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An *in vivo* proteomic study of the interaction between *Salmonella* Typhimurium and porcine ileum mucosa

Melania Collado-Romero[a], *Rodrigo P. Martins*[a], *Cristina Arce*[a], *Ángela Moreno*[a], *Concepción Lucena*[a], *Ana Carvajal*[b], *Juan J. Garrido*[a,*]

[a]*Grupo de Genómica y Mejora Animal, Departamento de Genética, Universidad de Córdoba, Campus de Rabanales, Edificio Gregor Mendel C5, 14071 Córdoba, Spain*

[b]*Departamento de Sanidad Animal, Facultad de Veterinaria, Universidad de León, 24071 León, Spain*

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ABSTRACT

The enteropathogen *Salmonella* Typhimurium is one of the main causes of porcine and human enterocolitis. We have used a 2-DE, MALDI-TOF/TOF-based approach to characterize *in vivo* proteome changes in porcine ileum mucosa after pathogen interaction. Ileum samples from non-infected and orally infected animals were collected at 2 days post infection and S. Typhimurium presence was confirmed by immunohistochemistry. Fifty one proteins, involved in immune response (acute phase response, inflammation and immune response regulation), apoptosis and pathogen-mediated cell invasion, were identified as being differentially expressed after pathogen challenge. Overall, anti-inflammatory signals and a possible down-regulation of dendritic cell maturation were observed. According to this, we identified the up-regulation of FK506-binding protein 4 (FKBP4), a negative regulator of the transcription factor IRF4 (interferon regulatory factor 4), implicated in Th2 and Th17 response. Transcriptional analysis using RT-qPCR indicated a general trend toward down-regulation of Th2 and Th17 cytokines genes, which would be in agreement with an IRF4 reduced transactivation activity. On the other hand, proteins that could be involved in maturation of *Salmonella*-containing vacuole and intracellular pathogen survival were up-regulated. Results derived from this study would be valuable to better characterize a possible pathogen led modulation of host responses *in vivo*.

1. Introduction

Salmonella enterica subsp. *enterica* serovar Typhimurium (S. Typhimurium) is a broad-host range serotype able to colonize the lower intestinal tract of a wide range of animals, including humans [1].

In swine, S. Typhimurium infection causes enterocolitis, frequently with sub-clinical symptoms, and can be maintained in a carrier-state by infected animals [2]. S. Typhimurium has become the most commonly non-typhoideal serotype isolated in pigs and recent data point pigs/pork as the main source of human salmonellosis in USA and European countries [2]. Although, in general, human salmonellosis is not a life-threatening disease, annually 3 million deaths due to non-typhoidal *Salmonella* are estimated occur worldwide [3]. Moreover, the prevalence of multidrug-resistant S. Typhimurium

Abbreviations: dpi, days post infection; Mw, molecular weight; ACN, acetonitrile; qPCR, quantitative real-time PCR; SCV, *Salmonella*-containing vacuole.

* *Corresponding author at: Grupo de Genómica y Mejora Animal, Departamento de Genética, Universidad de Córdoba, Campus de Rabanales, Edif.* C5 1ª planta, 14071 Córdoba, Spain. Tel.: +34 957 212692.
E-mail address: ge1gapaj@uco.es (J.J. Garrido).

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phagetypes in pigs, such DT104 used in this study, makes swine salmonellosis a major public-health concern [4,5]. Currently, the implementation of animal breeding projects and the design of effective vaccines are the bases for effective and sustainable disease control; therefore, a better understanding of host responses against *Salmonella* infection at the mucosal barrier is crucial.

Proteomics studies, including two-dimensional electrophoresis (2-DE), have been carried out in order to achieve a better understanding of *S.* Typhimurium physiology. Those studies include the proteomic characterization of different *Salmonella* spp. and strains [6], identification of translational changes occurring in the pathogen under *in vivo* mimicking conditions [7,8], and the characterization of the intracellular pathogen proteome [9,10]. However, little proteomic information is available from the host's point of view, and the few reported studies were carried out using *in vitro* infections of isolated cell lines [11]. In a recent work, we identified important differences in the inflammatory response at transcriptional level throughout the porcine gut upon *S.* Typhimurium infection [12]. Our results showed ileum mucosa as unable to up-regulate some pro-inflammatory cytokines, which could help to a more successful colonization of this site by the infecting bacteria. In view of these results, the aim of this present study was to identify changes in the porcine ileum proteome as a consequence of *S.* Typhimurium infection *in vivo*. Moreover, we sought to identify changes that could be directed by the pathogen in order to achieve a successful entrance and survival as intracellular pathogen in the intestine, i.e., we focused on the early response of colonized ileum mucosa to *S.* Typhimurium.

