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Pseudogenization of *sopA* and *sopE2* is functionally linked and contributes to virulence of *Salmonella enterica* serovar Typhi

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ABSTRACT

The difference in host range between *Salmonella enterica* serovar Typhimurium (*S.* Typhimurium) and *S. enterica* serovar Typhi (*S.* Typhi) can be partially attributed to pseudogenes. Pseudogenes are genomic segments homologous to functional genes that do not encode functional products due to the presence of genetic defects. *S.* Typhi lacks several protein effectors implicated in invasion or other important processes necessary for full virulence of *S.* Typhimurium. SopA and SopE2, effectors that have been lost by pseudogenization in *S.* Typhi, correspond to an ubiquitin ligase involved in cytokine production by infected cells, and to a guanine exchange factor necessary for invasion of epithelial cells, respectively. We hypothesized that *sopA* and/or *sopE* pseudogenization contributed to the virulence of *S.* Typhi. In this work, we found that *S.* Typhi expressing *S.* Typhimurium *sopE2* exhibited a decreased invasion in different epithelial cell lines compared with *S.* Typhi WT. *S.* Typhimurium *sopA* completely abolished the hypo-invasive phenotype observed in *S.* Typhi expressing *S.* Typhimurium *sopE2*, suggesting that functional SopA and SopE2 participate concertedly in the invasion process. Finally, the expression of *S.* Typhimurium *sopA* and/or *sopE2* in *S.* Typhi, determined changes in the secretion of IL-8 and IL-18 in infected epithelial cells.

1. Introduction

Salmonella enterica subspecies I serovars share over 90% of their genome sequences at nucleotide level. Nevertheless, several of these serovars exhibit major differences in the host range (Andino and Hanning, 2015). Regarding these features, *S. enterica* serovars have been classified into two main groups: Bacteria infecting a broad range of hosts, such as *S. enterica* serovar Typhimurium (*S.* Typhimurium), called "generalists"; and bacteria infecting only one or a small group of related hosts, such as the human-restricted pathogen *S. enterica* serovar Typhi (*S.* Typhi), called "host-restricted serovars" (Baumler and Fang, 2013). In addition, *S.* Typhimurium and *S.* Typhi produce different diseases in humans. While *S.* Typhimurium produces usually a self-limited gastroenteritis, *S.* Typhi is the etiologic agent of the typhoid fever, a deadly systemic infection (Baumler and Fang, 2013; Loetscher et al., 2012).

The evolution of a broad host pathogen to a host-restricted pathogen might have occurred by acquisition of new genes through horizontal transfer, by the loss of functions due to reductive evolution, or by a combination of these mechanisms (Feng et al., 2012; Hacker and Carniel, 2001). Reductive evolution is characterized by genome degradation produced by pseudogene accumulation. Pseudogenes (Ψ) are genetic segments, homologous to functional genes, encoding non-functional products because of the presence of mutations, deletions, or other molecular defects (Holt et al., 2009). Several studies comparing host-restricted pathogens with their host-generalist relatives, indicate that reductive evolution is a hallmark of host-restricted pathogenic bacteria, underlying the importance of pseudogenes in evolutionary

Abbreviations: $sopA_{STm}$, *S.* Typhimurium *sopA* gene; $\Psi sopA_{STy}$, *S.* Typhi *sopA* pseudogene; $sopE2_{STm}$, *S.* Typhimurium *sopE2* gene; $\Psi sopE2_{STy}$, *S.* Typhi *sopE2* pseudogene; GEF, Rho guanine nucleotide exchange factor; GAP, GTPase-activating protein.

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processes leading to host specificity (Andersson and Andersson, 2001; McClelland et al., 2004; Parkhill et al., 2003). Actually, the pseudogenization of genes encoding some important virulence factors has paradoxically determined an increased virulence in *S.* Typhi. For instance, the pseudogenization of *sseJ*, encoding an acyltransferase/lipase that contributes to intracellular proliferation in *S.* Typhimurium, resulted in a more cytotoxic *S.* Typhi strain (Trombert et al., 2010). On the other hand, the pseudogenization of *sopD2*, which encodes a known SPI-1-dependent effector involved in the *S.* Typhimurium pathogenesis, yields a more invasive *S.* Typhi strain (Trombert et al., 2011).

