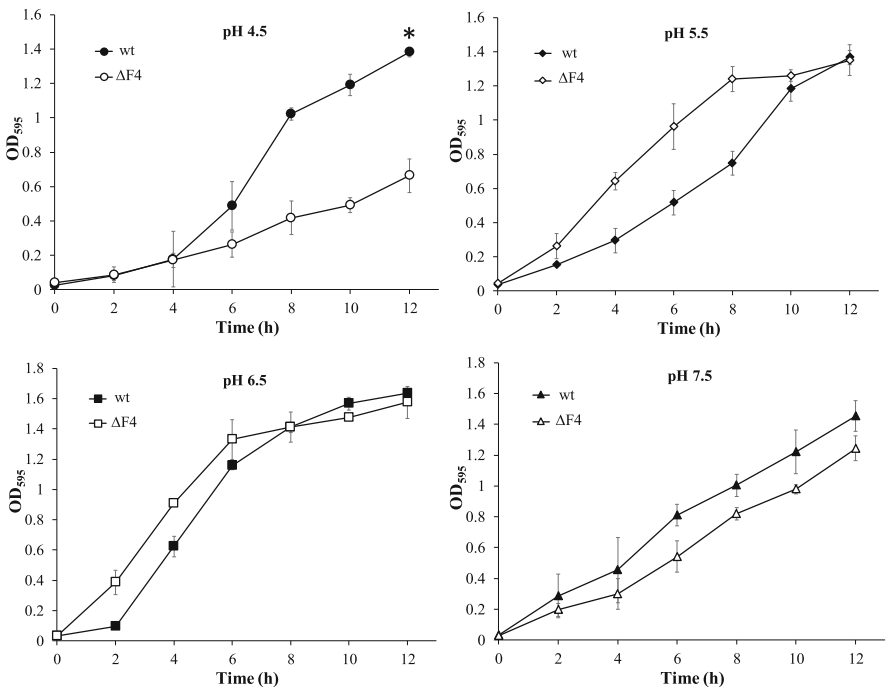
In the case of *S.* Typhi ΔF44 grown in LB pH 4.5, it showed a 76% of growth kinetics measured by OD_{595} and a 59% of CFU ml^{-1} at 12 h, with regard to the corresponding values of the parental IMSS-1 strain. This mutant strain also presented a clear deficiency to grow at pH 5.5, since its growth rate measured by OD_{595} was of 54% over the entire growth curve, and the number of viable cells obtained after 12 h of incubation was only of 43%, with regard to the corresponding values observed with *S.* Typhi wild type (Fig. 3). *S.* Typhi ΔF44 at pH 6.5 presented a growth rate of 79% at 8 h, 10 h, and 12 h of incubation, and the CFU ml^{-1} obtained after growing for 12 h was of 59%, as compared to the respective values presented by the wild-type strain. Finally, *S.* Typhi ΔF44 and *S.* Typhi IMSS-1 wild type presented a similar number of viable cells and growth kinetics in pH 7.5 (Fig. 3). These results suggested that the absent genes in *S.* Typhi ΔF44 have a relevant role in the resistance of *S.* Typhi to pH 5.5 and contribute to the bacterial acid tolerance of pH 4.5 and 6.5.

S. Typhi ΔF73 presented a defect in growth at pH 4.5, 5.5, 6.5, and 7.5, since its growth rates measured by OD_{595} were of 64%, 66%, 47%, and 74%, respectively, at 12 h of incubation, whereas the number of viable cells obtained were of 45%, 43%, 33%, and 54%, at the pH values mentioned. These data were relative to the corresponding results of the wild-type strain (Fig. 4). Therefore, *S.* Typhi ΔF73 lacks genetic elements for an efficient replication at pH 6.5, and they also participate for optimal growth at pH 4.5, 5.5, and 7.5 (Fig. 5).

The three mutant strains mentioned above displayed a similar growth rate kinetics as the wild type in LB broth, without the corresponding MES or Tris-HCl buffers used for maintaining acid or circumneutral pH in the growth experiments. Therefore, the results showed that *S.* Typhi utilized different types of genomic regions to survive in acid or circumneutral pH.

