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Research paper

Lose to win: *marT* pseudogenization in *Salmonella enterica* serovar Typhi contributed to the *surV*-dependent survival to H_2O_2, and inside human macrophage-like cells

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ABSTRACT

The difference in host range between *Salmonella enterica* serovar Typhimurium (*S.* Typhimurium) and *Salmonella enterica* serovar Typhi (*S.* Typhi) can be partially attributed to the gain of functions, to the loss of functions (i.e. pseudogenization), or to a combination of both processes. As previously reported, the loss of functions by pseudogenization may play a role in bacterial evolution, especially in host-restricted pathogens such as *S.* Typhi. The *marT-fidL* operon, located at the SPI-3, encodes the MarT transcriptional regulator and a hypothetical protein (i.e. FidL) with no significant similarities to known proteins, respectively. Even though predicted *S.* Typhimurium FidL exhibit 99.4% identity with *S.* Typhi FidL, *marT* has been annotated as a pseudogene in *S.* Typhi. In this work, we found that *S.* Typhi expressing *S.* Typhimurium *marT-fidL* exhibited an increased accumulation of reactive oxygen species (ROS), leading to a decreased survival in presence of H_2O_2. Moreover, we found that that the presence of a functional copy of *S.* Typhimurium *marT-fidL* in *S.* Typhi resulted in a repression of *surV* (STY4039), an ORF found in the *S.* Typhi SPI-3 but absent from *S.* Typhimurium SPI-3, that contribute to the resistance to H_2O_2 by decreasing the accumulation of ROS. Finally, we observed that the presence of *S.* Typhimurium *marT-fidL* in *S.* Typhi negatively affected the survival inside macrophage-like cells, but not in epithelial cells, after 24 h post infection. Therefore, this work provides evidence arguing that *marT* pseudogenization in *Salmonella* Typhi contributed to the *surV*-dependent survival against H_2O_2, and inside human macrophage-like cells. This is a good example of how the loss of functions (*marT* pseudogenization) and the gain of functions (presence of *surV*) might contribute to phenotypic changes improving virulence.

1. Introduction

Salmonella enterica subspecies *enterica* (*S. enterica*) includes serovars that commonly cause infections in warm-blooded animals (Baumler, 1997; Boyd et al., 1993; Groisman and Ochman, 1997). Genome sequences of closely related *S. enterica* serovars share >90% identity at the nucleotide level. Despite their remarkable similarity, *S. enterica* serovars differ in host specificity and in the distinct diseases produced (McClelland et al., 2001; Parkhill et al., 2001). Thus, some *S. enterica* serovars are considered "generalists" because they infect a broad range of hosts, such as. *S. enterica* serovar Typhimurium (*S.* Typhimurium) which is capable of infecting humans, mice, cattle, and other animals. By contrast, some host restricted serovars can infect one or few hosts; e.g. *S. enterica* serovar Typhi (*S.* Typhi), a strictly

Abbreviations: STm, *Salmonella enterica* serovar Typhimurium; STy, *Salmonella enterica* serovar Typhi; Ψ, Pseudogene; $marT_{STm}$, *S.* Typhimurium *marT* gene; $\Psi marT_{STy}$, *S.* Typhi *marT* pseudogene.

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human-restricted pathogen (Barrow and Duchet-Suchaux, 1997; Coburn et al., 2007; Collins, 1974; Parkhill et al., 2001; Parry et al., 2002; Soyer et al., 2009).

In humans, *S. enterica* infection begins with ingestion of contaminated water or food (Hook et al., 1990). In the intestine, bacteria adhere to the epithelium and promote their own internalization through intestinal epithelial cells and M cells of the Peyer's patches by using a subset of effector proteins secreted by a type III secretion system (T3SS) (Baumler et al., 1997; Galán, 1996). In the case of *S.* Typhimurium, bacteria can replicate inside the intestinal epithelium, without spreading systemically; therefore, *S.* Typhimurium produces only a self-limited gastroenteritis in humans (McClelland et al., 2001; Parkhill et al., 2001; Riquelme et al., 2011). By contrast, *S.* Typhi is able to pass through the M cells and reach the underlying lymphoid tissue, where bacteria are engulfed by macrophages and other phagocytic cells (Finlay and Falkow, 1989; Galán, 1996; Jones et al., 1994; Miao et al., 2003; Uchiya et al., 1999; Vázquez-Torres et al., 1999). Inside macrophages, *S. enterica* is able to resist the harmful effect of reactive oxygen species (ROS), such as hydrogen peroxide (H_2O_2) (van der Heijden et al., 2015). Infected macrophages migrate to regional lymph nodes and subsequently to deeper lymphoid organs such as the spleen and liver (Hensel et al., 1998; Rescigno et al., 2001). This process allows systemic dissemination of *S.* Typhi and determines the production of the typhoid fever.

It has been stated that the evolution of a broad host pathogen, such as *S.* Typhimurium, to a host-restricted pathogen, such as *S.* Typhi, might have occurred by (i) acquisition of new genes through horizontal transfer (e.g. *Salmonella* pathogenicity islands, SPI); (ii) genome degradation (i.e. loss of genetic information by deletion or pseudogene formation); or by a combination of these mechanisms (Andersson and Andersson, 1999; Hacker and Carniel, 2001; Moran and Plague, 2004). Pseudogenes (Ψ) are defined as fragments of once-functional genes that do not encode functional products. Pseudogenes usually arise by the presence of point mutations, insertions, deletions or genetic rearrangements producing premature stop codons, frameshifts, gene truncations or other genetic defects that preclude the proper expression of the gene and/or affect the final product (Dagan et al., 2006). It has been reported that loss of functions by pseudogenization may play a role in bacterial evolution, especially in host-restricted pathogens such as *S.* Typhi (Trombert et al., 2010; Trombert et al., 2011; Valenzuela et al., 2015). In this context, *S.* Typhi, a host-restricted serovar, presents >200 putative pseudogenes (5% of annotated coding sequences) (Deng et al., 2003), whereas the host-generalist pathogen *S.* Typhimurium have approximately 40 putative recognizable pseudogenes (only approximately 1% of their coding sequences) (Hibbing et al., 2010), suggesting that the pseudogene accumulation can explain, at least partially, the differences in virulence and host specificity found among *S. enterica* serovars. Thus, it has been proposed that pseudogenization of genes necessary for a gastrointestinal lifestyle ultimately leads to a systemic lifestyle and niche exclusion in the host-specific *S. enterica* serovars (Matthews et al., 2015; Trombert et al., 2010, 2011; Valenzuela et al., 2015).

SPI-3 is a pathogenicity island found in *S. enterica*. This island is relatively well conserved across different serovars, including *S.* Typhimurium and *S.* Typhi (Retamal et al., 2010) (Fig. 1A and B). *S.* Typhimurium SPI-3 (SPI-3_{STm}) harbors at least twelve open reading frames (ORF); some of them participating in virulence, e.g. the *mgtCB* operon contributes to intramacrophage survival and virulence in mice (Blanc-Potard and Groisman, 1997; Retamal et al., 2009). Other SPI-3 genes involved in *S.* Typhimurium virulence are *misL* and *marT*. MisL, encoded by *misL*, corresponds to a fibronectin-binding protein involved in bacterial adherence, thereby contributing to an increased invasiveness in human epithelial cells (Dorsey et al., 2005). MarT, encoded by the *marT* gene found in the *marT-fidL* operon, acts as a positive transcriptional activator that directly controls the expression of *misL*, indirectly contributing to virulence (Dorsey et al., 2005; Tukel et al., 2007). On the other hand, *fidL* encodes a hypothetical protein with no similarities with known proteins.

Although SPI-3 can be found in *S.* Typhimurium and *S.* Typhi, this genomic island presents conserved and non-conserved regions. For instance, *mgtCB* operon is functional in these two serovars (Blanc-Potard and Groisman, 1997; Retamal et al., 2009); whereas both *marT* and *misL* are annotated as pseudogenes in *S.* Typhi (Deng et al., 2003; Parkhill et al., 2001), suggesting that the MarT and MisL functions are negligible or detrimental in this serovar. Interestingly, *S.* Typhimurium *fidL* exhibits 98.3% identity at nucleotide level with *S.* Typhi *fidL*, whereas the predicted *S.* Typhimurium FidL protein exhibits 99.4% identity at amino acid level with the corresponding *S.* Typhi FidL protein. In addition, no premature stop codons or frameshifts were found in *S.* Typhi *fidL* with respect to *S.* Typhimurium *fidL*, explaining why *fidL* is not considered a pseudogene in *S.* Typhi (Deng et al., 2003; Parkhill et al., 2001; Valenzuela et al., 2014).

As previously reported, to study the role of different versions of the SPI-3 across some *S. enterica* serovars, the complete *S.* Typhi SPI-3 (SPI-3_{STy}) was replaced by the complete *S.* Typhimurium SPI-3 (SPI-3_{STm}) in *S.* Typhi genome, yielding the *S.* Typhi ΔSPI-3_{STy}::SPI-3_{STm} strain (Retamal et al., 2010). *S.* Typhi ΔSPI-3_{STy}::SPI-3_{STm} presents a higher susceptibility to oxidative stress induced by hydrogen peroxide (H_2O_2), with respect to the *S.* Typhi WT. This phenotype was attributed to the differences found in the SPI-3 when *S.* Typhi and *S.* Typhimurium were compared (Retamal et al., 2010), although the contribution of particular genes to H_2O_2 resistance was not explored.

In this work, we studied the effect of the heterologous expression of the fully functional *marT-fidL* operon from *S.* Typhimurium (STm) in *S.* Typhi (STy) regarding the resistance to H_2O_2. For that, a *S.* Typhi strain stably expressing *S.* Typhimurium *marT-fidL* operon in a single copy in *cis* was constructed. This genetic construction, achieved by a method previously described by our group (Valenzuela et al., 2015), allows studying the contribution of pseudogenes to virulence without the confounding effects of plasmids or artificially increased gene dosages. Since $FidL_{STy}$ and $FidL_{STm}$ predicted proteins present 99.4% identity, we simply called this strain as *S.* Typhi $marT^{+}_{STm}$. We found that *S.* Typhi $marT^{+}_{STm}$ exhibited a decreased survival in presence of 0.75 mM H_2O_2 and an increased accumulation of intracellular ROS. In addition, we found that STY4039 (Gene ID: 125027, named *surV* in this work), an ORF found in the *S.* Typhi SPI-3 but absent from *S.* Typhimurium SPI-3 (Fig. 1A and B), also contributed to the resistance to H_2O_2 by decreasing the accumulation of ROS. The presence of a functional copy of $marT_{STm}$-$fidL_{STm}$ in *S.* Typhi resulted in a repression of *surV*, negatively affecting the survival inside macrophage-like cells, but not in epithelial cells, after 24 h post-infection. This work provides evidence arguing that *marT* pseudogenization contributed to improve the *surV*-dependent survival inside macrophages in *S.* Typhi, presumably by increasing the resistance to H_2O_2. This is a good example of how the loss of functions (*marT* pseudogenization) and the gain of functions (presence of *surV*) can contribute to phenotypic changes that might improve virulence.

