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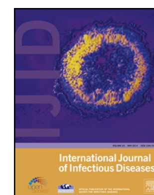

Comparative proteomics of *Shigella flexneri* 2a strain 301 using a rabbit ileal loop model reveals key proteins for bacterial adaptation in host niches

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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.
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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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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

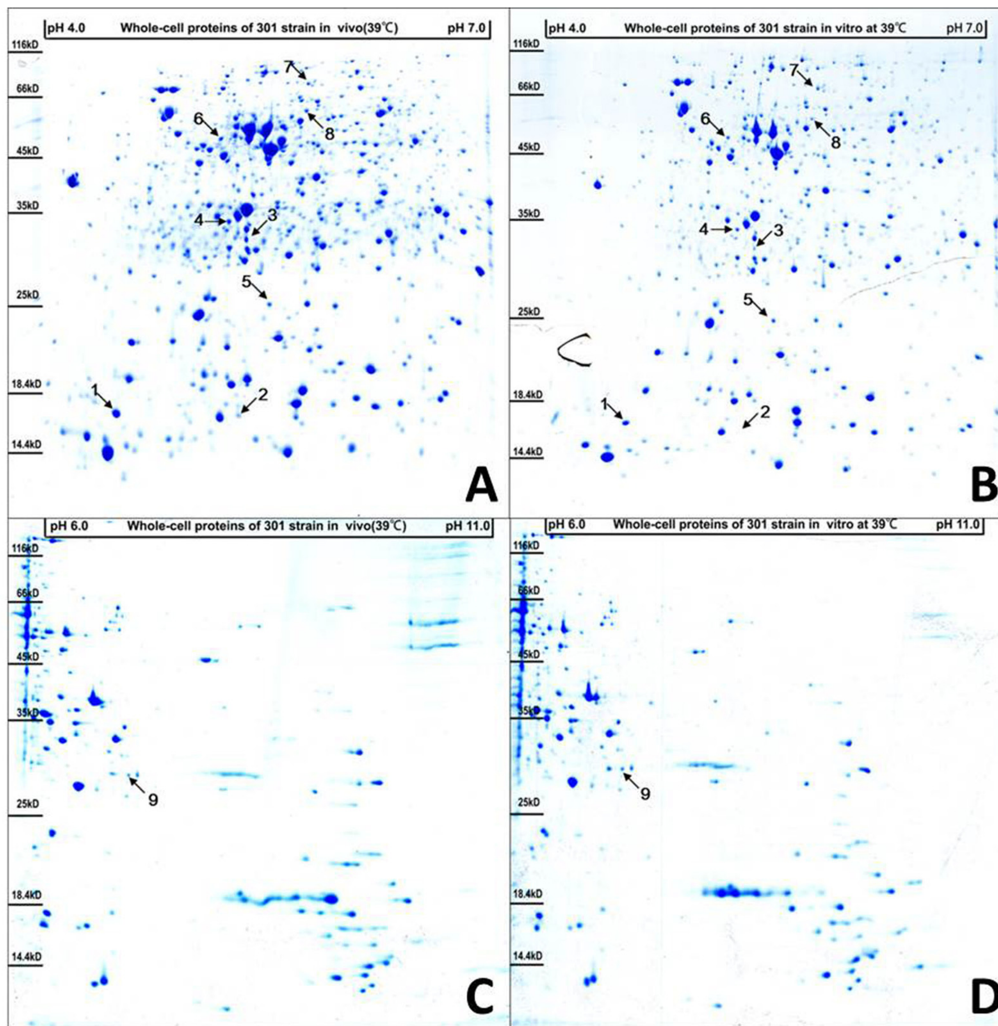

Figure 1. Comparison of two-dimensional gel electrophoresis profiles of bacteria recovered from the rabbit ileum (A and C) and bacteria grown in vitro (B and D). Acidic proteins (A and B) and basic proteins (C and D) were separated and analyzed. Identified proteins are indicated with arrows.

expression of seven other proteins (YgiW, YfiD, OmpA, MglB, MetK, TktA, and AhpF) was up-regulated.

3.2. The proteome of S. flexneri recovered from the rabbit cecum

Given the potential role of the cecum in Shigella infections, the protein samples from the rabbit cecum were also prepared in a second surgical operation. The whole-cell proteins were extracted, separated, and analyzed as described above (Figure 2). Only three proteins were differentially expressed in bacteria recovered in the cecum compared with those that were isolated from bacteria that were grown in vitro. Among these proteins, the abundance of two proteins (OmpA (spot ID 10) and YgiW (spot ID 11)) increased in vivo, while the abundance of the other protein (GlnH (spot ID 12)) decreased.