S. Typhi genomic regions involved in pH resistance are also relevant for invasion and replication in human macrophages

S. Typhi ΔF4 was evaluated in its capacity to invade, replicate, and survive inside macrophages. *S.* Typhi wild-type strain and *S.* Typhi ΔF4 were used for infection experiments in human monocyte cell line THP-1. In comparison to the wild-type strain, *S.* Typhi ΔF4 had a 91% decrease in its ability to invade macrophages (Fig. 6a) and was unable to replicate and survive inside these cells (Fig. 6b). Furthermore, Cohen's analysis (Cohen 1992; Sawilosky 2009) showed a large effect in the ability to invade and replicate inside macrophages between the *S.*



	OD_{595} 12 h	% OD ΔF4/wt	CFU ml^{-1} 12 h	% CFU ml^{-1} ΔF4/wt
wt pH 4.5	1.38		716 x 10^6	
ΔF4 pH 4.5	0.66	48	248 x 10^6	35
wt pH 5.5	1.37		696 x 10^6	
ΔF4 pH 5.5	1.35	99	672 x 10^6	97
wt pH 6.5	1.64		936 x 10^6	
ΔF4 pH 6.5	1.58	96	904 x 10^6	97
wt pH 7.5	1.46		724 x 10^6	
ΔF4 pH 7.5	1.24	85	548 x 10^6	76

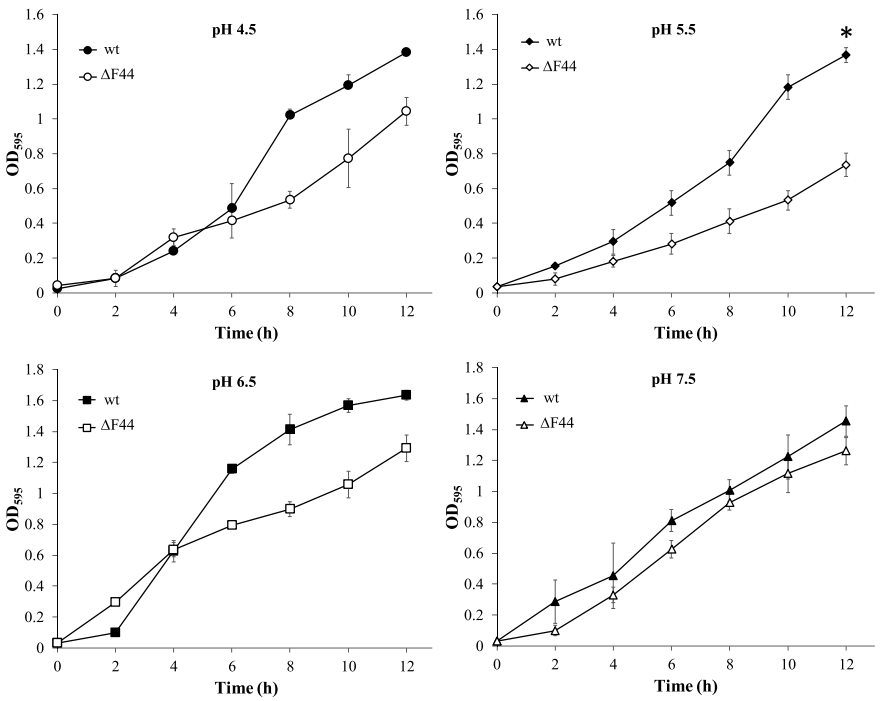
Fig. 2 Kinetics of pH resistance of the *S.* Typhi wild-type and *S.* Typhi ΔF4. Growth kinetics of *S.* Typhi wt and *S.* Typhi ΔF4 in LB at pH 4.5, 5.5, 6.5, and 7.5. The right columns indicate the OD_{595} obtained after 12 h of incubation, as well as the growth percentage measured by OD_{595} of the mutant versus wild-type strain (100%) at different pH values. The number of viable cells at 12 h (CFU ml^{-1}) and the growth percentage measured by CFU ml^{-1} of the deleted strain versus *S.* Typhi IMSS-1 (100%) at the pH values evaluated are also shown. Mean±standard deviation is presented. Statistical significance was determined using Student's *t*-test (*$P < 0.05$)

Typhi wild-type and the *S.* Typhi ΔF4 (Fig. 6c). Supporting the notion that the genes deleted in *S.* Typhi ΔF4 are fundamental to invade and replicate inside the eukaryotic cells. It is probable that the phenotypes mentioned are due to the lack of the *yabI* gene, which is involved in cell division (Boughner and Doerrler 2012) and is absent in *S.* Typhi ΔF4. This strain also lacks genes involved in thiamin transport and arabinose, fructose, and leucine metabolism, and it is well-known that enzymes for catabolism of sugars and amino acids show a pH dependence. During adaptation and challenge with acid, *S.* Typhimurium has shown an altered expression of a large proportion of its genome associated with metabolic pathways. Moreover, several genes involved in energy metabolism showed significant upregulation, including those for glycolysis, the citric acid cycle, and the pentose phosphate pathway (Ryan et al. 2015). Thus, the phenotype observed for *S.* Typhi ΔF4 in free living, and in the interaction with human cells, could be associated to more than one gene.

