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Salmonella Typhimurium infection primes a nutriprive mechanism in piglets

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ABSTRACT

Salmonella enterica serovar Typhimurium (*S.* Typhimurium) is an important cause of acute food- borne zoonoses worldwide, typically carried by pigs. It is well known that *Salmonella* has evolved a wide array of strategies enabling it to invade the host, but little information is available on the specific host responses to *Salmonella* infections. In the present study, we used an *in vivo* approach (involving piglets infected with a virulent or an attenuated *S.* Typhimurium strain) coupled to histological and proteomic analysis of the cecum mucosa, to highlight the host pathways activated during *S.* Typhimurium infection. We confirm the complex host-pathogen interaction. Our data showed that the metabolic and the cytoskeleton organization functions were the most significantly altered. In particular, the modifications of energy metabolic pathway could suggest a "nutriprive" mechanism, in which the host reduce its metabolic and energetic status to limit *Salmonella* infection. This study could represent a preliminary approach, providing information useful to better understand the host-*Salmonella* interaction.

1. Introduction

Salmonella enterica serovar Typhimurium (*S.* Typhimurium) is a Gram-negative bacterium able to colonize the lower intestinal tract of a wide range of animals, including humans (Fedorka-Cray et al., 1995). It is an important cause of acute food-borne zoonoses worldwide and responsible of numerous cases of human gastroenteritis and bacteremia annually. Pigs are typically asymptomatic carriers of *S.* Typhimurium and this commensal-like state establishes a significant reservoir of infection (Bearson and Bearson, 2011). Approximately 15% (range 7–20%) of all cases of enteric salmonellosis in industrialized countries originate from pork products (Burel et al., 2013), therefore, a better control of *Salmonella* infection in pigs is important to reduce health risks for humans. Proteome approaches are a useful tool to investigate the host–pathogen interaction, allowing the detection of pathophysiological alterations that occur during infection (Encheva et al., 2007; Arce et al., 2014). Most of previous studies have investigated the translational changes occurring in *S.* Typhimurium during infection (Sonck et al., 2009), while little information is available on the host response. However, recently, some studies have focused on the dynamic response of porcine mesenteric lymph nodes, ileum and intestinal mucosa to *S.* Typhimurium infection (Martins et al., 2012; Collado-Romero et al., 2012). The aim of the present study, was to highlight the alterations induced by two different strains of *Salmonella*, a fully virulent and an attenuated strain, in the porcine cecum proteome. The attenuated *S.* Typhimurium strain, lacking the high affinity zinc transporter ZnuABC, (Ammendola et al., 2007) was chosen because it represents a promising mucosal vaccine against salmonellosis in pigs (Gradassi et al., 2013; Pesciaroli et al., 2013; Ruggeri et al., 2014). The identified differentially expressed proteins were

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investigated by bioinformatic tools in order to identify the molecular pathways and the biological functions which are altered during infection. Our results provide general information that may be useful to better understand the host- *S.* Typhimurium interaction.

2. Materials and methods

2.1. Salmonella *spp. cultures*

Virulent *S.* Typhimurium ATCC 14028 (STM^{wt}) and its isogenic attenuated *znuABC* mutant ($STM^{\Delta znuABC}$) (Ammendola et al., 2007) were grown overnight at 37 °C in Brain Heart Infusion broth (Oxoid Ltd., Basingstoke, UK), harvested by centrifugation and washed twice in ice-cold phosphate buffer solution (PBS) (Sigma-Aldrich, St. Louis, MO).

2.2. Animals and samples collection

Thirty-one post-weaned piglets of 28 days of age were enrolled in the study. All were the offspring of *Salmonella*-free sows (negative for *Salmonella* by serological and microbiological tests); similarly, the study animals had proved to be *Salmonella*-free. Piglets were divided into three groups, each of which were allocated in separate rooms in biosafety level facility of the Istituto Zooprofilattico Sperimentale della Lombardia e dell'Emilia Romagna, and pellet fed was administered ad libitum. A total of 9 animals composed the naïve group (control) which was inoculated by oral route with sterile sodium bicarbonate buffer. Two groups of 11 piglets were orally infected with 2×10^9 CFU of $STM^{\Delta znuABC}$ ($STM^{\Delta znuABC}$) or 2×10^9 CFU of STM^{wt} (STM^{wt}), respectively.

Four naive control piglets and 5 animals of each of the two groups of infected piglets were euthanized using a captive bolt pistol and exsanguination at 1 day post infection (dpi); 5 piglets of naïve and 6 piglets of groups $STM^{\Delta znuABC}$ and STM^{wt} were euthanized at 12 dpi. Samples of ileum, cecum and colon were fixed in 10% neutral buffered formalin for subsequent histological analyses. In addition, sections of cecum were frozen by immersion in liquid nitrogen and stored at −80° for subsequent proteomic analyses. All the experiments were authorized by national authority and were conducted according to European Directive (2010/63/EU). The approval number is: 54/2012.

