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Global impact of *Salmonella* type III secretion effector SteA on host cells

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ABSTRACT

Salmonella enterica is a Gram-negative bacterium that causes gastroenteritis, bacteremia and typhoid fever in several animal species including humans. Its virulence is greatly dependent on two type III secretion systems, encoded in pathogenicity islands 1 and 2. These systems translocate proteins called effectors into eukaryotic host cell. Effectors interfere with host signal transduction pathways to allow the internalization of pathogens and their survival and proliferation inside vacuoles. SteA is one of the few *Salmonella* effectors that are substrates of both type III secretion systems. Here, we used gene arrays and bioinformatics analysis to study the genetic response of human epithelial cells to SteA. We found that constitutive synthesis of SteA in HeLa cells leads to induction of genes related to extracellular matrix organization and regulation of cell proliferation and serine/threonine kinase signaling pathways. SteA also causes repression of genes related to immune processes and regulation of purine nucleotide synthesis and pathway-restricted SMAD protein phosphorylation. In addition, a cell biology approach revealed that epithelial cells expressing *steA* show altered cell morphology, and decreased cytotoxicity, cell–cell adhesion and migration.

1. Introduction

Many pathogenic Gram-negative bacteria possess type III secretion systems (T3SSs) for their interaction with the host. These systems allow delivery into eukaryotic host cells of effector proteins that direct the different stages of the infection at the cellular level [1]. *Salmonella enterica* possesses two distinct virulence-related T3SSs, T3SS1 and T3SS2, that are encoded by genes located in *Salmonella* pathogenicity islands 1 and 2 (SPI1 and SPI2), respectively [2]. T3SS1 is necessary for the invasion of non-phagocytic cells [3], whereas T3SS2 is induced after invasion and is essential for survival and replication within macrophages [4,5]. *S. enterica* injects more than thirty T3SS effectors to their host cells and some of them have been shown to manipulate cellular processes such as actin cytoskeleton organization, tight junction alterations, biogenesis of the *Salmonella*-containing vacuole and cell death [6]. However, the functions for many effectors are still unknown.

SteA was identified as a *S. enterica* serovar Typhimurium T3SS effector [7] that can be secreted to culture media and translocated into epithelial cells and macrophages through T3SS1 and T3SS2, depending on culture conditions, infected cell types and infection times. The first 10 amino acids of SteA act as a signal sequence for its translocation into the eukaryotic cell [8]. The gene *steA* is located outside SPI1 and SPI2, and its low GC content (43%) suggests horizontal acquisition, common in virulence-associated genes. We have previously shown that its expression is transcriptionally controlled by the bacterial redox status in a PhoQ/PhoP-dependent manner [9]. A *steA* null mutant is threefold attenuated for BALB/c mice virulence after intraperitoneal infection [7] and SteA seems to be involved in the bacterial persistence during long time infection in 129X1/SvJ mice [10]. In the host cell, SteA localizes to the *trans*-Golgi network (TGN) and to *Salmonella*-induced membrane tubules containing the *trans*-Golgi marker GalT-mCherry [7,11]. A very recent report has shown that SteA participates in the control of *Salmonella*-containing vacuole membrane dynamics [12]. This study also suggests that SteA should have additional roles, which remain to be elucidated, at earlier times of host cell infection.

Similarity to proteins with known activities has been useful in some cases to determine the function of a specific effector [13],

Abbreviations: T3SS, type three secretion system; SPI, *Salmonella* pathogenicity island; NCBI, National Center for Biotechnology Information; LB, Luria-Bertani; FDR, false discovery rate; LDH, lactate dehydrogenase; qPCR, quantitative real-time PCR; HEMA, 2-hydroxyethyl methacrylate; GO, gene ontology.

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but in the case of SteA no sequence similarities have been detected. Expression of individual effectors, like AvrA or SopB, in the budding yeast *Saccharomyces cerevisiae*, that serves as a simplified heterologous model [14], has been a productive approach to study the effects of these proteins on host cells [15–17], thereby helping in the discovery of their activities. Here, we use mammalian cells as a relevant model to specifically analyze the effect on the host transcriptome of the effector SteA. We show that in epithelial HeLa cells SteA leads to changes in the expression of genes related to extracellular matrix organization, cell proliferation, serine/threonine kinase signaling pathways, immune processes, regulation of purine nucleotide synthesis and pathway-restricted SMAD protein phosphorylation, and produces significant changes in cell death, adhesion, and migration.

2. Materials and methods

2.1. Bacterial strains and bacterial culture

Bacterial strains used were *Escherichia coli* DH5α [18] and *S. enterica* serovar Typhimurium SV5846 [8], a derivative of strain 14028 carrying a *steA*::3xFLAG chromosomal fusion. The standard bacterial culture medium was Luria-Bertani (LB) broth. Solid LB contained agar 1.5% final concentration. Antibiotics were used at the following concentrations: kanamycin, 50 μg/ml; ampicillin, 100 μg/ml.