2. Materials and methods

2.1. Bacterial strains, media and culture conditions

S. Typhi STH2370 was obtained from the Infectious Diseases Hospital Lucio Córdova, Chile (Valenzuela et al., 2014). *S.* Typhimurium 14028s was obtained from the ISP, Chile. All the strains used in this study were grown routinely in liquid culture using Luria Bertani (LB) medium (Bacto peptone, 10 g/l; Bacto yeast extract, 5 g/l; NaCl, 5 g/l, prepared in phosphate buffer pH 7.0) at 37 °C, with aeration, or in microaerophilic conditions by adding an overlay of 500 μl of sterile mineral oil as a barrier to oxygen prior to performing infection assays with eukaryotic cells.

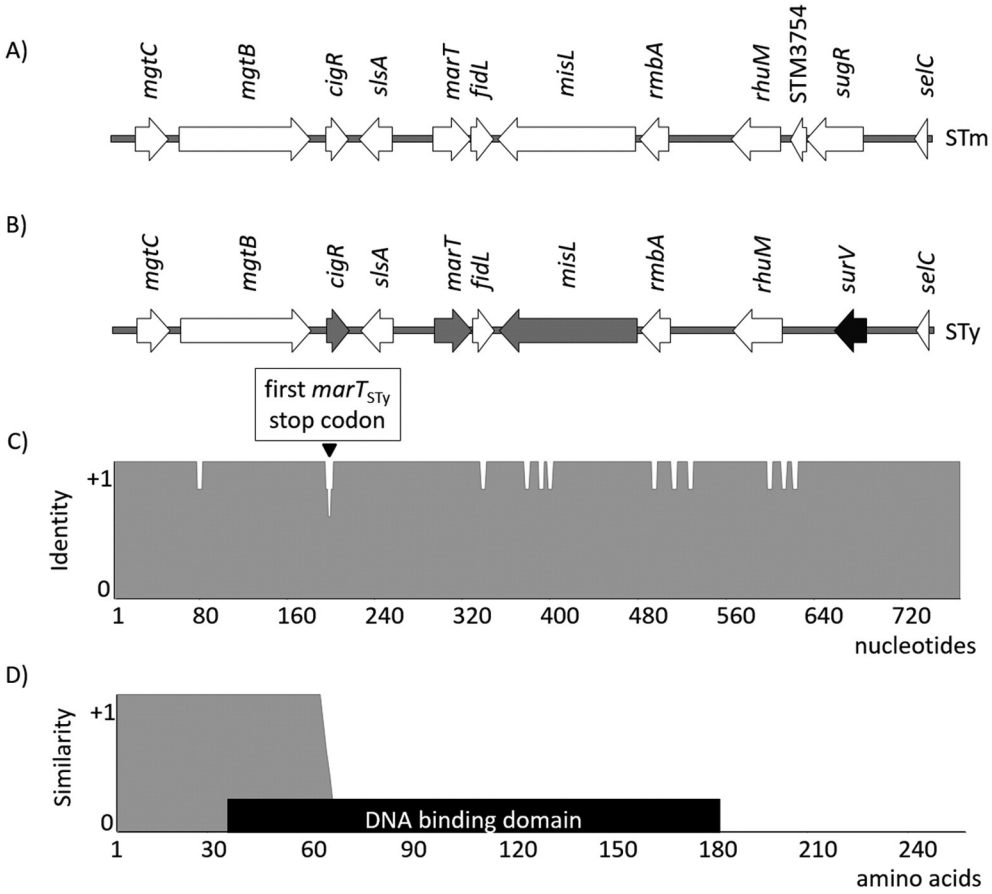

Fig. 1. Comparison of the *marT* loci between *S.* Typhi STH2370 and *S.* Typhimurium 14028s. SPI-3 in *S.* Typhimurium (STm, A) and *S.* Typhi (STy, B). Arrows represent open reading frames (ORFs). White arrows: Functional or putatively functional ORFs; grey arrows: ORFs annotated as pseudogenes in *S.* Typhi (Deng et al., 2003; Parkhill et al., 2001); black arrow: ORF present in *S.* Typhi but absent in *S.* Typhimurium. The *surV* ORF corresponds to STY4039 (Gene ID: 125027). (C) Nucleotide alignment between $marT_{STy}$ from *S.* Typhi STH2370 and $marT_{STm}$ from *S.* Typhimurium 14028s. The black, inverted triangle shows the first premature stop codon in $marT_{STy}$. (D) Predicted protein alignment between $MarT_{STy}$ from *S.* Typhi STH2370 and $MarT_{STm}$ from *S.* Typhimurium 14028s. The black box represents the putative DNA binding domain described for $MarT_{STm}$ (Tukel et al., 2007).

2.2. In silico analyses

Comparative sequence analyses were done with the SPI-3, *marT*, or *surV* sequences available at http://www.ncbi.nlm.nih.gov/ (*S.* Typhi strains STH2370, CT18, Ty2, Ty21a, and P-stx-12; and *S.* Typhimurium strains 14028s, KC14TY193, KC14TY44, USDA-ARS-USMARC-1899, ATCC 13311, VNP20009, L-3553, 138736, DT2, DT104, 08–1736, CFSAN001921, U288, 798, UK-1, ST4/74, T000240, SL1344, D23580, and LT2). $\Psi marT_{STy}$ and all the loci needed to confirm mutagenesis were sequenced at the Pontificia Universidad Católica, Chile. Sequences were analyzed using BLAST alignment and tools available at http://www.ncbi.nlm.nih.gov/, with visual inspection to improve the alignments.

2.3. Construction of S. Typhi STH2370 and S. Typhimurium 14028s mutant strains

To generate *S.* Typhimurium $\Delta marT_{STm}$, *S.* Typhi $\Delta\Psi marT_{STy}$, and *S.* Typhi Δ*surV*, we substituted the corresponding gene (or pseudogene) with the FRT scar (i.e. gaagttcctatactttctagagaataggaacttc) using the corresponding primers MarT[W2-H1P1] + MarT[W1-H2P2]; or SurV[H1P1] + SurV[H2P2] (Table 1) by lambda Red recombination (Datsenko and Wanner, 2000). The FRT scar from *S.* Typhimurium $\Delta marT_{STm}$::FRT, *S.* Typhi $\Delta\Psi marT_{STy}$::FRT, or *S.* Typhi Δ*surV*::FRT was alternatively used to transcriptionally fuse the *lacZ* reporter as described (Ellermeier et al., 2002) to construct the *S.* Typhimurium $\Delta marT_{STm}$::pCE36 (called $\Delta marT_{STm}$::*lacZ*), *S.* Typhi $\Delta\Psi marT_{STy}$::pCE36 (called $\Delta\Psi marT_{STy}$::*lacZ*), or *S.* Typhi Δ*surV*::pCE36 (called Δ*surV*::*lacZ*), respectively.

The *S.* Typhi $marT^{+}_{STm}$ strain [*S.* Typhi $\Delta(\Psi marT_{STy}$-$fidL_{STy})$::[$marT_{STm}$, $fidL_{STm}$, $\Delta misL_{STm}$::FRT]] was constructed as previously reported (Valenzuela et al., 2015) and detailed in Supplementary Fig. 1. Briefly, we generated the *S.* Typhimurium $\Delta misL_{STm}$::cam donor strain and the *S.* Typhi $\Delta(\Psi marT_{STy}$-$fidL_{STy})$::kan receptor strain by using the MisLW1 + MisLW2 and MarT[H1P1] + FidL[H2P2] primers, respectively, for lambda Red recombination (Datsenko and Wanner, 2000). Using the gDNA from the donor strain, we amplified the locus [$marT_{STm}$, $fidL_{STm}$, $\Delta misL_{STm}$::cam] by using the MarT-N + MisL-N primers. This amplicon was used to perform lambda Red recombination over the receptor strain to obtain *S.* Typhi $\Delta(\Psi marT_{STy}$-$fidL_{STy})$::[$marT_{STm}$, $fidL_{STm}$, $\Delta misL_{STm}$::cam]. The cam cassette was removed as described (Datsenko and Wanner, 2000), finally obtaining *S.* Typhi $\Delta(\Psi marT_{STy}$-$fidL_{STy})$::[$marT_{STm}$, $fidL_{STm}$, $\Delta misL_{STm}$::FRT]. Since $FidL_{STy}$ and $FidL_{STm}$ predicted proteins present 99.4% identity, and considering that *misL* is annotated as a pseudogene in *S.* Typhi, we called the obtained strain as *S.* Typhi $marT^{+}_{STm}$. All the mutants were confirmed by PCR using primers flanking the corresponding gene (Table 1) and by DNA sequencing of the corresponding loci. Furthermore, we tested at least two different clones of each construction in the experiments, always obtaining the same results.

S. Typhi $\Psi marT_{STy}$-3xFLAG and *S.* Typhimurium $marT_{STm}$-3xFLAG mutants were constructed using the primers MarT-Kan + MarT-FLAG (Table 1) following a procedure previously described (Uzzau et al., 2001).

All the strains used in this work are summarized in Table 2.

Table 1
List of primers used in this study.