The invasion of non-phagocytic cells, considered a fundamental step for *S. enterica* virulence, is governed by several translocated effectors with very specialized functions, including SopE and SopE2. These two effectors participates as Rho guanine nucleotide exchange factors (GEF) for Rho-GTPases. SopE specifically activates both Cdc42 and Rac1 RhoGTPases, whereas SopE2 activates only Cdc42 (Friebel et al., 2001). Activation of Rho-GTPases by GEF induces membrane rufflings and actin cytoskeleton rearrangement in the host cell, allowing bacterial invasion (Patel and Galan, 2005). *S. enterica* lacking either SopE or SopE2 exhibit only a slight defect in cellular invasion, whereas strains lacking both SopE and SopE2 present a clear reduction of invasion; thereby a functional redundancy *in vivo* has been proposed (Patel and Galan, 2006; Zhou et al., 2001). After internalization, host cells activate pro-inflammatory responses largely influenced by SopE and SopE2, and other effectors. SopE and SopE2 induce a Cdc42-dependent activation of the eukaryotic transcriptional factor NF-κB, a critical up-regulator of pro-inflammatory cytokine such as interleukin-8 (IL-8) (Bruno et al., 2009; Haraga et al., 2008; Hobbie et al., 1997; Patel and Galan, 2006, 2008). Moreover, SopE induces caspase-1 activation dependent mainly on Rac1, leading to the release of the pro-inflammatory interleukin-18 (IL-18) (Muller et al., 2009). IL-8 is a potent chemoattractant that promotes neutrophil transmigration in the infected intestine (Bignold et al., 1991), whereas IL-18 is essential for activating macrophages antimicrobial activities by increasing IFN-γ expression in the intestinal mucosa (Santos et al., 2009). SopA, another *S. enterica* effector, works along with SopE, SopE2, and other proteins to promote the production of chemoattractant cytokines leading to the infiltration of neutrophils (Wood et al., 2000; Zhang et al., 2003). Nevertheless, unlike SopE and SopE2, SopA induces neutrophil transepithelial migration due to its HECT-like E3 ubiquitin ligase activity (Zhang et al., 2006).

S. Typhi lacks some effector proteins that are crucial for the pathogenicity of the generalist serovar Typhimurium (Raffatellu et al., 2005b). In this context, both *sopA* and *sopE2* from *S.* Typhi are annotated as pseudogenes, suggesting that these genes are not relevant for the infection cycle of this host-restricted serovar (McClelland et al., 2004; Nuccio and Baumler, 2014; Parkhill et al., 2001). In contrast, *sopA* and *sopE2* are fully functional in *S.* Typhimurium (Raffatellu et al., 2005b; Wood et al., 2000; Zhang et al., 2002).

In this work, we studied the effect of the heterologous expression of the fully functional *sopA* and/or *sopE2* gene from *S.* Typhimurium in *S.* Typhi, to assess the phenotype in human cell lines. We found that the expression of functional *S.* Typhimurium *sopE2* gene in *S.* Typhi resulted in a decreased invasion in different epithelial cell lines compared with the *S.* Typhi WT. *S.* Typhimurium *sopA* completely abolished the hypo-invasive phenotype observed in *S.* Typhi expressing *S.* Typhimurium *sopE2*, suggesting that functional SopA and SopE2 participate concertedly in the invasion process. These data show that the pseudogenization of *sopA* and *sopE2* is functionally linked in *S.* Typhi. Finally, the expression of *S.* Typhimurium *sopA* and/or *sopE2* in *S.* Typhi, determined changes in the secretion of IL-8 and IL-18 by infected epithelial cells. The *sopA* and *sopE2* pseudogenization process and how it could have modulated the *S.* Typhi virulence are discussed in this work.