2. Materials and methods

2.1. Bacterial strain

The *Salmonella enterica* subs. *enterica* serovar Typhimurium phagetype DT104 was an isolate from a carrier pig [5]. Bacteria growing at 37 °C in LB broth to log stationary phase (OD_{600nm} of 0.8) was harvested by centrifugation at 8000 *g*, and adjusted to a final concentration of 10^8 CFU/ml in PBS.

2.2. Experimental infection

Eight 4-week old crossbreed weaned piglets were used. Before infection, all the animal's fecal samples were confirmed as being free of *Salmonella*. Pigs were housed in an environmentally controlled isolation facility at 25 °C and under constant light with *ad libitum* access to feed and water. After an acclimation period of 5 days, four pigs (one female and three males) were infected orally with 10^8 CFU of *S.* Typhimurium, whereas the control group (two females and two males) received sterile medium orally. The four non-infected control pigs were necropsied 2 h prior to experimental infection. The four infected pigs were necropsied at 2 dpi. The ileum was carefully emptied of its content and sectioned in pieces that were immediately snap frozen in liquid nitrogen and stored at −80 °C for protein and RNA isolation, or, alternatively, fixed in 10% neutral-buffered formalin for 24 h for subsequent immunostaining assays. All the infected animals were fecal-culture positive for *Salmonella* and developed similar clinical signs of gastrointestinal disease, including increased rectal temperature, diarrhea and lethargy. Piglets were housed in the experimental isolation facilities of the University of León (Spain). Animal care and procedures were in accordance with the guidelines of the Good Experimental Practices (GEP), under the supervision of the Ethical and Animal Welfare Committee of the University of León.

2.3. Immunohistochemistry using a *S. Typhimurium* specific antibody

Formalin-fixed tissues were embedded in paraffin wax following standard procedures. Sections of 5 μm were placed on slides coated with Poly-L-Lysine (Sigma-Aldrich) and kept at 55 °C for 45 min. Slides were dewaxed in xylene and rehydrated through graded alcohols to distilled water. Slides were then subjected to heat-mediated antigen retrieval in 0.01 M citric acid, incubated with a polyclonal antibody developed in our laboratory against the *Salmonella* strain used in this study and stained as described before [13].

2.4. Mucosa isolation, protein extraction and protein concentration determination

Ileum sections of around 2 cm were thawed onto an ice-cold plate and opened by means of a longitudinal cut. The luminal surface was thoroughly cleaned using sterile gauze and PBS to eliminate mucus, and blotted dry onto dried gauze. Mucosa scrapings were obtained using a razor, weighed and homogenized using a glass tissue-lyser and lysis buffer in a proportion of 1 ml of buffer per 500 mg of mucosa. Lysis buffer composition was: 7 M urea, 2 M thiourea, CHAPS 4% (Sigma-Aldrich), DTT 1% (Sigma-Aldrich), 50 U DNAse I (Roche) per ml of lysis buffer, Proteinase Inhibitor Cocktail P8340 (Sigma-Aldrich), Bio-Lyte ampholytes 0.8% (Bio-Rad) and Milli-Q water. The whole homogenization process was carried out in ice. Samples were incubated by 20 min in an orbital shaker and finally centrifuged at 16,000 *g* for 15 min at 4 °C. Supernatants were individually recovered and used for subsequent analysis. Protein concentration was determined using Bradford Protein Assay (Bio-Rad) according to the manufacturer's instructions.

2.5. Two-dimensional gel electrophoresis (2-DE) and image analysis

IPG strips (17 cm, 4–7 linear pH gradient) (Bio-Rad) were rehydrated with 600 μg of the protein solution in a total volume of 300 μl. Control and infected samples were focused simultaneously in a Protean IEF Cell (Bio-Rad), using the following parameters: 1) active rehydration at 50 V for 12 h; 2) at 250 V for 15 min without pause after rehydratation; 3) rapid ramp until reaching 10,000 V h and 4) until 60,000 V h with slow ramped voltage. After IEF, the IPG strips were equilibrated by soaking first for 10 min in 50 mM Tris–HCl, pH 8.8, 6 M Urea, 2% SDS, 30% glycerol and 2% (w/v) DTT (Sigma-Aldrich) and then for 10 min in the solution containing 2.5% (w/v) iodoacetamide (Sigma-Aldrich). Second dimension was performed on 12% SDS-polyacrylamide gels using Protean Plus Dodeca Cell (Bio-Rad). Gels were stained with SYPRO Ruby protein gel