Primer	Sequence
MarT[W2-H1P1]	5′CCATAACATCGCATTGTTACCATGCCAAAACATAACGTAGCATATGAATATCCTCCTTA3′
MarT[W1-H2P2]	5′TTTTATAAGGTTGCTGACAAATCAATGCCGTAACCGCTGTGTAGGCTGGAGCTGCTTCG3′
SurV[H1P1]	5′GTGACGCTTCTGCTCAATGAGCAATATCATCTTTGCTGTT GTAGGCTGGAGCTGCTTCG3′
SurV[H2P2]	5′GCTCCGTTTGAAGGAATCCGCTGTTCAGGTTCAGGTGATCCATATGAATATCCTCCTTAG3′
MisLW1	5′CAAATGAAAGCGTTTCCGGGTAAAAGCCGCTGAAGATCAGTGTAGGCTGGAGCTGCTTCG3′
MisLW2	5′ATTTACGCAGCATCCTCCTCCAGCACCGAGCTTGGCAACACATATGAATATCCTCCTTAG3′
MarT[H1P1]	5′GTCTATTAACCCTCTCCAACTACGTTATGTTTTGGCATGGTGTAGGCTGGAGCTGCTTCG3′
FidL[H2P2]	5′ATATTGGTGAACTGAAAGTGGGATTTGAAGGACAAATTACCATATGAATATCCTCCTTAG3′
MarT-Kan	5′CTTCTCCGTATACAAATGGAAGAGATGTCCAAGTCCTACCCATATGAATATCCTCCTTAG3′
MarT-FLAG	5′AGGCTGCGTGACGCTTTTTTTCCGCGGGGTGACACATGGGGACTACAAAGACCATGACGG3′
MarT-N	5′AACTGCAGCAATACAAAACAGGAGACTT3′
MisL-N	5′GCATAAGCTGCGGCTTGAAA3
MarT-NEcoRI	5′TTGAATTCCAATACAAAACAGGAGACTT3′
FID2	5′GAATTCTGATCTTCAGCGGCTTTTAC3′
SurV-N	5′GTGGATGGTGTTCATACGAG3′
SurV-C	5′ATGCTGTCGCCCTGAATCAG3′
RT-SurVR	5′GGCAAAATAACGAGAACAGTC3′
RT-SurVF	5′GCTGTTACAGCGAAATCCGT3′
16sFW	5′GTAGAATTCCAGGTGTAGCG3′
16sRV	5′TTATCACTGGCAGTCTCCTT3′

2.4. PCR amplifications and plasmid construction (cloning)

PCR amplifications were performed using an Eppendorf thermal cycler and Taq polymerase recombinant (Promega) DNA polymerase. Reaction mixtures contained 1× PCR master mix, primers (1 μM), and 100 ng of template DNA. Standard conditions for amplification were 30 cycles at 94 °C for 30 s, 60 °C for 30 s, and 72 °C for 2–3 min, followed by a final extension step at 72 °C for 10 min. Template *S.* Typhi STH2370, *S.* Typhimurium 14,028 s, and mutant derivatives gDNA were prepared with Wizard® Genomic DNA Purification Kit, according to the manufacturer's instructions. Products generated by PCR amplification were resolved in 1.0% agarose gels. pTMART was constructed by cloning the $marT_{STm}$-$fidL_{STm}$ locus and its promoter region (220 bp), using the MarT-NEcoRI + FID2 primers, into the pCR TOPO TA 2.1® plasmid. pTSURV was constructed by cloning $surV_{STy}$ and its promoter region (252 bp), using the SurV-N + SurV-C primers, into the pCR TOPO TA 2.1® plasmid.

Table 2
Strains used in this work.

Strain name	Genotype	*marT*	*surV*
STy WT	*Salmonella enterica* serovar Typhi strain STH2370 WT	Ψ	+
STy Δ$\Psi marT_{STy}$	Δ$\Psi marT_{STy}$::FRT	−	+
STy Δ*surV*	Δ*surV*::FRT (ΔSTY4039::FRT)	Ψ	−
STy Δ$\Psi marT_{STy}$::*lacZ*	Δ$\Psi marT_{STy}$::pCE36	−	+
STy Δ*surV*::*lacZ*	Δ*surV*::pCE36	Ψ	−
STy $marT^{+}_{STm}$	*S.* Typhi Δ($\Psi marT_{STy}$-$fidL_{STy}$)::[$marT_{STm}$, $fidL_{STm}$, Δ$misL_{STm}$::FRT]	+	+
STy Ψ$marT_{STy}$-3xFLAG	Ψ$marT_{STy}$-3xFLAG	Ψ	+
STm WT	*Salmonella enterica* serovar Typhimurium strain 14028s WT	+	−
STm Δ$marT_{STm}$	Δ$marT_{STm}$::FRT	−	−
STm Δ$marT_{STm}$::*lacZ*	Δ$marT_{STm}$::pCE36	−	−
STm $marT_{STm}$-3xFLAG	$marT_{STm}$-3xFLAG	+	−

STy: *S.* Typhi STH2370; STm: *S.* Typhimurium 14028s.
+: The gene is present and functional.
−: The gene is absent.
Ψ: Pseudogene (non-functional), phenotypically equivalent to the absence of the gene.

2.5. Measurement of β-galactosidase activity

S. Typhi and *S.* Typhimurium mutant strains with *lacZ* fusions were grown in LB at 37 °C with shaking to logarithmic (OD_{600} = 0.5) or to stationary phase (OD_{600} = 1.4), and then chilled to 4 °C. β-Galactosidase activities were measured by a modification of the method of Miller (Miller and Hershberger, 1984). Briefly, each bacterial culture was suspended in 900 μl of Z buffer (0.6 M Na_2HPO_4, 40 mM NaH_2PO_4, 10 mM KCl, 1 mM $MgSO_4$, 50 mM β-mercaptoethanol; pH 7.0). Bacteria were permeabilized with 10 μl chloroform, 10 μl 0.1% SDS, vortexed for 10 s, and incubated at 30 °C for 10 min, and 200 μl of *o*-nitrophenyl-D-galactopyranoside (4 mg/ml) was added. Reactions were stopped by adding 500 μl 1 M Na_2CO_3. β-Galactosidase activities are expressed in Miller units, according to the formula $10^3 \times (OD_{420} - 1.75 \times OD_{550})$ ml^{-1} min^{-1} OD_{600}^{-1}. All these experiment were repeated at least three times.

2.6. Western blot assays

Strains carrying the epitope-tagged genes were grown in LB at 37 °C with shaking to logarithmic (OD_{600} = 0.5) or stationary phase (OD_{600} = 1.4). Bacterial pellets were resuspended in 1 ml of Tris-HCl pH 8.0 and sonicated in ice during 100 s with pulses. Bacterial proteins (60 μg), previously quantified following the Bradford method (Bradford, 1976), were resolved by 12% SDS PAGE, transferred to poly(vinylidene difluoride) membranes and stained with Ponceau S to confirm the protein load as previously described (Stochaj et al., 2006). The membranes were washed with distilled water and probed with a primary mouse anti-FLAG mAbs (1:1000), and subsequently probed by a secondary horseradish peroxidase-conjugated goat anti-mouse IgG [1:5000 (Sigma)]. As control, we used primary mouse anti-Hsp60 mAbs (1:10,000) subsequently probed with a secondary horseradish peroxidase-conjugated goat anti-mouse IgG [1:5000 (Sigma)] to detect the Hsp60 protein (60 kDa). In all the cases, detection was performed by enhanced chemioluminescence (ECL, Amersham Pharmacia).

2.7. Hydrogen peroxide killing assay

This assay was carried out according to a procedure previously reported (Tu et al., 2006) with some modifications. Briefly, bacteria were grown to logarithmic phase (OD_{600} = 0.5) in LB at 37 °C with shaking. Then, bacteria were diluted 1:100 into LB (untreated) or

LB + 0.75 mM H_2O_2. After 0, 5, 10, 20, 30, and 60 min incubation at 37 °C with aeration, cultures were serially diluted in cold LB and plated onto LB agar plates and incubated overnight at 37 °C to determine the number of colony-forming units (cfu). The percentage survival was calculated as follows: (cfu of hydrogen peroxide-treated culture/cfu of untreated culture) × 100. The experiment was repeated at least 6 times, each time in technical triplicate.

2.8. Determination of intracellular reactive oxygen species (ROS)

Measurement of intracellular ROS was performed using the 2′,7′-dichlorodihydrofluorescein diacetate (H_2DCFDA) probe, according to a procedure previously described (Calderon et al., 2014) with some modifications. Briefly, cells grown aerobically to OD_{600} of 0.6 in LB at 37 °C were incubated with 10 μM H_2DCFDA for 20 min. Then cells were washed and suspended in PBS, and treated with 0.75 mM H_2O_2. Fluorescence was measured at short time intervals using a TECAN Infinite 200 PRO Nanoquant microplate reader (excitation, 480 nm; emission, 520 nm). Emission values were normalized by protein concentration. The experiment was performed in biological triplicate.

2.9. RNA isolation, reverse transcription and real-time PCR (RT-PCR)

Total mRNA from strains grown to an OD_{600} of approximately 0.5 was extracted using TRIzol reagent (Invitrogen). RNA was precipitated with isopropanol for 10 min at room temperature, washed with ice-cold 70% v/v ethanol and resuspended in DEPC-treated water prior to treatment with DNase I to remove any trace of DNA. Purity of extracted RNA was determined by spectrophotometry. Reverse transcription was performed with 2 μg of DNase-treated RNA using Superscript II RT (Invitrogen) at 50 °C for 50 min followed by 70 °C for 10 min in 20 μl with 2.5 mM of the reverse primer RT-SurVR. As previously described, the 16sRV reverse primer for 16 s rRNA was used to normalize against a reference transcript (Jofre et al., 2014). Relative quantification of each mRNA was performed using Brilliant II SYBR Green QPCR Master Reagent Kit and the Mx3000P detection system (Stratagene). The reaction mixture was carried out in a final volume of 20 μl containing 1 μl of diluted cDNA (1:1000), 0.24 μl of each primer (120 nM) (RT-SurVR + RT-SurVF for *surV* mRNA detection, and 16sFW + 16sRV for 16 s rRNA detection), 10 μl of 10× Master Mix, 0.14 μl of diluted ROX (1:200) and 8.38 μl of nuclease-free H_2O. The reaction was performed under the following conditions: 10 min at 95 °C followed by 40 cycles of 30 s at 95 °C, 30 s at 53 °C and 45 s at 72 °C. Finally, a melting cycle from 53 to 95 °C was performed to check for amplification specificity. Amplification efficiency was calculated from a standard curve constructed by amplifying serial dilutions of RT-PCR products for each gene. These values were used to obtain the fold change in expression for the gene of interest normalized with 16 s levels as previously described (Jofre et al., 2014; Pfaffl, 2001). Experiments were performed in three biological and technical replicates.

2.10. Assays of adherence, invasion, and proliferation in eukaryotic cells

Macrophage-like human cell line (U937), and the epithelial cell line (HEp-2) were maintained in RPMI 1640 (Invitrogen) containing 10% fetal calf serum pre-treated for 30 min at 60 °C, at 37 °C in a 5% CO_2/95% air mixture. For the adherence assay, we followed a procedure previously described (Berrocal et al., 2015). To determine the invasion (3 h post infection) and proliferation rate (24 h post infection) into HEp-2 epithelial cells, the gentamicin protection assay was performed as previously described (Contreras et al., 1997; Valenzuela et al., 2015). To determine the invasion (3 h post infection) and proliferation rate (24 h post infection) into U937 macrophage-like cells, the gentamicin protection assay was performed as described (Retamal et al., 2010), but with an additional step where the mixture of U937 cells and bacteria was centrifuged for 5 min at 1000 x g to synchronize the infection, as previously described (Berrocal et al., 2015). In all cases, experiments were performed in 6 full biological replicates, each time in technical triplicate.

2.11. Statistics

p values were calculated according to the Student's *t*-test. Values of $p < 0.05$ (*) were considered statistically significant.

