International Journal of Infectious Diseases 40 (2015) 28–33

Contents lists available at ScienceDirect

International Journal of Infectious Diseases

journal homepage: www.elsevier.com/locate/ijid

Comparative proteomics of *Shigella flexneri* 2a strain 301 using a rabbit ileal loop model reveals key proteins for bacterial adaptation in host niches

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ARTICLE INFO

Article history:
Received 23 March 2015
Received in revised form 17 September 2015
Accepted 19 September 2015

Corresponding Editor: Eskild Petersen, Aarhus, Denmark

Keywords:
Comparative proteomics
Mass spectrometry
Rabbit ileal loop model
Shigella flexneri
Two-dimensional gel electrophoresis

SUMMARY

Objectives: Many studies focusing on changes in the host following *Shigella spp* invasion have been reported in recent years. However, the key factors required for the adaptation of these pathogens to host niches have usually been neglected.

Methods: In this study, a comparative proteomic analysis was performed to examine changes in the protein expression profile of *Shigella flexneri* within the host using a rabbit ileal loop model to reveal proteins that are associated with pathogenic adaptation.

Results: The protein expression profiles of bacteria isolated from the ileum and colon were very similar, although they differed slightly from that of bacteria isolated from the cecum. When compared with the sample in vitro, the expressions of seven proteins were found to be upshifted in vivo (OmpA, YgiW, MglB, YfiD, MetK, TktA, and AhpF), while two proteins were down-regulated (ElaB and GlnH).

Conclusions: The abundance of nine proteins changed in vivo, suggesting that these proteins may contribute to adaptation to the intestinal lumen.

1. Introduction

Four species of *Shigella* (*S. flexneri*, *S. sonnei*, *S. dysenteriae*, and *S. boydii*) cause bacillary dysentery in humans and primates.[1] Within each species, there is a large variety of serotypes based on the structure of the O-antigen repeats that comprise the polysaccharide moiety of the lipopolysaccharide.[2] The endemic form of shigellosis is primarily caused by *S. flexneri* and *S. sonnei*.[3] Given that the genome of the *S. flexneri* 2a strain 301 has been sequenced[4] and that it has the ability to invade epithelial cells,[5] this strain is an ideal candidate for microbial proteomic analyses in an animal model.

Extensive studies have examined the interactions between *Shigella spp* and different types of host, including piglets,[6,7] guinea pigs,[8] rabbits,[9,10] macaques,[11,12] and mice.[13] However, these studies have mostly focused on the disease phenotypes of the host, such as infection assessments, weight loss, diarrhea, fever,[8] and pathological changes in the intestinal mucosa.[9,10] In fact, the physiological changes in pathogens in the intestinal environment are equally important for understanding the interactions between bacteria and their hosts. Pieper et al. reported a comprehensive proteomic analysis of *S. dysenteriae* and found that the abundance of 1061 distinct gene products changed in a bacterial sample isolated from the large bowel of infected gnotobiotic piglets.[6] Pieper et al. also analyzed the proteome of *S. flexneri* within the epithelial cell cytoplasm and discovered that the levels of glycogen biosynthesis enzymes and mixed acid fermentation enzymes were much higher than they were in vitro.[14] These data indicate that many intracellular proteins play a crucial role in Shigella pathogenicity, in addition to key effectors encoded by the large virulence plasmid.

Regarding animal models, rabbits are more commonly used than gnotobiotic piglets because of their convenience and affordability. For example, using a ligated gastrointestinal (GI) loop model, Marteyn et al. demonstrated that available oxygen in vivo could activate the type III secretion system (T3SS) of *S. flexneri* at the tips of intestinal villi.[9] Interestingly, a virulent *S. flexneri* strain could not successfully colonize the colon or cause colitis in

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http://dx.doi.org/10.1016/j.ijid.2015.09.014

rabbits following colonic inoculation without cecal bypass (ligation of the distal cecum).[15] This result suggests that the cecum plays an important role in Shigella infections.

As an enteric pathogen, Shigella can sense environmental changes, including pH, osmolarity, temperature, oxygen tension, magnesium, reactive oxygen species (ROS), and nitrogen oxide (NO), and adjust its own proteome to adapt to different environmental niches. In this study, a modified rabbit ligated GI loop model was adopted to analyze the changes in bacterial protein expression profiles in vivo on a global level. In particular, three parts of the rabbit intestinal tract (the ileum, cecum, and colon) were included in these experiments. The biological functions of differentially expressed proteins are discussed.

2. Materials and methods

2.1. Bacterial strains and culture conditions

S. flexneri 2a strain 301 was grown routinely on Luria–Bertani (LB; 10 g tryptone, 5 g yeast extract, and 10 g of NaCl per liter) agar plates. The inoculum for animal experiments was prepared by selecting a typical colony from LB plates and inoculating it into liquid LB medium, followed by incubation at 37 °C with shaking. One hundred milliliters of bacterial culture (optical density at 600 nm (OD_{600}) = 3.0) was harvested by centrifugation at 5000 × *g*, washed twice with ice-cold phosphate-buffered saline (PBS), and then suspended in 40 ml of PBS. Half of the suspension was used for animal experiments and the other half was left in a sealed centrifuge tube, which was maintained at 39 °C, as a control.