The capacity of *S.* Typhi ΔF44 to invade, replicate, and survive in phagocytic cells was also evaluated in this work. This strain had a reduced capacity to invade macrophages, since the CFU ml^{-1} obtained at 2-h post-infection corresponded to 31% with respect to the values observed in *S.* Typhi IMSS-1 (Fig. 6a). *S.* Typhi ΔF44 was unable to replicate inside phagocytic cells. Moreover, of the 9.7×10^4 bacteria that invaded the THP-1 cells, only 5.5×10^4 (56%) were obtained at 16-h post-infection, demonstrating that this mutant strain is defective in its persistence inside macrophages (Fig. 6b). The data mentioned before is supported by a Cohen's analysis (Cohen 1992; Sawilosky 2009), showing that the *S.* Typhi ΔF44 presented a dramatic inability to invade and replicate inside macrophages compared to the *S.* Typhi wild-type strain (Fig. 6c).

It is probable that the deficiency in growth of *S.* Typhi ΔF44 at pH 4.5, 5.5, and 6.5, as well as its reduced capacity to invade and survive in the THP-1 cells, is due to the loss of the Hya hydrogenase component genes and of the *marA*, *marB*, or *marR* genes. However, it is relevant to note that other genes included in this fragment, such as *osmC*, are expressed during infection and replication inside macrophages.

S. Typhi ΔF73 have a deletion of 64 kb, and since some ORFs contained in this fragment are expressed when *S.* Typhi interacts with eukaryotic cells, human macrophage infection assays were performed with *S.* Typhi and *S.* Typhi ΔF73 strains and revealed a 94% decrease in invasion of *S.* Typhi ΔF73 as compared with the wild-type strain (Fig. 6a). At 16-h post-infection, the number of viable cells was like those that invaded macrophages at 2-h post-infection, indicating that the mutant strain had the ability to survive inside phagocytic



	OD 12 h	% OD ΔF44/wt	CFU ml^{-1} 12 h	% CFU ml^{-1} ΔF44/wt
wt pH 4.5	1.38		660 x 10^6	
ΔF44 pH 4.5	1.05	76	392 x 10^6	59
wt pH 5.5	1.37		724 x 10^6	
ΔF44 pH 5.5	0.74	54	312 x 10^6	43
wt pH 6.5	1.64		884 x 10^6	
ΔF44 pH 6.5	1.29	79	524 x 10^6	59
wt pH 7.5	1.45		732 x 10^6	
ΔF44 pH 7.5	1.26	87	564 x 10^6	77

Fig. 3 Kinetics of pH resistance of the *S.* Typhi wild-type and *S.* Typhi ΔF44. Growth kinetics of *S.* Typhi wt and *S.* Typhi ΔF44 in LB at pH 4.5, 5.5, 6.5, and 7.5. The right columns indicate the OD_{595} obtained after 12 h of incubation, as well as the growth percentage measured by OD_{595} of the mutant versus wild-type strain (100%) at different pH values. The number of viable cells at 12 h (CFU ml^{-1}) and the growth percentage measured by CFU ml^{-1} of the deleted strain versus *S.* Typhi IMSS-1 (100%) at the pH values evaluated are also shown. Mean±standard deviation is presented. Statistical significance was determined using Student's *t*-test (*$P < 0.05$)

cells but is unable to replicate (Fig. 6b). In addition, Cohen's analysis (Cohen 1992; Sawilosky 2009) reflects the incapacity of *S.* Typhi ΔF73 to efficiently invade and replicate inside macrophages, comparing to *S.* Typhi wild-type strain (Fig. 6c).