3. Results

3.1. S. Typhi marT ($\Psi marT_{STy}$) corresponds to a pseudogene

To assess whether *marT* presents characteristics commonly associated to pseudogenes (Kuo and Ochman, 2010) in the serovar Typhi, we compared the *marT* sequence obtained from *S.* Typhimurium 14028s ($marT_{STm}$, 799 bp) (Fig. 1A) and *S.* Typhi STH2370 ($marT_{STy}$, 788 bp) (Fig. 1B). We observed that $marT_{STy}$ presented substitutions in 12 nucleotides compared with $marT_{STm}$. The G200A transition exhibited by $marT_{STy}$ generated the first premature stop codon (8 in total) in *S.* Typhi (Fig. 1C). In addition, $marT_{STy}$ also presented a deletion at the nucleotide 392, resulting in a frameshift downstream the first premature stop codon. A comparison of the sequences of the predicted proteins showed that $MarT_{STy}$ corresponded to a truncated version of $MarT_{STm}$, where the putative DNA-binding winged-helix-turn-helix domain, found between amino acids 32 and 180 in $MarT_{STm}$ (Tukel et al., 2007), is partially lost. These genetic defects strongly suggest that $MarT_{STy}$ is non-functional (Fig. 1D) since $marT_{STy}$ presents features commonly associated to pseudogenes (i.e. frame-shifts and premature stop codons) when compared with the functional $marT_{STm}$.

To assess whether these features are characteristic to serovar Typhi, we compared *marT* from *S.* Typhi STH2370, the Chilean strain, with available nucleotide sequences of *marT* from other *S.* Typhi strains (CT18, Ty2, Ty21a, and P-stx-12). We found that all the sequences tested were 100% identical among them. On the other hand, we also compared available sequences of *marT* from 20 *S.* Typhimurium strains (14,028 s, KC14TY193, KC14TY44, USDA-ARS-USMARC-1899, ATCC 13311, VNP20009, L-3553, 138736, DT2, DT104, 08-1736, CFSAN001921, U288, 798, UK-1, ST4/74, T000240, SL1344, D23580, and LT2, available in NCBI databases). As well as with $marT_{STy}$, all the $marT_{STm}$ sequences were 100% identical among them. Therefore, the substitutions in 12 nucleotides, the deletion of 1 nucleotide, and the premature stop codons found in $marT_{STy}$ seems to be features that distinguish the serovar Typhi from the serovar Typhimurium. All these genetic features, including the presence of a truncated DNA binding domain (Fig. 1D), explain why *marT* from *S.* Typhi has been considered a non-functional gene (i.e. a pseudogene) (Deng et al., 2003; Parkhill et al., 2001). From here, we will refer to $marT_{STy}$ as $\Psi marT_{STy}$ to denote its pseudogene nature.

Recently, it has been stated that experimental research is needed to unequivocally identify a pseudogene (Urrutia et al., 2014). In this context, we first assessed whether the $\Psi marT_{STy}$ promoter region is functional. For that, we constructed a single-copy, *cis*-transcriptional *lacZ* fusion to $\Psi marT_{STy}$ in *S.* Typhi as previously reported (Ellermeier et al., 2002), yielding the *S.* Typhi $\Delta\Psi marT_{STy}$::*lacZ* strain. As control, the same procedure was used to generate *S.* Typhimurium $\Delta marT_{STm}$::*lacZ*. As shown in Fig. 2A, *S.* Typhi $\Delta\Psi marT_{STy}$::*lacZ* exhibited an increased β-galactosidase activity at stationary phase, showing a similar expression pattern as that of *S.* Typhimurium $\Delta marT_{STm}$::*lacZ*. This result showed the $marT_{STy}$ promoter region is functional, assertion that is supported by the fact that $marT_{STy}$ promoter region is 100% identical to $marT_{STm}$ promoter region if considered 200 bp upstream of the start codon, while $marT_{STy}$ promoter region is 99% identical to $marT_{STm}$ promoter region if considered 300 bp upstream of the start codon (data not shown). Since a functional promoter does not necessarily imply protein

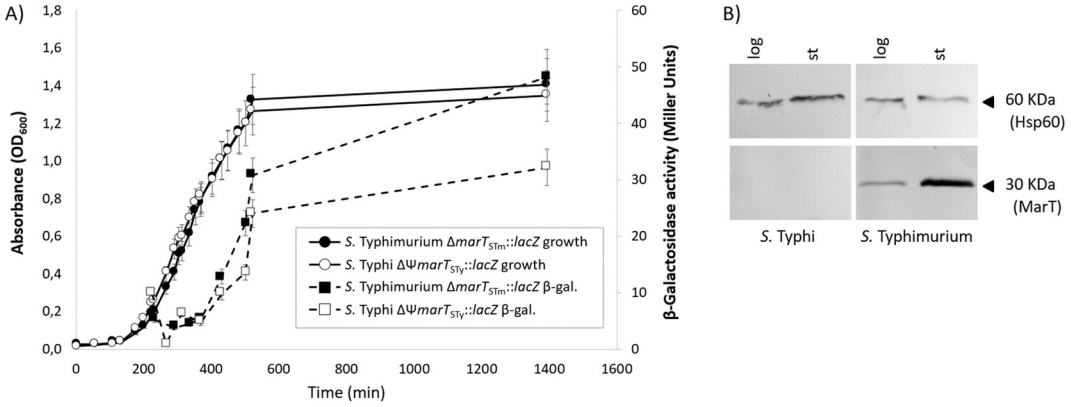

Fig. 2. *marT* expression in *S.* Typhi and *S.* Typhimurium. (A) β-Galactosidase activity (squares, dashed lines, left Y axis) through the growth curve (circles, continuous lines, right Y axis) of *S.* Typhimurium Δ$marT_{STm}$::*lacZ* (black) or *S.* Typhi Δ$\Psi marT_{STy}$::*lacZ* (white). Bacteria were grown in LB at 37 °C with shaking. (B) Detection of MarT-3xFLAG by Western blot in the strains *S.* Typhimurium 14028s $marT_{STm}$-3xFLAG and *S.* Typhi STH2370 $\Psi marT_{STy}$-3xFLAG. Bacteria were cultured to logarithmic phase (log, $OD_{600} = 0.5$) or stationary phase (st, $OD_{600} = 1.4$) in LB prior to extracting total proteins and perform the Western blot. In all the cases, the 3xFLAG epitope was placed replacing the codon corresponding to the stop codon found in the $marT_{STm}$. Detection of the Hsp60 protein was used as loading control.

production, we assessed whether $marT_{STy}$ encodes a protein comparable to $MarT_{STm}$. For that, we constructed the *S.* Typhi $\Psi marT_{STy}$-3xFLAG strain by replacing the codon corresponding to the stop codon found in $marT_{STm}$ by a 3xFLAG epitope, as previously described (Urrutia et al., 2014; Valenzuela et al., 2015). As control, we generated the *S.* Typhimurium $marT_{STm}$-3xFLAG strain by using

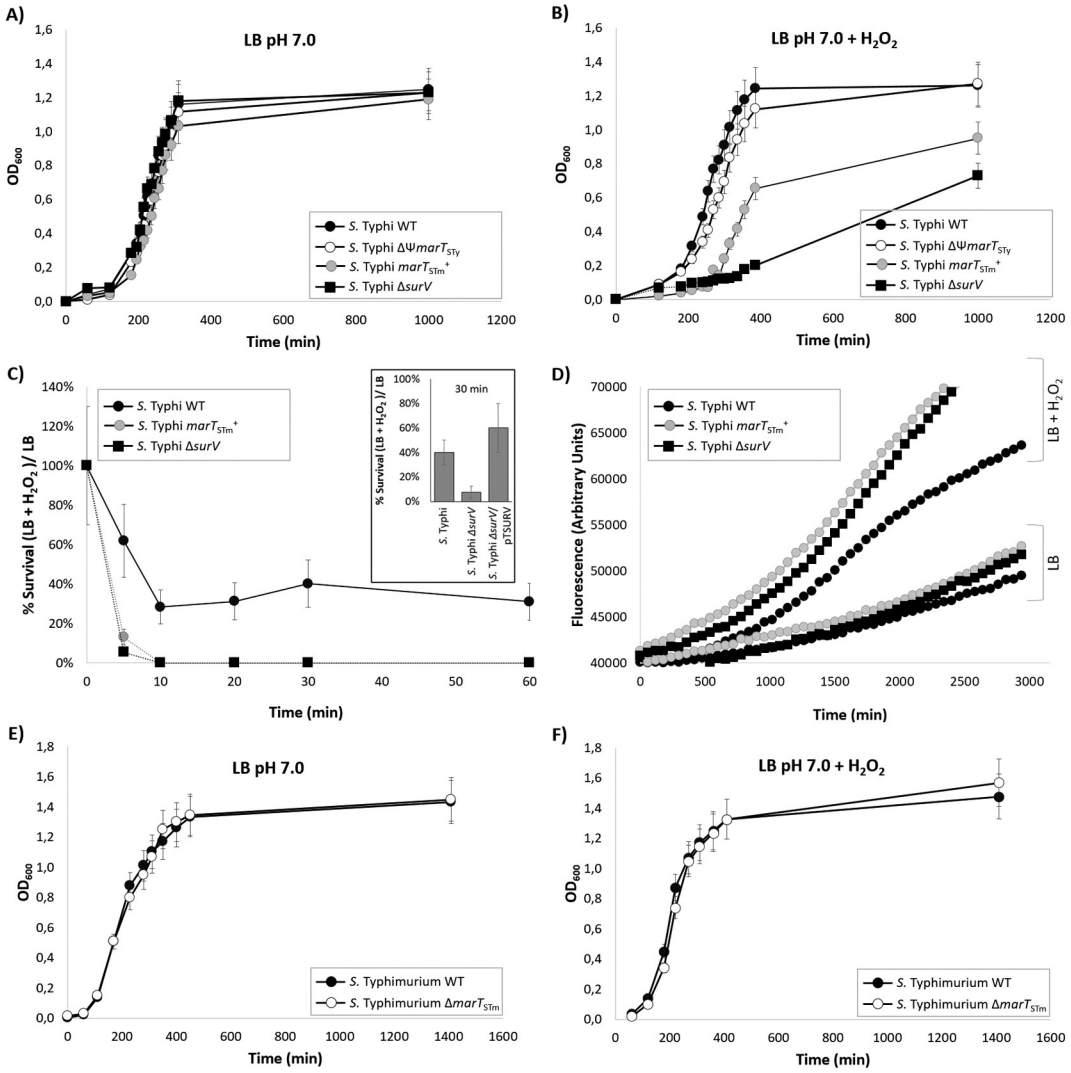

Fig. 3. *S.* Typhi Δ*surV* and *S.* Typhi $marT^{+}_{STm}$ present a decreased fitness in presence of H_2O_2. (A) and (E) Growth curves performed in LB at 37 °C with shaking. (B) and (F) Growth curves performed in LB + 0.75 mM H_2O_2 at 37 °C with shaking. (C) Hydrogen peroxide killing assay as described in Methods. The figure shows a representative experiment performed in technical triplicate. (D) Bacterial total ROS in LB and in LB + 0.75 mM H_2O_2 inferred by the H_2DCFDA assay. The figure shows a representative experiment.

the same procedure. As shown in Fig. 2B, $MarT_{STm}$ was detected as a 30 kDa protein in both logarithmic and stationary phase, where $MarT_{STm}$ accumulated at stationary phase, as expected from the β-galactosidase assays. By contrast, $MarT_{STy}$ was undetectable with the same assay (Fig. 2B); supporting that $marT_{STy}$ corresponds to a pseudogene (Fig. 1). Finally, *S.* Typhi WT and *S.* Typhi Δ$\Psi marT_{STy}$ were undistinguishable with respect to the survival to H_2O_2 or inside the macrophage-like human cell line U937 (see below), demonstrating that, at least under the tested conditions, $\Psi marT_{STy}$ is dispensable. All these data show that $\Psi marT_{STy}$ exhibits features commonly associated with pseudogenes.