2.2. Animal experiments and isolation of S. flexneri from rabbit intestines

Japanese white rabbits were anesthetized with 10% (w/v) chloral hydrate (2 ml/kg) via the auricular vein. Dialysis bags (molecular weight cutoff 15 000 Da) filled with bacteria (suspended in 20 ml of PBS) were surgically placed into the ileum, cecum, and colon. As a control, the same volume of bacteria was placed in a sealed centrifuge tube that was incubated at 39 °C, which is equal to the normal rectal temperature of the rabbits, without shaking. About 7 h after the bacterial inoculation, the animals were killed and the dialysis bags were removed for bacterial protein extraction.

2.3. Preparation of cell lysates

S. flexneri cell pellets were harvested by centrifugation at 5000 × *g*, washed twice with ice-cold PBS, and then suspended in lysis buffer (7 M urea, 2 M thiourea, 4% (w/v) CHAPS, and 1% (w/v) DL-dithiothreitol (DTT)) containing Complete Protease Inhibitor Cocktail (Roche Applied Science, Penzberg, Germany). After ultrasonic lysis, to digest nucleic acids, 0.5% immobilized pH gradient (IPG) buffer, DNase I (10 μg/ml), and RNase A (10 μg/ml) were added and incubated with gentle agitation for 1 h at 20 °C. The lysate was centrifuged at 40 000 × *g* for 30 min at 4 °C, and the supernatant was collected as the protein sample for the subsequent analyses. To standardize the protein contents of different samples, the protein concentration of all samples was measured using the PlusOne 2-D Quant kit (GE Healthcare, Chalfont St. Giles, UK), and 800-μg aliquots were stored at −80 °C.

2.4. Two-dimensional gel electrophoresis (2-DE) of the protein samples

The 2-DE procedure and in-gel protein digestion were performed as described previously.[16] IPG strips of pH 4–7 and pH 6–11 were used for loading acidic and basic proteins, respectively. Briefly, each 800-μg protein sample was used to rehydrate an 18-cm IPG strip for 12 h at 20 °C. After focusing, the strips were equilibrated with DTT and iodoacetamide for 15 min in equilibrium buffer (2% sodium dodecyl sulfate (SDS), 50 mM Tris–HCl (pH 8.8), 6 M urea, and 30% glycerol). Then, 12.5% SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was used for the second dimension. Protein spots were carefully excised from Coomassie-stained 2-DE gels, destained, washed, and then digested for 13 h with modified sequencing grade trypsin (Roche Applied Science). Peptides from the digested proteins were used for the matrix-assisted laser desorption/ionization dual time-of-flight (MALDI–TOF/TOF) analysis.

2.5. Mass spectrometry analysis

MALDI–TOF/TOF mass spectrometry (MS) measurements were performed on a Bruker Ultraflex III MALDI–TOF/TOF MS (Bruker Daltonics, Billerica, MA, USA) operating in reflectron mode. A saturated solution of α-cyano-4-hydroxycinnamic acid in 50% acetonitrile and 0.1% trifluoroacetic acid was used as the matrix. The SNAP algorithm in FlexAnalysis 3.4 was used to identify the 150 most prominent peaks. The subsequent tandem MS (MS/MS) analysis was performed in a data-dependent manner, and the five most abundant ions were subjected to high-energy, collision-induced dissociation analysis. The collision energy was set to 1 keV; nitrogen was used as the collision gas.

2.6. Data interpretation and database searching

The program Mascot 2.1 (Matrix Science Ltd, Boston, MA, USA) was used to search the MS data against the *S. flexneri* 2a strain 301 database to eliminate redundancies resulting from multiple members of the same protein family, and the results were checked against the non-redundant database of the National Center for Biotechnology Information. The search parameters were as follows: trypsin digestion with one missed cleavage, carbamidomethyl modification of cysteine as a fixed modification, oxidation of methionine as a variable modification, +0.2 Da maximum peptide tolerance, +0.6 Da maximum MS/MS tolerance, a peptide charge of 1, and monoisotopic mass.

3. Results

3.1. The proteome of S. flexneri recovered from the rabbit ileum

A preliminary proteomic analysis of acidic proteins showed that the protein expression profiles of bacteria recovered from the rabbit ileum and the colon were so similar that no significant difference could be detected (data not shown). Thus, the difference in expression between the protein samples isolated from bacteria recovered from the terminal ileum and those that were isolated from bacteria that were incubated in vitro were mainly compared and analyzed. The acidic (loaded onto the pH 4–7 strips) and basic (loaded onto the pH 6–11 strips) proteins expressed by bacteria recovered from the ileum and those that were expressed by bacteria that were grown in vitro were separated by isoelectric focusing (18 cm) in the first dimension, and then by 12.5% SDS-PAGE in the second dimension. As shown in Figure 1, the 2-DE gels of the two samples are comparable to each other, and more than 1000 spots were detected in each sample by colloidal Coomassie staining. After an abundance comparison, the expression of two proteins (ElaB (spot ID 5) and GlnH (spot ID 9)) was found to be down-regulated in vivo, while the