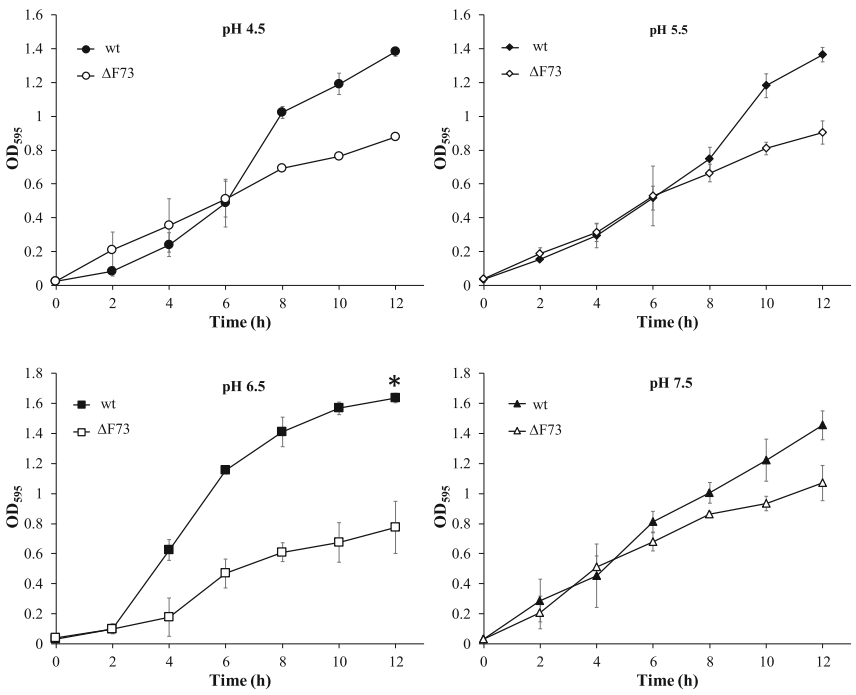
The *S.* Typhi ΔF73 strain lacks the STY0332-STY0337 and STY0345-STY0348 clusters that correspond to the *safAEBCD* and *tcfABCD* chaperone-usher fimbriae operons, respectively. The *S.* Typhi ISP1820 strain deleted in *saf* (Δ*saf*) or *tcf* (Δ*tcf*) fimbrial operons had a reduced ability towards invading intestinal epithelial INT-407 cells, as compared with the wild type. Interaction of *S.* Typhi ISP1820 Δ*saf* or Δ*tcf* with THP-1 macrophages was assessed for phagocytosis or uptake (20 min). The deletion of *saf* or *tcf* cluster decreased phagocytosis to 65% of the values observed with the wild-type strain (Dufresne et al. 2018). The data mentioned above agree with the phenotype reported here for the invasion of THP-1 cells by *S.* Typhi ΔF73.

Since other genes present in the 64 kb region deleted in this mutant are expressed when *S.* Typhi infects macrophages, such as genes that encode the hypothetical proteins STY0300, STY0313, STY0323, STY0326, and STY0338, the defect of *S.* Typhi ΔF73 to efficiently grow at several pH values and to invade and replicate in eukaryotic cells needs further analysis to identify the specific genes involved in these processes.

Discussion

Enteric pathogens are often exposed to environmental stresses, among which fluctuations in pH are the most frequent. Acid, circumneutral, or alkaline environments are encountered in the saliva (6.3–7.3), stomach (1.5–3.5), gastrointestinal track (7.0–7.5), or macrophages (4.5) (Booijink et al. 2007; Evans et al. 1988; Steele-Mortimer 2008; Sutor and Wilkie 1976). Thus, it is expected that human pathogenic bacteria have developed different pH-protective mechanisms. *Salmonella* modulates pH homeostasis by modifications of the lipid content of the membrane (Alvarez-Ordóñez et al. 2008). Additionally, the ATR protects *Salmonella* spp. at pH levels of 3.0, although it is activated when environmental pH values are between 4.8 and 6.0 and when pH homeostasis fails. Furthermore, this human pathogen contains 43 acid shock proteins that act to prevent and repair the damage occasioned to macromolecules by the acids (Keerthirathne et al. 2016) Therefore, *Salmonella* contains different responses to survive at different levels of acidic pH.

In this sense, previously, we reported that *S.* Typhi IMSS-1 at pH of 7.5 induces the expression of the LysR-



	OD 12 h	% OD ΔF73/wt	CFU ml^{-1} 12 h	% CFU ml^{-1} ΔF73/wt
wt pH 4.5	1.38		660 x 10^6	
ΔF73 pH 4.5	0.88	64	300 x 10^6	45
wt pH 5.5	1.37		724 x 10^6	
ΔF73 pH 5.5	0.91	66	256 x 10^6	43
wt pH 6.5	1.64		884 x 10^6	
ΔF73 pH 6.5	0.77	47	296 x 10^6	33
wt pH 7.5	1.45		732 x 10^6	
ΔF73 pH 7.5	1.07	74	392 x 10^6	54

Fig. 4 Kinetics of pH resistance of the *S.* Typhi wild-type and *S.* Typhi ΔF73. Growth kinetics of *S.* Typhi wt and *S.* Typhi ΔF73 in LB at pH 4.5, 5.5, 6.5, and 7.5. The right columns indicate the OD_{595} obtained after 12 h of incubation, as well as the growth percentage measured by OD_{595} of the mutant versus wild-type strain (100%) at different pH values. The number of viable cells at 12 h (CFU ml^{-1}) and the growth percentage measured by CFU ml^{-1} of the deleted strain versus *S.* Typhi IMSS-1 (100%) at the pH values evaluated are also shown. Mean±standard deviation is presented. Statistical significance was determined using Student's *t*-test (*$P < 0.05$)

type transcriptional regulator LtrR and the CRISPR-*cas* locus (Medina-Aparicio et al. 2017; Rebollar-Flores et al. 2020). Nevertheless, the absence of these genetic elements did not affect the growth of the bacteria at pH 7.5. Furthermore, *S.* Typhi at pH 4.5 induces the expression of the flagellin FliC (Jindal et al. 2012), but its role in the resistance to acid pH has not been described. Therefore, we decided to identify genetic determinants involved in pH resistance in this human pathogen, since these genes could be essential for *S.* Typhi pathogenic process.