3.2. The presence of a functional copy of marT contributed to increase the susceptibility to H_2O_2 in S. Typhi

A previous work performed in our laboratory showed that *S.* Typhi ΔSPI-3_{STy}::SPI-3_{STm} (i.e. a *S.* Typhi hybrid strain carrying the SPI-3 region of *S.* Typhimurium instead of its own SPI-3) exhibited a decreased survival against a challenge of 3 mM H_2O_2 for 30 min (Retamal et al., 2010). This phenotype was attributed to differences between SPI-3_{STy} and SPI-3_{STm}, particularly to *marT*, although the role of *marT* in the survival to H_2O_2 was not directly tested (Retamal et al., 2010). To assess the role of *marT* in H_2O_2 resistance, the bacterial growth pattern was monitored in presence and absence of H_2O_2. Since the H_2O_2 concentrations found inside macrophages and other phagocytic cells range between 0.0125 mM and 1 mM, we decided test 0.75 mM H_2O_2, a sub lethal concentration for *S. enterica* (Tang et al., 2007; Wong and McClelland, 1994). We observed that *S.* Typhi WT and *S.* Typhi Δ$\Psi marT_{STy}$ growth was undistinguishable in LB and LB + 0.75 mM H_2O_2. This result shows that $\Psi marT_{STy}$ is dispensable under the tested conditions, as expected for a pseudogene (Fig. 3A and B, compare black and white circles). To determine whether *marT* pseudogenization involves phenotypic changes in *S.* Typhi, we complemented *S.* Typhi with the functional allele $marT_{STm}$ taken from *S.* Typhimurium, following a similar strategy to that reported in studies of other pseudogenes (Trombert et al., 2010; Trombert et al., 2011; Urrutia et al., 2014; Valenzuela et al., 2015). For that, we used a previously reported procedure aimed to exchange pseudogenes by the corresponding functional allele, in a single chromosomal copy, and without the presence of vehicle plasmids (Valenzuela et al., 2015). This procedure yielded the *S.* Typhi $marT^{+}_{STm}$ strain (see Materials and methods and Supplementary Fig. 1) As shown in Fig. 3A, *S.* Typhi $marT^{+}_{STm}$ showed similar growth pattern as that of otherwise isogenic *S.* Typhi WT in LB. Nevertheless, in presence of 0.75 mM H_2O_2, *S.* Typhi $marT^{+}_{STm}$ exhibited a growth impairment compared with the WT strain (Fig. 3B, compare black and grey circles). To further explore this result, we performed a H_2O_2 killing assay. *S.* Typhi and mutant derivatives were grown in LB pH 7.0 prior to performing a subculture in LB + 0.75 mM H_2O_2 (treatment) or in LB (control), and the percentage of survival was calculated at different times. We observed that the percentage of survival of *S.* Typhi $marT^{+}_{STm}$ was <20% at 5 min with H_2O_2, whereas <10% were observed after 10 min of exposure to H_2O_2. By contrast, *S.* Typhi WT presented approximately 40% of survival, even after 60 min of exposure to H_2O_2 (Fig. 3 C). These results suggest that the levels of intracellular reactive oxygen species (ROS) in *S.* Typhi are increased when the functional $marT_{STm}$ is present. Thus, the ROS-sensitive probe H_2DCFDA was used to monitor by fluorescence the production of intracellular ROS at different time intervals after the treatment of exponentially growing cells with 0.75 mM H_2O_2. H_2DCFDA is a chemically reduced form of fluorescein used as an indicator of ROS inside cells. Upon cleavage of the acetate groups by intracellular esterases and oxidation, the non-fluorescent H_2DCFDA is converted to the highly fluorescent 2′,7′-dichlorofluorescein that can be easily detected. As expected, *S.* Typhi $marT^{+}_{STm}$ exhibited higher levels of total ROS compared with *S.* Typhi WT when bacteria were exposed to H_2O_2, as inferred by the increased fluorescence (Fig. 3D). These results show that the presence of a functional copy of *marT* contributed to increase the susceptibility to H_2O_2 in *S.* Typhi by increasing the intracellular ROS.

Taking these results into account, we hypothesized that the presence of a functional copy of *marT* in *S.* Typhi is somehow detrimental under conditions involving the presence of ROS, such as H_2O_2. If this is the case, *S.* Typhimurium Δ$marT_{STm}$ should be more resistant to H_2O_2 than the *S.* Typhimurium WT. Unexpectedly, these two strains presented the same growth both in LB and in LB + 0.75 mM H_2O_2 (Fig. 3C and D), or even in LB + 3 mM H_2O_2 (data not shown). From these results, we inferred that the genetic context is important to reveal the putative role of $marT_{STm}$ in the survival to H_2O_2. Since $MarT_{STm}$ is a transcriptional factor (Tukel et al., 2007), we hypothesized that the increased susceptibility to H_2O_2 observed in *S.* Typhi harboring a functional copy of $marT_{STm}$ depends on another effector gene, absent from *S.* Typhimurium, that could be regulated by $MarT_{STm}$.

3.3. The surV gene contributes to the H_2O_2 resistance in S. Typhi

It has been previously reported that $MarT_{STm}$, overexpressed from a multicopy vector harboring both $marT_{STm}$ and $fidL_{STm}$ genes, contributes to the repression of the *t3766* ORF in *S.* Typhi (Retamal et al., 2010), albeit the role of *t3766* in the H_2O_2 resistance was not previously explored. The *t3766* ORF (corresponding to STY4039 Gene ID: 125027 in *S.* Typhi, henceforth called *surV*), is located in the non-conserved region of SPI-3 (Fig. 1B, right). The *surV* gene, present in *S.* Typhi but absent in *S.* Typhimurium (Fig. 1A and B), is a 597 bp ORF that encodes a putative protein of 50 kDa with a predicted HNHc domain, exhibiting no significant identity with previously characterized proteins. HNHc (His-Asn-His) proteins are a very common family of small nucleic acid-binding proteins that are generally associated with endonuclease activity and are found in all kingdoms of life (i.e. bacteria, viruses and eukaryotes). The HNHc domain can be found in several proteins from different organisms, including yeast intron 1 protein, MutS, bacterial colicins, pyocins, and endonucleases (Zhang et al., 2016). We observed that the *S.* Typhi Δ*surV* mutant exhibited an increased susceptibility to H_2O_2 compared with *S.* Typhi WT, although we found no differences in LB (Fig. 3A and B). As well as for *S.* Typhi $marT^{+}_{STm}$, *S.* Typhi Δ*surV* survival presented a similar trend in the H_2O_2 killing assay, phenotype that was reversed by the presence of pTSURV (a plasmid harboring *S.* Typhi *surV* and its promoter region) (Fig. 3C). Furthermore, *S.* Typhi Δ*surV* showed increased levels of intracellular ROS (similar to those exhibited by *S.* Typhi $marT^{+}_{STm}$), compared with *S.* Typhi WT (Fig. 3D).

All these data show that *surV* gene contributes to the H_2O_2 resistance in *S.* Typhi.

3.4. A single copy of the $marT_{STm}$ gene contributes to repress the surV transcription in S. Typhi

The heterologous expression of the $marT_{STm}$-$fidL_{STm}$ operon from a multicopy vector resulted in repression of *surV*, as determined by RT-PCR (Retamal et al., 2010). Since $marT_{STm}$ encodes a functional transcriptional factor (Tukel et al., 2007), we assessed whether the presence of $marT_{STm}$ affects the expression of *surV* in *S.* Typhi at the transcriptional level. Firstly, we constructed a chromosomal *S.* Typhi Δ*surV*::*lacZ* transcriptional fusion strain as described (Ellermeier et al., 2002). Then, we transformed the *S.* Typhi Δ*surV*::*lacZ* strain with the pTMART, a plasmid constructed by cloning $marT_{STm}$-$fidL_{STm}$ and its promoter region (220 bp) into pCR TOPO TA 2.1®. We compared the *S.* Typhi Δ*surV*::*lacZ* strain with *S.* Typhi Δ*surV*::*lacZ*/pTMART by assessing the β-galactosidase both in

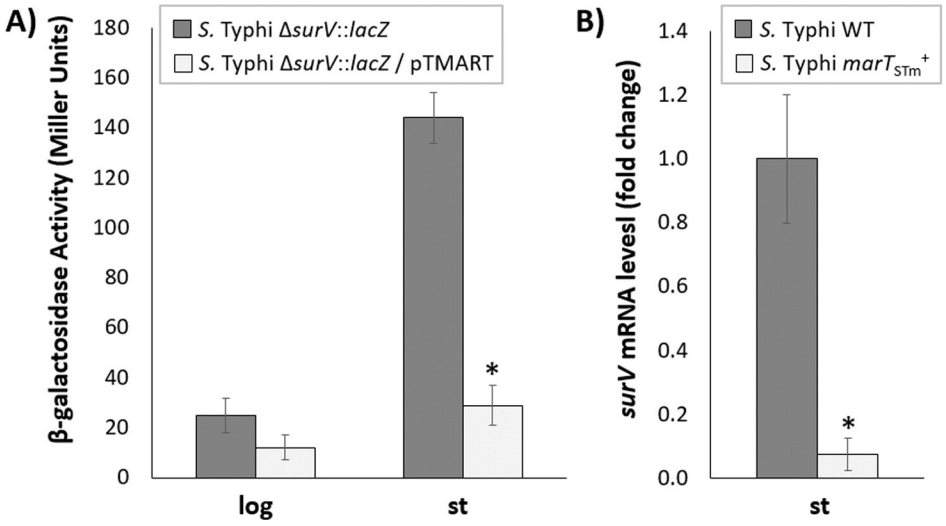

Fig. 4. A single copy of $marT_{STm}$ contributes to repress *surV* expression in *S.* Typhi. A) β-Galactosidase assay comparing *S.* Typhi Δ*surV*::*lacZ* and *S.* Typhi Δ*surV*::*lacZ*/pTMART grown in LB, measured at logarithmic (log) and stationary phase (st). B) RT-PCR comparing *S.* Typhi WT and *S.* Typhi $marT^{+}_{STm}$ grown to stationary phase (st) in LB. $^{*}p < 0.05$.