2.2. Plasmid construction, DNA amplification, and sequencing

Plasmid pIZ1963 is a derivative of pBABEpuro [19] coding for SteA with a C-terminal 3xFLAG tag. To construct this plasmid, *steA*::3xFLAG was amplified from strain SV5846 using primers steApcdnadir and steAflagxho3, digested with *Bam*HI and *Xho*I and ligated together with vector pBABEpuro previously digested with *Bam*HI and *Sal*I. Amplification reactions and confirmation by sequencing were carried out as previously described [8]. Primers are listed in Table S1.

2.3. Mammalian cell culture, transfection and lysis

HeLa cells (ECAC No. 93021013) were cultured, transfected, and lysed as previously described [20]. For stable transfection, HeLa cells were electroporated with pBABEpuro or its derivative and selection was started 24 h after electroporation in medium containing 1 μg/ml puromycin (InvivoGen).

2.4. Bacterial infection of cultured cells and analysis of SteA translocation

HeLa cells were plated in 6-well plates at 6×10^5 cells per well and incubated for 24 h. *Salmonella* infections were carried out as previously described [8]. The cell culture was washed twice with PBS 1 h post-infection and overlaid with DMEM containing 100 μg/ml gentamicin. One hour later the concentration of gentamicin was lowered to 16 μg/ml after an additional wash with PBS. Infected mammalian cells were lysed 6 h post-infection with Nonidet P-40 buffer as described [8]. The extract was centrifuged at 13,000 rpm for 20 min and the supernatant was filtered and analyzed by immunoblot.

2.5. Electrophoresis, immunoblot, and antibodies

Proteins in cell extracts were resolved by SDS–PAGE. The gel was blotted onto a nitrocellulose membrane (Amersham) and probed with mouse monoclonal anti-FLAG M2 primary antibodies (1:5000; Sigma) and mouse monoclonal anti-β-actin C4 (1:5000; Santa Cruz Biotechnology) as loading control. Goat anti-mouse horseradish peroxidase-conjugated antibodies (1:5000; BioRad) were used as secondary antibodies. Detection was via chemiluminescence procedures (Pierce).

2.6. RNA preparation, gene array processing, and statistical analysis

Total RNA from HeLa cells stably transfected with pBABEpuro or its derivative encoding the SteA-3xFLAG fusion was isolated in triplicate using 1 ml of TRIzol reagent (Invitrogen) according to the protocol supplied by the manufacturer. An additional purification step was carried out by using the RNeasy Min Elute Cleanup Kit (Qiagen). Biotinylated single-stranded cDNA was prepared from 100 ng per sample of total intact RNA extracted from 9 independent samples (3 from HeLa pBABEpuro, 3 from HeLa pBABEpuro-SteA-3xFLAG L2, and 3 from HeLa pBABEpuro-SteA-3xFLAG L4). Labeled cDNA was hybridized to GeneChip Human Gene 1.0 ST Arrays (Affymetrix) following the manufacturer's instructions. The arrays were scanned in a 3000 7G Scanner from Affymetrix. Image analysis, fluorescent data quantification and quality control was carried out with Affymetrix software. All procedures and preliminary data analysis, including fluorescent data processing, normalization using Robust Multi-array Average (RMA) algorithms, and annotations, were performed at the Genomics Unit of the Andalusian Center for Molecular Biology and Regenerative Medicine (CABIMER, Seville, Spain). Fold change was calculated for each cell line relative to the control line transfected with pBABEpuro. Statistical significance (p value) was calculated by empirical Bayes moderated t-test based on the results of three arrays per condition. The false discovery rate (FDR) for each p value was also calculated. Genes that changed with an FDR-adjusted p value higher than 0.05 were removed from subsequent analysis. The microarray data used in this analysis is available from NCBI's Gene Expression Omnibus at http://www.ncbi.nlm.nih.gov/geo/ under accession number GSE51043.

2.7. Function prediction of gene sets

To predict the functional association networks of the activated and repressed genes from the microarray, we used the web-based software application tool GeneMANIA [21]. Gene ontology (GO) terms associated to genes in Table 1 were searched using AmiGO version 1.8 [22].

2.8. Quantitative real-time PCR (qPCR)

The protocol for qPCR was previously described [9]. DNA primers are indicated in Table S1. Gene expression levels were normalized to transcripts of *BCAT1*, gene that appeared not affected by the presence of SteA in the microarray.

2.9. Proliferation assays

HeLa cells were seeded in 6-well plates at a density of 6×10^4 cells per well and grown at 37 °C, 5% CO_2 in DMEM. At 24 h intervals, the cells from a well per cell line were trypsinized and counted on a haemocytometer.

2.10. Cell death, cell adhesion, and cell migration assays

Cell death was measured using a previously described protocol based on lactate dehydrogenase (LDH) release in the cultures [20]. Cell adhesion was determined as previously described [23]. For cell–cell adhesion assays, 6×10^4 cells/ml were seeded onto 6-well plates coated with poly-(2-hydroxyethyl methacrylate) (poly-HEMA) (Sigma) in order to prevent cell-matrix interactions. After

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