Initially, a collection of individual mutants in genes coding for global regulators was evaluated for growth and survival in acid and circumneutral pH. Thus, the role of nucleoid-like proteins, two-component systems, and LysR-type and LuxR-type regulatory proteins (Table 1) in pH resistance was analyzed, finding that these genetic elements by themselves are not involved in the acid or circumneutral pH response (data not shown). These results suggest that pH resistance is regulated by novel transcriptional factors or signals not described in *S.* Typhi.

These data also indicated that the pH response between *Salmonella* serovars could be different, since it is well-known that the *S.* Typhimurium *phoP* mutant showed greatly increased sensitivity to acid pH, suggesting that this system may have a role in detecting a decrease in environmental pH (Bearson et al. 1998). Furthermore, mutations in the *fur* locus eliminate induction of several acid pH-inducible genes, prevent synthesis of the inducible pH homeostasis system, and thus confer an extremely acid-sensitive phenotype in *S.* Typhimurium (Foster and Hall 1992). Additionally, OmpR induces the genetic expression of acid shock proteins in *S.* Typhimurium (Bang et al. 2000). However, the *S.* Typhi *phoP*, *ompR*, and *fur* mutants grew like the wild-type strain at pH 4.5 and 7.5, suggesting that *S.* Typhi uses different genetic regulatory proteins than *S.* Typhimurium to sense and respond to environmental pH.

Moreover, *S.* Typhi is 200–2000 times more susceptible to lethal acidity as compared to *S.* Typhimurium (Tiwari et al. 2004). In this respect, *S.* Typhi is a restricted human host pathogen, and its requirements to survive in pH are different to those of *S.* Typhimurium, since the latter is able to colonize vegetables, fruits, animals, and humans. Therefore, the knowledge of *S.* Typhi genetic determinants involved in pH survival is fundamental.

In this work, by analysis of bacterial strains deleted of specific regions of the genome, we reported specific genetic fragments involved in *S.* Typhi pH resistance and in

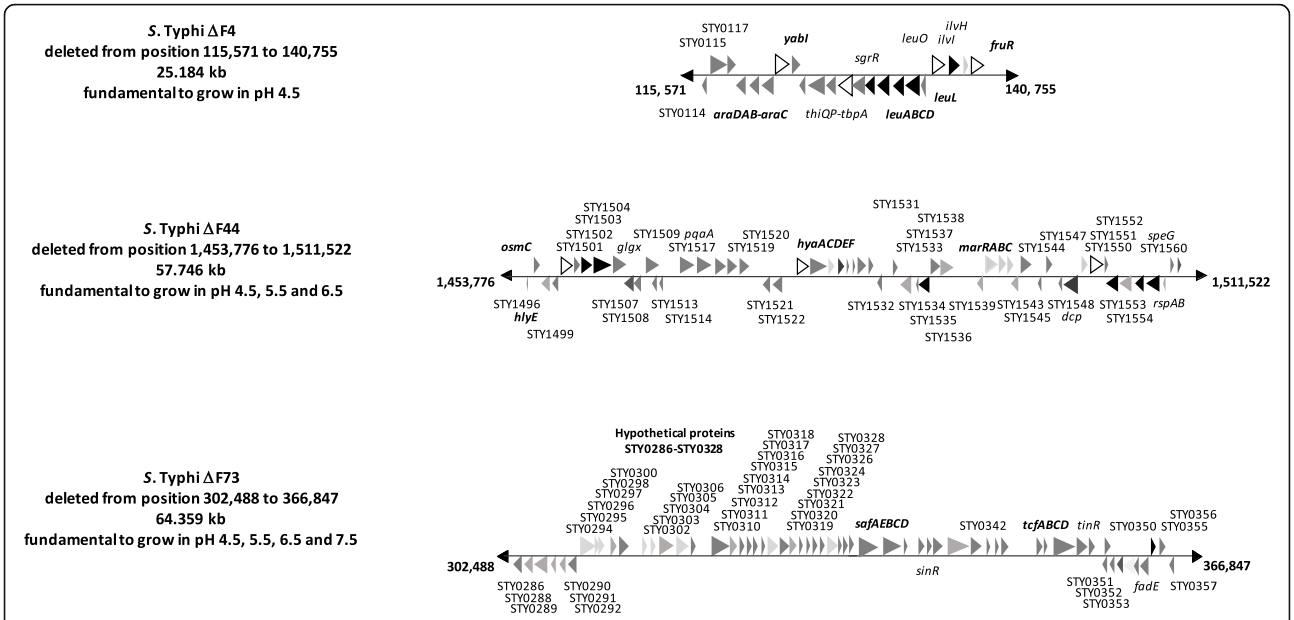
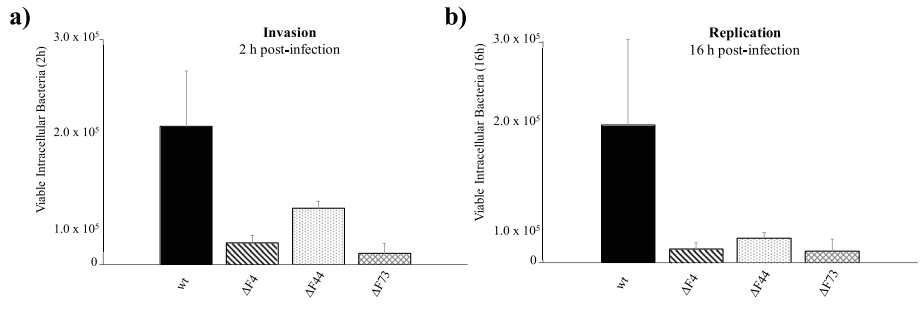