logarithmic ($OD_{600} = 0.5$) and stationary ($OD_{600} = 1.4$) phase in bacteria cultured in LB. The *surV* gene increased its expression at stationary phase but the presence of pTMART significantly decreased the β-galactosidase activity, suggesting that $MarT_{STm}$ is directly or indirectly involved in the repression of *surV* expression at transcriptional level (Fig. 4A). To assess these results in an independent way and without the presence of vehicle plasmids, we evaluated the *surV* transcription in *S.* Typhi WT and *S.* Typhi $marT^{+}_{STm}$

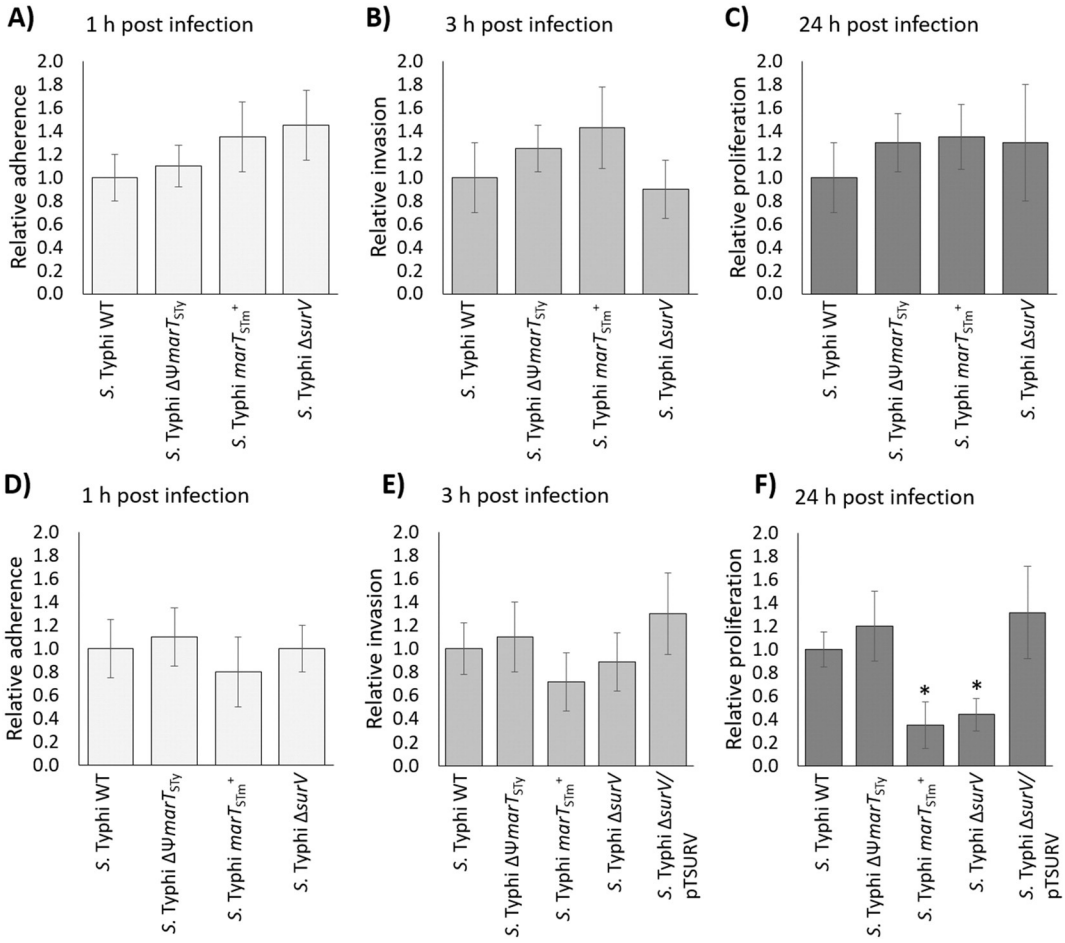

Fig. 5. *S.* Typhi Δ*surV* and *S.* Typhi $marT^{+}_{STm}$ present a decreased survival inside U937 cells. Adherence (A and D, 1 h post infection), invasion (B and E, 3 h post infection), and proliferation (C and F, 24 h post infection) assays performed in the HEp-2 epithelial cells (A, B, and C) and U937 (D, E, and F). All the results shown are relative to the WT in the respective experiment. All the experiments were repeated at least three times. $^{*}p < 0.05$.

by qRT-PCR. When the strains were cultured in LB, we observed that the *surV* expression was significantly repressed in *S*. Typhi $marT^{+}_{STm}$ compared with *S*. Typhi WT (Fig. 4B). Altogether, our results suggest that a single, functional copy of $marT_{STm}$ contributes to repress *surV* transcription in *S*. Typhi under the tested conditions.

3.5. The presence of $marT_{STm}$ or the absence of surV in S. Typhi resulted in proliferation defects in U937 cells

Since *S*. Typhi $marT^{+}_{STm}$ exhibited a decreased fitness in presence of H_2O_2 due to an increased intracellular accumulation of ROS, we hypothesized that the presence of a single functional copy of $marT_{STm}$ in *S*. Typhi might contribute to exert a detrimental effect on the bacterial fitness inside ROS producing cells. To test this, we compared adherence, invasion and proliferation of *S*. Typhi WT and mutant derivatives, in the macrophage-like human cell line U937. We also included assays in HEp-2, an epithelial cell line that is not associated to ROS production. The Fig. 5A, B, and C show that neither the presence of $marT_{STm}$ nor the absence of *surV* affected the interaction with HEp-2 at any level, since all the bacterial strain exhibited the same behavior than *S*. Typhi WT. On the other hand, we found that *S*. Typhi harboring $marT_{STm}$ exhibited no defects in adherence or invasion in U937 cells (Fig. 5D and E). By contrast, we observed that both *S*. Typhi $marT^{+}_{STm}$ and *S*. Typhi Δ*surV* exhibited a similar proliferation defect, in comparison with the WT (Fig. 5F). The presence of the pTSURV plasmid restored the WT phenotype in the *S*. Typhi Δ*surV*/pTSURV strain (Fig. 5F). In all cases, *S*. Typhi $\Delta\Psi marT_{STy}$ presented an undistinguishable phenotype compared with the WT strain (Fig. 5), reinforcing the fact that *marT* is a pseudogene in *S*. Typhi.

Altogether, the data presented in this study show that the *marT* pseudogenization in *S*. Typhi contributes to the *surV*-dependent survival to H_2O_2, and to the proliferation inside U937 cells.

4. Discussion

Genes encoding irrelevant functions in a determined niche accumulate mutations finally leading to the loss of functions. On the other hand, genes whose encoded functions are no longer compatible with a determined lifestyle (i.e. detrimental genes) are selectively inactivated to increase the fitness. In pathogenic bacteria, the inactivation of genes encoding the detrimental functions results in an increased adaptation to the host, giving rise to pseudogenes (Maurelli, 2007; Ochman and Davalos, 2006). In this work, we showed that *marT* is indeed a pseudogene in *S*. Typhi due to several genetic defects found in the open reading frame (ORF), including deletions. There are some examples showing that the pseudogenization of genes encoding some important virulence factors in generalist *S. enterica* serovars has paradoxically determined an increased virulence in the host restricted serovar Typhi. Pseudogenization of *sseJ*, which encodes an acyltransferase/lipase contributing to intracellular proliferation in *S*. Typhimurium, resulted in a more cytotoxic *S*. Typhi strain (Trombert et al., 2010). On the other hand, pseudogenization of *sopD2* or *sopE2*, two genes encoding known SPI-1-dependent effectors involved in the *S*. Typhimurium pathogenesis, yielded more invasive *S*. Typhi strains (Trombert et al., 2011; Valenzuela et al., 2015). In addition, pseudogenization of *sopA*, a gene encoding a ubiquitin ligase in *S*. Typhimurium, apparently contributed to *sopE2* pseudogenization in *S*. Typhi (Valenzuela et al., 2015).

Usually, pseudogenes exhibit genetic erosion even in the promoter region, precluding the production of mRNA (e.g. $\Psi sopA_{STy}$ and $\Psi sopE2_{STy}$) (Valenzuela et al., 2015). By contrast, in some cases the presence of a functional promoter may be an indicator suggesting that the mutations or deletions found in the corresponding ORF do not lead to a pseudogene formation, but to a functional allelic variant (e.g. $shdA_{STy}$) (Urrutia et al., 2014). In the case of $\Psi marT_{STy}$, we found a functional promoter (Fig. 2A). Since $\Psi marT_{STy}$ is indeed a pseudogene (and not an allelic variant of $marT_{STm}$), the functional promoter can be explained by the fact that *marT* is part of an operon along with *fidL* (Retamal et al., 2010). Actually, both *S*. Typhi WT and *S*. Typhi $marT^{+}_{STm}$ express *fidL* in a similar way (Supplementary Fig. 3). Considering that predicted $FidL_{STy}$ and $FidL_{STm}$ proteins exhibit 99.4% identity, we inferred that the *marT* promoter region is functional in *S*. Typhi in order to produce FidL, a protein presumably important for the *S*. Typhi life cycle. In the case of $marT_{STm}$ promoter region, it has been already described that β-galactosidase activity is detectable in bacteria harboring the *lac* reporter fused to *marT* in *S*. Typhimurium grown in LB (Blanc-Potard et al., 1999), albeit a dependency on the growth phase was not previously reported. Nevertheless, other growth conditions (400 mM NaCl, pH 5.0, 0.75 mM H_2O_2, 1.5 mM H_2O_2) were unable to increase *marT* expression in both *S*. Typhi and *S*. Typhimurium, as determined by β-galactosidase assays (data not shown).

Previously, it has been disclosed that *S*. Typhi $\Delta SPI\text{-}3_{STy}::SPI\text{-}3_{STm}$ exhibited a decreased survival to a challenge of 3 mM H_2O_2 for 30 min. Nevertheless, the role of *marT* or *surV* in H_2O_2 survival was not explored (Retamal et al., 2010). Here, we showed that the presence of one functional copy of $marT_{STm}$ contributes to decrease the fitness in presence of 0.75 mM H_2O_2 (a physiological concentration) in *S*. Typhi, due to the repression of *surV*. It is not uncommon that regulators encoded in genomic islands regulates genes located in the same genomic islands (e.g. HilA in SPI-1) (Kuo and Ochman, 2010), remarking the selfish nature of some genomic segments acquired by horizontal transfer (Lawrence, 2003). In the case of SPI-3, $MarT_{STm}$ regulates the transcription of *misL* (Tukel et al., 2007), and contribute to the downregulation of *surV* when heterologously expressed in *S*. Typhi. Nevertheless, whether $MarT_{STm}$ is directly or indirectly regulating *surV* remains to be elucidated.