2. Materials and methods

2.1. Bacterial strains, media and culture conditions

S. Typhi strain STH2370 (STy) was obtained from the Infectious Diseases Hospital Lucio Córdova, Chile. *S.* Typhimurium ATCC14028s (STm) was obtained from the ISP, Chile. The strains 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) at 37 °C, with aeration, or under microaerophilic conditions by adding an overlay of 500 μl of sterile mineral oil as a barrier to oxygen prior to cell assays with cultured human cells HEp-2, HT-29 or T84. When required, medium was supplemented with kanamycin (Kan; 50 mg/ml), chloramphenicol (Cam; 20 mg/ml), or ampicillin (Amp; 50 mg/ml). Media were solidified by adding agar (15 g/L).

2.2. In silico analyses

Comparative sequence analyses were made with the *sopA* and *sopE2* sequences available at http://www.ncbi.nlm.nih.gov/ (*S.* Typhi strains STH2370, CT18, Ty2, Ty21a, and P-stx-12; and *S.* Typhimurium strain 14028s). *S.* Typhi STH2370 $\Psi sopA_{STy}$ and $\Psi sopE2_{STy}$ genes, 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. Typhimurium 14028s and S. Typhi STH2370 mutant strains

To generate the *S.* Typhimurium $\Delta sopA_{STm}$, *S.* Typhimurium $\Delta sopE2_{STm}$, *S.* Typhi $\Delta\Psi sopA_{STy}$, *S.* Typhi $\Delta\Psi sopE2_{STy}$, and *S.* Typhi Δ*sopE* deletion mutants, we substituted the corresponding gene with the FRT scar (i.e. gaagttcctatactttctagagaataggaacttc) using the primers SopA (H1 + P1)Del + SopA (H2 + P2)Del; SopE2 (H1 + P1)Del + SopE2 (H2 + P2)Del; or SopE (H1 + P1)Del + SopE (H2 + P2)Del (Table 1) following the Red/Swap technique (Datsenko and Wanner, 2000).

To generate the *S.* Typhi strains heterologously expressing a stable single copy, in *cis*, of either *S.* Typhimurium *sopA* ($sopA_{STm}$) or *S.* Typhimurium *sopE2* ($sopE2_{STm}$) we performed the following procedure. First, we constructed the *S.* Typhimurium donor strains by replacing the 3′UTR region of $sopA_{STm}$ or $sopE2_{STm}$ by a chloramphenicol (cam) cassette using the Red/Swap technique (Datsenko and Wanner, 2000) with the primers SopA(H1 + P1)3′UTR + SopA(H2 + P2)3′UTR; or SopE2(H1 + P1)3′UTR + SopE2(H2 + P2)3′UTR, respectively (Table 1). The substitutions produced no disruptions in the corresponding open reading frame since only the 3′UTR was replaced. This procedure yielded the *S.* Typhimurium $sopA_{STm}$3′UTR::cam and *S.* Typhimurium $sopE2_{STm}$3′UTR::cam donor strains, respectively. As the second step, we constructed the *S.* Typhi receptor strains. For that, we substituted the corresponding *S.* Typhi pseudogene with a kanamycin (kan) cassette using the Red/Swap technique (Datsenko and Wanner, 2000) with the corresponding primers SopA (H1 + P1)Del + SopA (H2 + P2)Del; or SopE2(H1 + P1)Del + SopE2(H2 + P2)Del (Table 1). With this procedure, we obtained the *S.* Typhi $\Delta\Psi sopA_{STy}$::kan and *S.* Typhi $\Delta\Psi sopE2_{STy}$:: kan receptor strains, respectively. Third, we amplified the $sopA_{STm}$3′UTR::cam or $sopE2_{STm}$3′UTR::cam loci using gDNA from the donor strains as template. For that, the flanking primers used