Fig. 5 Schematic representation of the DNA fragments deleted in *S.* Typhi ΔF4, *S.* Typhi ΔF44, or *S.* Typhi ΔF73 strains that were less resistant to different pH values. Genes probably involved in invasion and replication of macrophages are indicated in bold. *S.* Typhi ΔF4 lack two putative IS element transposases (STY0114, STY0115), one hypothetical protein (STY0117), an operon involved in L-arabinose metabolism (*araDAB-araC* or STY0118-STY0121), a DedA-family integral membrane protein (*yabI* or STY0122), the thiamine operon (a thiamine transporter ATP-binding subunit, thiamine ABC transporter membrane component and thiamine-binding periplasmic protein precursor, *thiQP-tbpA*, or STY0123-STY0124-STY0125), a transcriptional regulator *sgrR* (STY0127), the enzymes responsible for the biosynthesis of leucine from valine (*leuABCD* or STY0129 to STY0132), the *leu* operon leader peptide (*leuL* or STY0133), the leucine transcriptional activator LeuO (*leuO* or STY0134), the acetolactate synthase III large and small subunits (*ilvI* and *ilvH* or STY0135 and STY0136), and the gene that encodes a fructose repressor (*fruR* or STY0138). *S.* Typhi ΔF44, this fragment lacks genes such as a hypothetical protein (STY1496), the *osmC* osmotically inducible protein C (*osmC* or STY1497), HlyE hemolysin (*hylE* or STY1498), a hypothetical protein (STY1499), two putative secreted protein (STY1501 and STY1502), putative glycogen debranching protein (*glgx* or STY1505), the putative aminotransferase (STY1507), a hypothetical protein (STY1509), putative isomerase (STY1513), putative regulatory protein (STY1514), putative multidrug efflux protein (STY1517), hypothetical protein (STY1518), membrane transport protein (STY1519), putative alcohol dehydrogenase (STY1520), putative regulatory protein (STY1521), putative secreted hydrolase (STY1522), the hydrogenases (*hyaACDEF* or STY1523-STY1530), putative ATP/GTP-binding protein (STY1531), hypothetical proteins (STY1533-STY1535), putative aldehyde-dehydrogenase (STY1536), putative regulatory protein (STY1537), sugar efflux transporter (STY1538), hypothetical protein (STY1539), the multiple antibiotic resistance protein *marR*, *marA*, and *marB* (STY1540-STY1542), two hypothetical proteins (STY1543-STY1544), putative periplasmic (STY1545), a competence damage-inducible protein A (STY1547), hypothetical protein (STY1548), dipeptidyl carboxypeptidase II (*dcp* or STY1549), putative oxydoreductase (STY1550), putative regulatory protein (STY1551), hypothetical protein (STY1552), putative membrane transport protein (STYSTY1554), starvation-sensing protein (*rspAB* or STY1555-STY1556), hypothetical protein (STY1558), putative secreted protein (STY1560), and a spermidine N1-acetyltransferase (*speG* or STY1561). *S.* Typhi ΔF73, genetic elements deleted in this fragment are hypothetical proteins (STY0286-STY0328), *safAEBCD* fimbrial assembly (STY0332-STY0337), *sinR* transcriptional regulator (STY0341), hypothetical protein (STY0342), *tcfABCD* fimbrial assembly (STY0345-STY0348), *tinR* transcriptional regulator (STY0349), hypothetical protein (STY0350), possible outer membrane adhesion (STY0351), probable secreted protein (STY0352), possible hydrolase (STY0353), possible acyl-CoA dehydrogenase (*fadE* or STY0354), phosphopentose isomerase (STY0355), and hypothetical proteins (STY0356-STY0357)

the invasion, replication, and survival inside human macrophages.