3.3. Identification of selected proteins

A total of 12 protein spots representing nine differentially expressed proteins were identified reproducibly by MS. Enlarged images of 2-DE gels highlighting selected differentially expressed proteins are shown in Figure 3. The database search results of these proteins are summarized in Table 1. The detailed functional categories and cellular localization of these proteins, as predicted by the PSORTb algorithm, are also described.

4. Discussion

Because the colon and terminal ileum are sites of colonization and invasion for Shigella, it is reasonable that the whole-cell protein samples recovered from bacteria isolated from these tissues exhibited similar expression profiles according to the present 2-DE results, while the difference between the protein profiles of bacteria recovered from the ileum and cecum indicated that there might be small differences in the microenvironmental niche between different parts of the intestinal tract. Although the cecum is located at the junction between the colon and terminal ileum, some special environmental signals might suppress the activation of Shigella. In particular, more differentially expressed proteins were identified in the samples isolated from the colon and terminal ileum. The functions of these proteins are related to oxygen tension and oxidative stress (discussed below), which reflect the different microenvironments in the cecum and ileum.

S. flexneri is a facultative intracellular pathogen that invades and propagates in epithelial cells. To discriminate between proteins associated with bacterial adaptation in different host niches and those that are required for intracellular survival, the bacteria were encased in a dialysis bag before placing them into the gut, rather than directly incubating them in the gut. Thus, known virulence-related proteins, such as the key effectors of the T3SS, were not