It has been reported that the overexpression of $marT_{STm}$-$fidL_{STm}$ from a multicopy vector led to a decreased survival inside U937 compared with *S*. Typhi WT. However, this difference was only observed in invasion (2 h post-infection), but not in proliferation (24 h post-infection) (Retamal et al., 2010). By contrast, we found here that *S*. Typhi $marT^{+}_{STm}$ exhibited proliferation defects after 24 h post-infection. To heterologously express $marT_{STm}$ in *S*. Typhi (i.e. to generate *S*. Typhi $marT^{+}_{STm}$), we used a previously described method aimed to produce a stable, single-copy, in *cis* complementation of the corresponding functional allele (Valenzuela et al., 2015). Thus, the results presented in this work can be attributed directly to the heterologous expression of genes of interest rather than to issues derived from the use of multicopy vectors, i.e. the presence of unrelated foreigner genes, an inappropriate gene dosage leading to artificial higher expression levels, titration of regulatory factors due to the high copy number of vectors, or even deleterious effects produced by the antibiotics used to maintain the plasmids. Actually, even though plasmid vectors are commonly used in the study of bacterial pathogenicity, in some cases they can affect the ability of *S. enterica* to survive in either cultured mammalian cells or even in mice (Knodler et al., 2005). As shown in this work, a single functional copy of $marT_{STm}$ contribute to proliferation defects in *S*. Typhi inside U937. These proliferation defects might be conceivably due to the repression of *surV*, since *S*. Typhi Δ*surV* showed a similar defect than *S*. Typhi $marT^{+}_{STm}$.

In this work, we showed that *surV* contributes to decrease intracellular ROS when bacteria were cultured in presence of H_2O_2, suggesting that *surV* might be contributing somehow to increase the antioxidant activity, e.g. the catalase activity. Nevertheless, it has been postulated that DNA repair is more important than catalases for *S. enterica* virulence, since classic *S*. Typhimurium catalases KatE, KatG or KatN do not contribute to the survival inside macrophages, or even in mice, due to the presence of other genes encoding redundant functions (Buchmeier et al., 1995; Hebrard et al., 2009). Considering that *surV* exhibits a HNHc nucleases domain, found in DNA mismatch repair proteins such as MutS (Ogata et al., 2011), it is possible that SurV not only contributes to virulence by diminishing the ROS, but also by

participating in the DNA repair or in other unknown processes. ROS, such as hydrogen peroxide, inactivates iron containing proteins that possess [Fe—S] catalytic centers. The [Fe—S]-containing proteins have prominent roles in multiple important cellular processes, including respiration, central metabolism, gene regulation, RNA modification, and DNA repair and replication. The [Fe—S] centers are oxidized generating an unstable intermediate which quickly releases Fe^{2+}. This loss can fuel Fenton chemistry, which produces the highly reactive hydroxyl radical which in turn can oxidize DNA, lipids and proteins, then exacerbating oxidative stress. Due to its potential damaging effects, in bacteria, iron solubilization and metabolism is strictly regulated. Currently we are analyzing the possibility that this ROS-scavenging role of *surV* is indirect by the participation in the maintenance of the iron homeostasis. Additionally, we do not discharge that *surV* may help to reduce ROS levels by the participation in the maintenance of a proper intracellular reducing environment with non-enzymatic antioxidants such as NADPH and NADH pools, and glutathione (GSH).

From an evolutionary standpoint, bacterial genomes both expand and contract, depending upon the rate of acquisition of new genes (and phenotypes) and the deletion of redundant sequences (Goodhead and Darby, 2015). As described above, in *S.* Typhimurium, the fully functional *marT* gene encodes a positive transcriptional activator of *misL*, a gene encoding a fibronectin binding protein that is required for intestinal colonization in chicks and mice inoculated orally with this serovar (Dorsey et al., 2005; Morgan et al., 2004; Tukel et al., 2007). In this context, *marT* pseudogenization could arise because of the loss of function of *misL* in *S.* Typhi. A previous work already postulated that, in the case of genes whose products are functionally linked, the pseudogenization of one of the corresponding genes could facilitate the pseudogenization of the second gene (Valenzuela et al., 2015). However, in this study, the presence of *surV* in *S.* Typhi, a gain of function compared with *S.* Typhimurium, could have also contributed to fix the loss of function of *marT* in the population, by increasing the bacterial fitness. Thus, the gain of new functions might be also a factor giving rise to new of pseudogenes.

The data presented here demonstrate that *surV* contributes to the survival to H_2O_2 by decreasing the total ROS in *S.* Typhi, but also to the survival inside macrophages. Moreover, a single functional copy of $marT_{STm}$ contributed to repress *surV*, strongly suggesting that the *marT* pseudogenization improved the *surV*-dependent survival to H_2O_2, and the proliferation in human macrophages. Finally, since Infected macrophages migrate to regional lymph nodes and subsequently to deeper lymphoid organs such as the spleen and liver contributing to systemic dissemination of *S.* Typhi (Hensel et al., 1998; Rescigno et al., 2001), we propose that *marT* pseudogenization conceivable contributed to the *S.* Typhi adaptation to the systemic infection in humans.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.meegid.2016.08.029.

References

Andersson, J.O., Andersson, S.G., 1999. Insights into the evolutionary process of genome degradation. Curr. Opin. Genet. Dev. 9, 664–671.
Barrow, P.A., Duchet-Suchaux, M., 1997. *Salmonella* and *Salmonellosis* '97 proceedings. *Salmonella* Carriage and the Carrier State. Zoopôle, Ploufragan, France, pp. 241–250.
Baumler, A.J., 1997. The record of horizontal gene transfer in *Salmonella*. Trends Microbiol. 5, 318–322.
Baumler, A.J., Tsolis, R.M., Heffron, F., 1997. Fimbrial adhesins of *Salmonella typhimurium*. Role in bacterial interactions with epithelial cells. Adv. Exp. Med. Biol. 412, 149–158.
Berrocal, L., Fuentes, J.A., Trombert, A.N., Jofre, M.R., Villagra, N.A., Valenzuela, L.M., Mora, G.C., 2015. *stg* fimbrial operon from *S.* Typhi STH2370 contributes to association and cell disruption of epithelial and macrophage-like cells. Biol. Res. 48, 34.
Blanc-Potard, A.B., Groisman, E.A., 1997. The *Salmonella selC* locus contains a pathogenicity island mediating intramacrophage survival. EMBO J. 16, 5376–5385.
Blanc-Potard, A.B., Solomon, F., Kayser, J., Groisman, E.A., 1999. The SPI-3 pathogenicity island of *Salmonella enterica*. J. Bacteriol. 181, 998–1004.
Boyd, E.F., Wang, F.S., Beltran, P., Plock, S.A., Nelson, K., Selander, R.K., 1993. *Salmonella* reference collection B (SARB): strains of 37 serovars of subspecies I. J. Gen. Microbiol. 139 (Pt 6), 1125–1132.
Bradford, M.M., 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72, 248–254.
Buchmeier, N.A., Libby, S.J., Xu, Y., Loewen, P.C., Switala, J., Guiney, D.G., Fang, F.C., 1995. DNA repair is more important than catalase for *Salmonella* virulence in mice. J. Clin. Invest. 95, 1047–1053.
Calderon, I.L., Morales, E.H., Collao, B., Calderon, P.F., Chahuan, C.A., Acuna, L.G., Gil, F., Saavedra, C.P., 2014. Role of *Salmonella* Typhimurium small RNAs RyhB-1 and RyhB-2 in the oxidative stress response. Res. Microbiol. 165, 30–40.
Coburn, B., Grassl, G.A., Finlay, B.B., 2007. *Salmonella*, the host and disease: a brief review. Immunol. Cell Biol. 85, 112–118.
Collins, F.M., 1974. Vaccines and cell-mediated immunity. Bacteriol. Rev. 38, 371–402.
Contreras, I., Toro, C.S., Troncoso, G., Mora, G.C., 1997. *Salmonella typhi* mutants defective in anaerobic respiration are impaired in their ability to replicate within epithelial cells. Microbiology 143 (Pt 8), 2665–2672.
Dagan, T., Blekhman, R., Graur, D., 2006. The "domino theory" of gene death: gradual and mass gene extinction events in three lineages of obligate symbiotic bacterial pathogens. Mol. Biol. Evol. 23, 310–316.
Datsenko, K.A., Wanner, B.L., 2000. One-step inactivation of chromosomal genes in *Escherichia coli* K-12 using PCR products. Proc. Natl. Acad. Sci. U. S. A. 97, 6640–6645.
Deng, W., Liou, S.R., Plunkett 3rd, G., Mayhew, G.F., Rose, D.J., Burland, V., Kodoyianni, V., Schwartz, D.C., Blattner, F.R., 2003a. Comparative genomics of *Salmonella enterica* serovar Typhi strains Ty2 and CT18. J. Bacteriol. 185, 2330–2337.
Dorsey, C.W., Laarakker, M.C., Humphries, A.D., Weening, E.H., Baumler, A.J., 2005. *Salmonella enterica* serotype Typhimurium MisL is an intestinal colonization factor that binds fibronectin. Mol. Microbiol. 57, 196–211.
Ellermeier, C.D., Janakiraman, A., Slauch, J.M., 2002. Construction of targeted single copy *lac* fusions using lambda Red and FLP-mediated site-specific recombination in bacteria. Gene 290, 153–161.
Finlay, B.B., Falkow, S., 1989. *Salmonella* as an intracellular parasite. Mol. Microbiol. 3, 1833–1841.
Galán, J.E., 1996. Molecular genetic bases of *Salmonella* entry into host cells. Mol. Microbiol. 20, 263–271.
Goodhead, I., Darby, A.C., 2015. Taking the pseudo out of pseudogenes. Curr. Opin. Microbiol. 23, 102–109.
Groisman, E.A., Ochman, H., 1997. How *Salmonella* became a pathogen. Trends Microbiol. 5, 343–349.
Hacker, J., Carniel, E., 2001. Ecological fitness, genomic islands and bacterial pathogenicity. A Darwinian view of the evolution of microbes. EMBO Rep. 2, 376–381.
Hebrard, M., Viala, J.P., Meresse, S., Barras, F., Aussel, L., 2009. Redundant hydrogen peroxide scavengers contribute to *Salmonella* virulence and oxidative stress resistance. J. Bacteriol. 191, 4605–4614.
Hensel, M., Shea, J.E., Waterman, S.R., Mundy, R., Nikolaus, T., Banks, G., Vazquez-Torres, A., Gleeson, C., Fang, F.C., Holden, D.W., 1998. Genes encoding putative effector proteins of the type III secretion system of *Salmonella* pathogenicity island 2 are required for bacterial virulence and proliferation in macrophages. Mol. Microbiol. 30, 163–174.
Hibbing, M.E., Fuqua, C., Parsek, M.R., Peterson, S.B., 2010. Bacterial competition: surviving and thriving in the microbial jungle. Nat. Rev. Microbiol. 8, 15–25.
Hook, M., McGavin, M.J., Switalski, L.M., Raja, R., Raucci, G., Lindgren, P.E., Lindberg, M., Signas, C., 1990. Interactions of bacteria with extracellular matrix proteins. Cell Differ. Dev. 32, 433–438.
Jofre, M.R., Rodriguez, L.M., Villagra, N.A., Hidalgo, A.A., Mora, G.C., Fuentes, J.A., 2014. RpoS integrates CRP, Fis, and PhoP signaling pathways to control *Salmonella* Typhi hlyE expression. BMC Microbiol. 14, 139.
Jones, B.D., Ghori, N., Falkow, S., 1994. *Salmonella* Typhimurium initiates murine infection by penetrating and destroying the specialized epithelial M cells of the Peyer's patches. J. Exp. Med. 180, 15–23.
Knodler, L.A., Bestor, A., Ma, C., Hansen-Wester, I., Hensel, M., Vallance, B.A., Steele-Mortimer, O., 2005. Cloning vectors and fluorescent proteins can significantly inhibit *Salmonella enterica* virulence in both epithelial cells and macrophages: implications for bacterial pathogenesis studies. Infect. Immun. 73, 7027–7031.
Kuo, C.H., Ochman, H., 2010. The extinction dynamics of bacterial pseudogenes. PLoS Genet. 6.
Lawrence, J.G., 2003. Gene organization: selection, selfishness, and serendipity. Annu. Rev. Microbiol. 57, 419–440.
Matthews, T.D., Schmieder, R., Silva, G.G., Busch, J., Cassman, N., Dutilh, B.E., Green, D., Matlock, B., Heffernan, B., Olsen, G.J., Farris Hanna, L., Schifferli, D.M., Maloy, S., Dinsdale, E.A., Edwards, R.A., 2015. Genomic comparison of the closely-related *Salmonella enterica* serovars Enteritidis, Dublin and Gallinarum. PLoS One 10, e0126883.
Maurelli, A.T., 2007. Black holes, antivirulence genes, and gene inactivation in the evolution of bacterial pathogens. FEMS Microbiol. Lett. 267, 1–8.