S. Typhi ΔF4 had a 25 kb fragment deleted, from position 115,571 to 140,755 (21 ORFs) according to the *S.* Typhi CT18 genome (Parkhill et al. 2001) (Fig. 5), and was unable to grow like the wild type in LB at pH 4.5. Some of the genes contained in this fragment corresponded to transposases, an operon involved in arabinose metabolism, the DedA-family integral membrane protein *yabI*, the thiamine operon *thiQP-tbpA*, the enzymes responsible for the biosynthesis of leucine *leuABCD*, the leucine transcriptional activator LeuO, and a fructose repressor (Fig. 5).

Since LeuO is the only global transcriptional regulator coded in the 25 kb deleted fragment of *S.* Typhi ΔF4, and LeuO is involved in different biological processes including detoxification, virulence, porin synthesis, and regulation of the CRISPR-Cas system in *E. coli* and *Salmonella* (De la Cruz et al. 2007; Dillon et al. 2012; Espinosa and Casadesús 2014; Guadarrama et al. 2014; Hernández-Lucas et al. 2008; Medina-Aparicio et al. 2011; Westra et al. 2010), a *leuO* mutant was obtained. Interestingly, the *S.* Typhi ΔF4 also lacks genes involved in thiamine transport (*thiQP-tbpA*). Since ABC transporters produce ATP hydrolysis that is a source of energy used by the acid resistance systems (Fath and



c)

Strain	Effect size Hedges's g for invasion	Effect size Hedges's g for replication	Cohen´s effect size for invasión and replication
wt/ΔF4	2.1856	2.0022	Large
wt/ΔF44	1.5981	1.8387	Large
wt/ΔF73	1.4757	2.0182	Large

Fig. 6 Genomic regions involved in pH resistance are also involved in *S.* Typhi invasion, replication, and survival inside human macrophages. THP-1 cells were infected with *S.* Typhi IMSS-1 (wt), *S.* Typhi ΔF4 (ΔF4), *S.* Typhi ΔF44 (ΔF44), and *S.* Typhi ΔF73 (ΔF73). The viable intracellular bacteria (CFU ml^{-1}) were determined at 2-h post-infection to evaluate invasion (**a**) and after 16 h to determine replication and survival inside macrophages (**b**). Mean±standard deviation is presented. The Hedges's g (Hedges 1981) effect size was calculated using the esc package in Rstudio (**c**) and Cohen's rule of thumb. Hedges's values of 0.2 indicate small effect, 0.5 indicate medium effect, and 0.8 indicate large effect, shows a large difference between the ability of the wild-type versus the mutant strains to invade and replicate inside macrophages

Kolter 1993), we also generated a *S.* Typhi strain devoid of the *thiQP-tpbA* cluster. The Δ*leuO* and Δ*thiQP-tpbA* strains were evaluated independently in LB at pH 4.5. The results showed that these genes are not involved in pH resistance, since the corresponding mutant strains grew as well as the wild-type strain (data not shown).

S. Typhi ΔF4 also lacks the *yabI* gene that encodes a DedA protein that belongs to a conserved family of inner-membrane proteins. *E. coli* deletions of the *yabI* gene display defects in cell division (Thompkins et al. 2008). Furthermore, transcriptomic analysis performed when *S.* Typhi infects human macrophages THP-1 showed that *yabI* is upregulated at 2-h (invasion) and 24-h (replication) post-infection (Faucher et al. 2006). Additionally, microarray-based studies demonstrated that the *yabI* gene homolog STM0105 increased its expression when *S.* Typhimurium SL1344 infects murine macrophage-like J774-A.1 cells (Eriksson et al. 2003). Based on this data, probably, the *yabI* gene is fundamental for macrophage infection and replication of *S.* Typhi.

The bacterial strain-denominated *S.* Typhi ΔF44 had a clearly reduced growth at pH 5.5 and showed a slight defect in growth at pH 4.5 and 6.5. This strain lacks a 57.746 kb fragment, which includes 52 genes, from position 1,453,776 to 1,511,522 with respect to the *S.* Typhi CT18 genome (Parkhill et al. 2001) (Fig. 5). Some of the genes present in this fragment, such as *osmC* osmotically inducible protein C, *hlyE* hemolysin, hypothetical protein, or putative hydrolase, were induced when *S.* Typhi invades (2-h post-infection) and replicates (24-h post-infection) inside human macrophages (Faucher et al. 2006). Remarkably, the upregulation of the *osmC* homolog, STM1563, was also detected when *S.* Typhimurium SL1344 infected murine macrophages (Eriksson et al. 2003).