2.3. Histology

Formalin-fixed tissues were embedded in paraffin wax and stained with haematoxylin and eosin according to standard procedures. Features taken in consideration: the presence of eosinophilic, neutrophilic and lympho-plasmacytic infiltrate in the lamina propria and in the submucosa. It was also evaluated the severity of the intestinal epithelial damage.

2.4. Protein extraction, two dimensional gel electrophoresis and image analysis

Pools containing equal amounts (50 mg) of each of the cecum samples were prepared for the three experimental groups, immediately after thawing, generating 6 sample pools. Protein extraction was performed as previously described (Signorelli et al., 2012), with minor modifications. Briefly, samples were homogenized in lysis buffer (7 M urea, 2 M thiourea, 2% CHAPS, 1% DTT, and inhibitor cocktail Sigma-Aldrich, St. Louis, MO), sonicated on ice, shaken for 1 h at 15 °C and centrifuged for 5 min at 20000*g* at 15 °C. Total protein concentration was quantified by 2D Quant Kit (GE Heathcare, Niskayuna, NY). Pellet was precipitated using cold acetone; then it was dried and resuspended in lysis buffer.

Samples (100 μg/strip) were loaded on rehydrated IPG strips (11 cm, pH 3–10, NL, Bio-Rad) for isoelectric focusing using a Protean IEF cell (Bio-Rad Laboratories, Inc., Hercules, CA) for a total of approximately 27 kVh. After focusing, the IPG strips were gently soaked for a total of 15 min in equilibration solution (6 M urea, 50 mM Tris-HCl buffer pH 8.8, 30% v/v glycerol, 2% w/v SDS and 0.002% bromophenol blue) containing 2%, w/v DTT, followed by 2 step incubation in equilibration solution containing 2.5% w/v iodoacetamide for a total of 15 min. Second-dimension gel electrophoresis was carried out in a 4–15% polyacrylamide Ready Gel precast gels using the CRITERION™ Cell (Bio-Rad). Equilibrated strips were placed onto gels and run at a constant voltage of 200 V for about an hour. Gels were stained overnight in cCBB—colloidal Coomassie Brilliant Blue (Neuhoff et al., 1988). Each pool was analyzed in triplicate.

The proteins were visualized by gel scanning with the Molecular Imager Pharos FX scanner (Bio-Rad Laboratories). To identify valid spots, PD Quest advanced program software (Bio-Rad Laboratories) was used. Spots were automatically detected on the basis of the spot parameters chosen such as the faintest, smallest, and largest spot on the gel scan, and only those well-resolved were taken into account. Spot photodensity was normalized for the total quantity of all valid spots. Spots photodensities of the $STM^{\Delta znuABC}$ and STM^{wt} groups were compared with the control group, separately.

2.5. Statistical analysis of spot data

The spots with a photodensity fold change larger than 2 and a *p*-value below 0.05 (*t*-test), between at least a paired spot (control and sample, at 1 dpi or at 12 dpi), were considered differentially expressed and identified by mass spectrometry (MS). Significant differences in spot photodensity were confirmed by one-way analysis of variance of data (ANOVA). ANOVA was performed with GLM procedure, using the SAS 9.1 statistical software (SAS, Statistical Analysis with SAS/STAT® Software V9.1. SAS Institute Inc. 2009), considering each animal group as factor. Data are expressed as estimate mean and compared by Duncan's test with $p < 0.05$ as significant level.

2.6. Protein identification by mass spectrometry

Protein spots were excised manually from the gels, and destained with 50 mM ammonium bicarbonate pH 8.0 in 50% acetonitrile. Gel pieces were re-suspended in 50 mM ammonium bicarbonate pH 8.0 containing 100 ng of trypsin, incubated for 2 h at 4 °C and then overnight at 37 °C. The supernatant containing the resulting peptide mixtures was removed and the gel pieces were re-extracted with acetonitrile. The 2 fractions were then collected, freeze-dried, and analyzed by LC–MS/MS using the LC/MSD Trap XCT Ultra (Agilent Technologies, Palo Alto, CA) equipped with an 1100HPLC system and a chip cube (Agilent Technologies). Mass spectral data were used to search a non-redundant protein database (National Center for Biotecnology Information—NCBI mammals database) using an in-house version of the Mascot software (Matrix Science, Boston, MA, USA). Each spectrum from mass spectrometry was searched against the mammalian protein database. Proteins of the bacterial origin were not identified.

2.7. System biology analysis

To gain information about the biological significance of the identified proteins, we used the bioinformatic tools. Integrated functions and interactions of the proteins were explored using