McClelland, M., Sanderson, K.E., Spieth, J., Clifton, S.W., Latreille, P., Courtney, L., Porwollik, S., Ali, J., Dante, M., Du, F., Hou, S., Layman, D., Leonard, S., Nguyen, C., Scott, K., Holmes, A., Grewal, N., Mulvaney, E., Ryan, E., Sun, H., Florea, L., Miller, W., Stoneking, T., Nhan, M., Waterston, R., Wilson, R.K., 2001. Complete genome sequence of *Salmonella enterica* serovar Typhimurium LT2. Nature 413, 852–856.

Miao, E.A., Brittnacher, M., Haraga, A., Jeng, R.L., Welch, M.D., Miller, S.I., 2003. *Salmonella* effectors translocated across the vacuolar membrane interact with the actin cytoskeleton. Mol. Microbiol. 48, 401–415.

Miller, F.D., Hershberger, C.L., 1984. A quantitative beta-galactosidase alpha-complementation assay for fusion proteins containing human insulin B-chain peptides. Gene 29, 247–250.

Moran, N.A., Plague, G.R., 2004. Genomic changes following host restriction in bacteria. Curr. Opin. Genet. Dev. 14, 627–633.

Morgan, E., Campbell, J.D., Rowe, S.C., Bispham, J., Stevens, M.P., Bowen, A.J., Barrow, P.A., Maskell, D.J., Wallis, T.S., 2004. Identification of host-specific colonization factors of *Salmonella enterica* serovar Typhimurium. Mol. Microbiol. 54, 994–1010.

Ochman, H., Davalos, L.M., 2006. The nature and dynamics of bacterial genomes. Science 311, 1730–1733.

Ogata, H., Ray, J., Toyoda, K., Sandaa, R.A., Nagasaki, K., Bratbak, G., Claverie, J.M., 2011. Two new subfamilies of DNA mismatch repair proteins (MutS) specifically abundant in the marine environment. ISME J. 5, 1143–1151.

Parkhill, J., Dougan, G., James, K.D., Thomson, N.R., Pickard, D., Wain, J., Churcher, C., Mungall, K.L., Bentley, S.D., Holden, M.T., Sebaihia, M., Baker, S., Basham, D., Brooks, K., Chillingworth, T., Connerton, P., Cronin, A., Davis, P., Davies, R.M., Dowd, L., White, N., Farrar, J., Feltwell, T., Hamlin, N., Haque, A., Hien, T.T., Holroyd, S., Jagels, K., Krogh, A., Larsen, T.S., Leather, S., Moule, S., O'Gaora, P., Parry, C., Quail, M., Rutherford, K., Simmonds, M., Skelton, J., Stevens, K., Whitehead, S., Barrell, B.G., 2001. Complete genome sequence of a multiple drug resistant *Salmonella enterica* serovar Typhi CT18. Nature 413, 848–852.

Parry, C.M., Hien, T.T., Dougan, G., White, N.J., Farrar, J.J., 2002. Typhoid fever. N. Engl. J. Med. 347, 1770–1782.

Pfaffl, M.W., 2001. A new mathematical model for relative quantification in real-time RT-PCR. Nucleic Acids Res. 29, e45.

Rescigno, M., Rotta, G., Valzasina, B., Ricciardi-Castagnoli, P., 2001. Dendritic cells shuttle microbes across gut epithelial monolayers. Immunobiology 204, 572–581.

Retamal, P., Castillo-Ruiz, M., Mora, G.C., 2009. Characterization of MgtC, a virulence factor of *Salmonella enterica* Serovar Typhi. PLoS One 4, e5551.

Retamal, P., Castillo-Ruiz, M., Villagra, N.A., Morgado, J., Mora, G.C., 2010. Modified intracellular-associated phenotypes in a recombinant *Salmonella* Typhi expressing *S.* Typhimurium SPI-3 sequences. PLoS One 5, e9394.

Riquelme, S.A., Wozniak, A., Kalergis, A.M., Bueno, S.M., 2011. Evasion of host immunity by virulent *Salmonella*: implications for vaccine design. Curr. Med. Chem. 18, 5666–5675.

Soyer, Y., Orsi, R.H., Rodriguez-Rivera, L.D., Sun, Q., Wiedmann, M., 2009. Genome wide evolutionary analyses reveal serotype specific patterns of positive selection in selected *Salmonella* serotypes. BMC Evol. Biol. 9, 264.

Stochaj, W.R., Berkelman, T., Laird, N., 2006. Staining membrane-bound proteins with ponceau s. CSH Protocols 2006.

Tang, D., Shi, Y., Kang, R., Li, T., Xiao, W., Wang, H., Xiao, X., 2007. Hydrogen peroxide stimulates macrophages and monocytes to actively release HMGB1. J. Leukoc. Biol. 81, 741–747.

Trombert, A.N., Berrocal, L., Fuentes, J.A., Mora, G.C., 2010. *S.* Typhimurium *sseJ* gene decreases the *S.* Typhi cytotoxicity toward cultured epithelial cells. BMC Microbiol. 10, 312.

Trombert, A.N., Rodas, P.I., Mora, G.C., 2011. Reduced invasion to human epithelial cell lines of *Salmonella enterica* serovar Typhi carrying *S.* Typhimurium *sopD2*. FEMS Microbiol. Lett. 322, 150–156.

Tu, X., Latifi, T., Bougdour, A., Gottesman, S., Groisman, E.A., 2006. The PhoP/PhoQ two-component system stabilizes the alternative sigma factor RpoS in *Salmonella enterica*. Proc. Natl. Acad. Sci. U. S. A. 103, 13503–13508.

Tukel, C., Akcelik, M., de Jong, M.F., Simsek, O., Tsolis, R.M., Baumler, A.J., 2007. MarT activates expression of the MisL autotransporter protein of *Salmonella enterica* serotype Typhimurium. J. Bacteriol. 189, 3922–3926.

Uchiya, K., Barbieri, M.A., Funato, K., Shah, A.H., Stahl, P.D., Groisman, E.A., 1999. A *Salmonella* virulence protein that inhibits cellular trafficking. EMBO J. 18, 3924–3933.

Urrutia, I.M., Fuentes, J.A., Valenzuela, L.M., Ortega, A.P., Hidalgo, A.A., Mora, G.C., 2014. *Salmonella* Typhi *shdA*: pseudogene or allelic variant? Infect. Genet. Evol. 26, 146–152.

Uzzau, S., Figueroa-Bossi, N., Rubino, S., Bossi, L., 2001. Epitope tagging of chromosomal genes in Salmonella. Proc. Natl. Acad. Sci. U. S. A 98, 15264–15269.

Valenzuela, C., Ugalde, J.A., Mora, G.C., Alvarez, S., Contreras, I., Santiviago, C.A., 2014. Draft genome sequence of *Salmonella enterica* serovar Typhi strain STH2370. Genome Announc. 2.

Valenzuela, L.M., Hidalgo, A.A., Rodriguez, L., Urrutia, I.M., Ortega, A.P., Villagra, N.A., Paredes-Sabja, D., Calderon, I.L., Gil, F., Saavedra, C.P., Mora, G.C., Fuentes, J.A., 2015. Pseudogenization of *sopA* and *sopE2* is functionally linked and contributes to virulence of *Salmonella enterica* serovar Typhi. Infect. Genet. Evol. 33, 131–142.

van der Heijden, J., Bosman, E.S., Reynolds, L.A., Finlay, B.B., 2015. Direct measurement of oxidative and nitrosative stress dynamics in *Salmonella* inside macrophages. Proc. Natl. Acad. Sci. U. S. A. 112, 560–565.

Vázquez-Torres, A., Jones-Carson, J., Baumler, A.J., Falkow, S., Valdivia, R., Brown, W., Le, M., Berggren, R., Parks, W.T., Fang, F.C., 1999. Extraintestinal dissemination of *Salmonella* by CD18-expressing phagocytes. Nature 401, 804–808.

Wong, K.K., McClelland, M., 1994. Stress-inducible gene of *Salmonella* Typhimurium identified by arbitrarily primed PCR of RNA. Proc. Natl. Acad. Sci. U. S. A. 91, 639–643.

Zhang, L., Huang, Y., Xu, D., Yang, L., Qian, K., Chang, G., Gong, Y., Zhou, X., Ma, K., 2016. Biochemical characterization of a thermostable HNH endonuclease from deep-sea thermophilic bacteriophage GVE2. Appl. Microbiol. Biotechnol.