The *S.* Typhi ΔF44 strain also lacks different Hya hydrogenase components such as *hyaC*, *hyaE*, and *hyaF*. These genetic elements are essential for the biosynthesis and functionality of the Hya hydrogenase in *E. coli* (Friedrich and Schwartz 1993; Vignais and Toussaint 1994). *Salmonella enterica* serovar Typhimurium possesses three hydrogenases: Hya, Hyb, and Hyd. *S.* Typhimurium Δ*hya* exposed to LB medium pH 4.0 showed lower tolerance for acid stress, with about 90% fewer viable cells than the wild type, after 24 h of incubation. Moreover, in a previous study, RAW 264.7 murine macrophages were infected with *S.* Typhimurium Δ*hya*, and bacterial numbers were counted at various time points post-infection (2, 4, 12, and 24 h). At all these time points, there were 22 times less Δ*hya* bacteria than the wild type. Thus, Hya plays an important role in *S.* Typhimurium acid tolerance and survival in macrophages (Zbell et al. 2008).

S. Typhi ΔF44 strain is also deficient of the *marR*, *marA*, and *marB* genes, and these genetic elements together with *hya* are induced by H_2O_2 and acid conditions. This suggests a strong connection between acid and oxidative stress. It has also been proposed that low pH amplifies the toxicity of oxygen radicals, which generate oxidative stress for bacteria (Maurer et al. 2005). Interestingly, these conditions are present inside macrophages. Since *S.* Typhi ΔF44 has a deficiency in growth in acid pH and a reduced efficiency in invading and

replicating inside human macrophages, it is possible that the lack of *marA*, *marB*, *marR*, and *hya* prevents a synergic mechanism to combat both acid and oxidative stress present in the macrophage.

In comparison to the wild type, the *S.* Typhi ΔF73 strain showed a decrease in growth rate at pH 4.5, 5.5, 6.5, and 7.5. Notably, this strain was severely affected in its growth at pH 6.5. The 64 kb deletion characterizing this strain (nucleotides 302, 488 to 366,847) according to the *S.* Typhi CT18 genome (Parkhill et al. 2001) contains 55 genes (Fig. 5). Transcriptional experiments reported in another study demonstrated that genetic elements located in this fragment such as *safE*, *safB* (periplasmic fimbrial chaperone proteins), *safC* (outer-membrane fimbrial usher protein), probable secreted protein STY0352, and possible acyl-CoA dehydrogenase *fadE* or STY0357 are upregulated when *S.* Typhi invades (2-h post-infection) and replicates (24-h post-infection) in THP-1 macrophages.

This strain lacks several *Salmonella* genes induced during infection or replication inside macrophages, such as fimbriae. For instance, bacterial adhesion plays a critical role in the ability of pathogen to infect human cells. Adhesins, like fimbriae, promote internalization into epithelial cells. Thus, fimbriae-mediated adhesion to macrophages helps *E. coli* to avoid clearance by the innate immune system. Fimbriae also stabilize adhesion to host urinary epithelium. Therefore, fimbriae are essential for the virulence of uropathogenic *E. coli* strains (Anderson et al. 2003). Moreover, *E. coli* overexpressing type 1 fimbriae is more efficient for invasion (Avalos Vizcarra et al. 2016). Since *S.* Typhi ΔF73 strain does not have two fimbriae operons (*saf* and *tcf*), it is probable that their absence prevents *S.* Typhi ΔF73 invasion and replication inside macrophages. Additionally, *S.* Typhi ΔF73 also lacks many hypothetical proteins, and these could be responsible for the growth defect at pH 4.5, 5.5, 6.5, and 7.5. Future studies are needed to validate this hypothesis.

Conclusions

In this work, we presented three *S.* Typhi deleted strains that had defects towards the survival at specific pH values and were also unable to invade and replicate in human macrophages. Thus, the ability to survive at diverse pH is probably linked to virulence, since an efficient response to the pH present in the saliva, stomach, small intestine, gallbladder, and macrophages allows the bacteria to perform an efficient pathogenic infection.

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Authors' contributions

BDM-M carried out the experiments and drafted the manuscript. LM-A made contributions to analysis of experimental data and revised the manuscript. IS-F performed THP-1 infection experiments. AV generated the *S.* Typhi mutant strains. EC revised the manuscript. IH-L supervised the project, designed the experiments, and wrote the manuscript. All authors read and approved the final manuscript.

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Availability of data and materials

The authors declare that all materials and data are available within the article

Declarations

Ethics approval and consent to participate

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