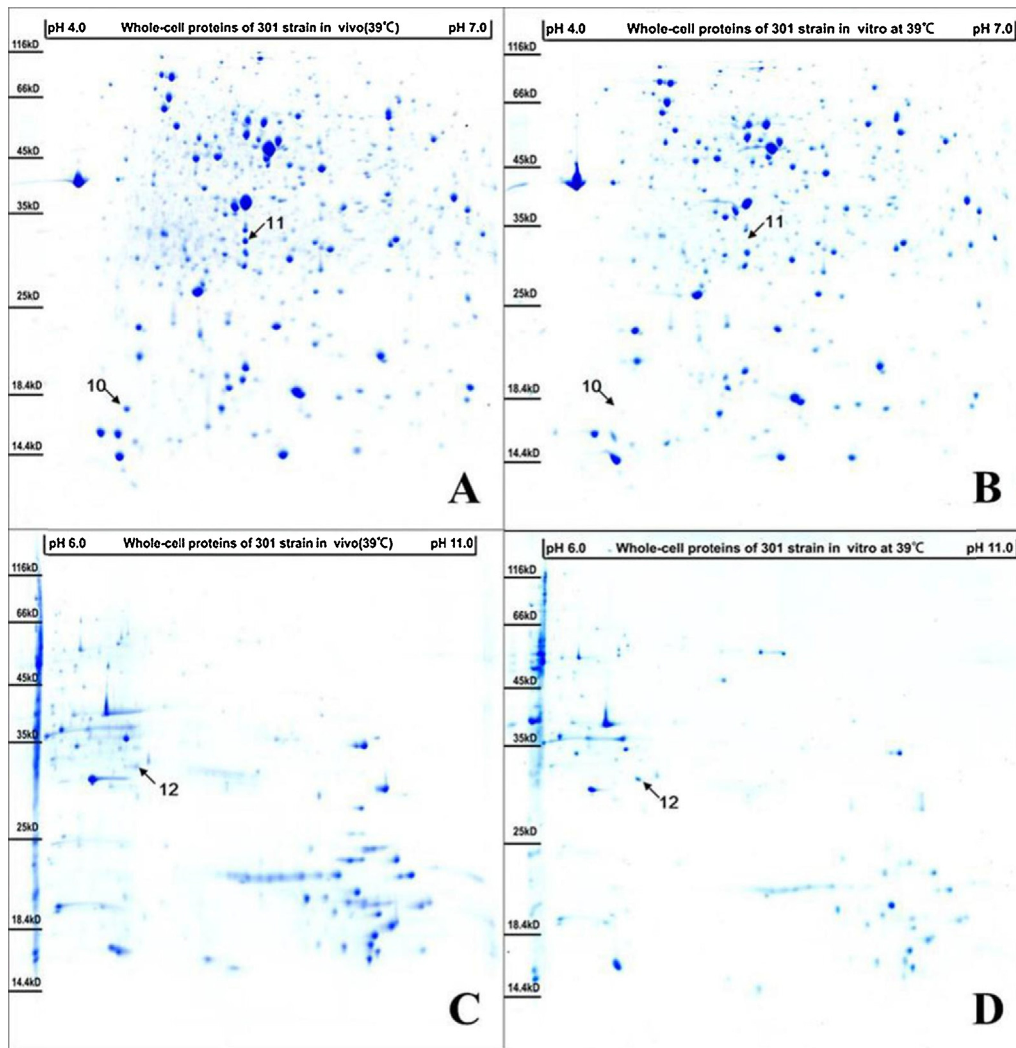

Figure 2. Comparison of two-dimensional gel electrophoresis profiles of bacteria recovered from the rabbit cecum (A and C) and bacteria grown in vitro (B and D). Acidic proteins (A and B) and basic proteins (C and D) were separated and analyzed. Identified proteins are indicated with arrows.

identified in this study because of the lack of direct contact between the bacteria and the intestinal epithelium.

The microenvironment, including the oxygen tension, osmotic pressure, and ROS, of the intestinal tract differs from that in vitro. Importantly, oxygen is limited in the intestinal lumen. Thus, pathogens should rely mainly on anaerobic respiration rather than aerobic respiration to obtain energy. In this study, under low oxygen tension conditions in the intestinal lumen, FNR,[9] one of the main global regulators at the aerobic/anaerobic interface, was activated, and the expression of a series of anaerobic respiration-related enzymes, including YfiD, a pyruvate–formate lyase, whose expression was very low in vitro, increased by several hundred-fold in vivo. This phenomenon is consistent with a previous study of *S. dysenteriae* in the piglet model.[6] Meanwhile, YfiD might function to maintain acid homeostasis in bacteria growing under hypoxic conditions.[17] Furthermore, the transketolase TktA, which was identified as an up-regulated protein, is a key enzyme that connects glycolysis and the pentose phosphate pathway,[18] and it plays an important role in anaerobic fermentation in hypoxic environments.

Although the *S. flexneri* incubated in the dialysis bag could not directly contact intestinal epithelium cells, they could still secrete some harmful low-molecular weight substances, such as antigens or virulence factors. These could pass through the dialysis membrane and stimulate the host intestinal tract, thereby causing epithelial cells to release ROS and NO,[19] which in turn places oxidative stress on the pathogens. As a result, the expression of hydrogen peroxide resistance-associated proteins, such as YgiW and AhpF, also increased in this study. The former is a periplasmic protein with an amino-terminal signal peptide, and it is reported to be involved in the cellular response to hydrogen peroxide and cadmium stress.[20] The AhpF component of alkyl hydroperoxide reductase belongs to the family of pyridine nucleotide-disulfide oxidoreductases, which are thought to have a role in the reduction of hydroperoxide.

Additionally, three proteins (OmpA,[21] MetK,[22] and YgiW[20]) that were up-regulated in vivo have been reported to be associated with biofilm formation in *Escherichia coli*. The outer membrane protein OmpA is believed to be a non-specific diffusion channel, whose function may be very important for bacteria to sense extracellular signals and stimuli.[23] YgiW is a member of the bacterial oligonucleotide/oligosaccharide binding fold family.[24] A homologous protein, VisP, of *Salmonella enterica* serovar Typhimurium is a virulence-related protein that binds to peptidoglycan and interacts with the lipid A-modifying enzyme LpxO.[25] Methionine adenosyltransferase (MetK) is an important and unique bacterial enzyme that catalyzes the formation of the sulfonium compound *S*-adenosylmethionine.[26] All of the above three proteins have been

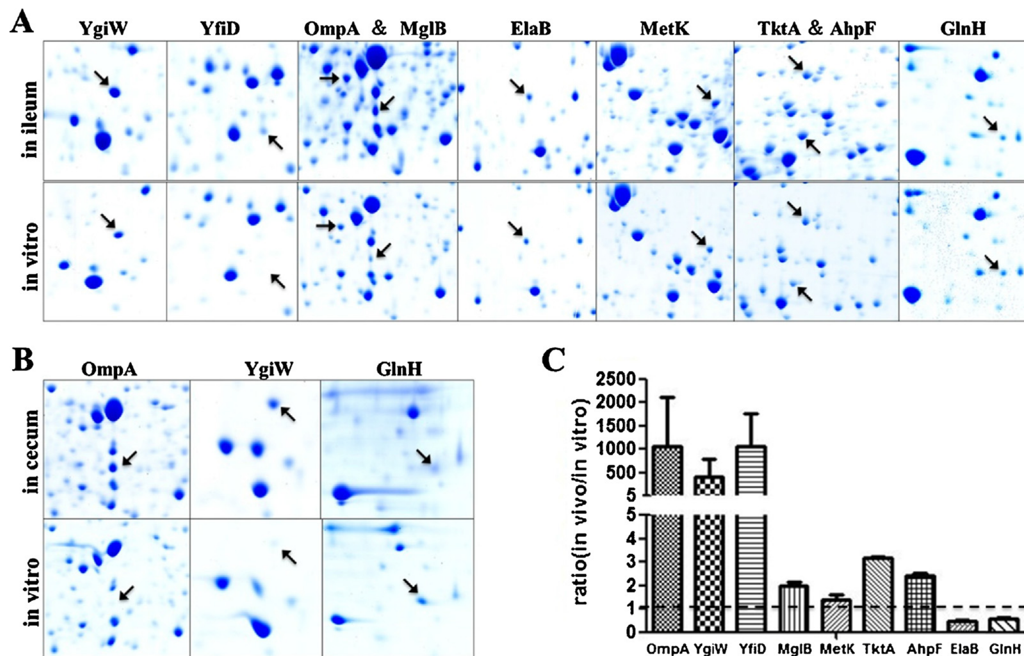

Figure 3. Abundance comparison of selected differentially expressed proteins. (A) Enlarged images of two-dimensional electrophoresis gels of proteins from bacteria isolated from the rabbit ileum. (B) Enlarged images of two-dimensional electrophoresis gels of proteins from bacteria isolated from the rabbit cecum. (C) Bar graph representing the abundance ratios (in vivo/in vitro) of selected proteins.

Table 1
Detailed information for the proteins identified

Sample	ID	Score	gi	Gene	Localization	COG(s)	Expression pattern[a]
Ileum	1	166	gi|30042631	*ygiW*	Periplasmic	COG3111S	+
	2	134	gi|30042236	*yfiD*	Cytoplasmic	COG3445R	+
	3	465	gi|30040740	*ompA*	Outer membrane	COG2885M	+
	4	447	gi|30041846	*mglB*	Periplasmic	COG1879G	+
	5	265	gi|30041952	*elaB*	Cytoplasmic	COG4575S	–
	6	185	gi|30042515	*metK*	Cytoplasmic	COG0192H	+
	7	185	gi|30042505	*tktA*	Cytoplasmic	COG0021G	+
	8	185	gi|30040308	*ahpF*	Cytoplasmic	COG3634O	+
	9	602	gi|30040540	*glnH*	Periplasmic	COG0834ET	–
Cecum	10	468	gi|30040740	*ompA*	Outer membrane	COG2885M	+
	11	162	gi|30042631	*ygiW*	Periplasmic	COG3111S	+
	12	90	gi|30040540	*glnH*	Periplasmic	COG0834ET	–

COGs, clusters of orthologous groups.
[a] The expression pattern of the proteins identified refers to the relative abundance of the protein in the in vivo sample compared to that in the in vitro sample. A '+' symbol means that the protein had a higher abundance in vivo than in vitro; a '–' symbol means the opposite.

reported to be associated with biofilm formation. Although Shigella is very closely related to *E. coli*, it is rarely reported to generate biofilms. The mechanism underlying the changes in abundance of these proteins is still not clear, although it is possible that pathogenic bacteria experience several types of environmental pressures in vivo and activate a self-protective process that is similar to biofilm formation.

In conclusion, this is the first extensive proteomic survey of *S. flexneri* recovered from different parts of the rabbit intestinal tract. First, it was demonstrated that *S. flexneri* efficiently sensed and responded to different microenvironments in the cecum, colon, and terminal ileum. Second, the abundance of nine proteins differed between cells recovered from the rabbit intestine and those grown in vitro. The bacterial response to hypoxic conditions required higher expression levels of enzymes involved in anaerobic fermentation, such as YfiD and TktA. Proteins associated with resistance to ROS (YgiW and AhpF) also had a role in the adaptation of bacteria to environmental niches of the host. YgiW, MetK, and OmpA might participate in a self-protective process that is similar to biofilm formation. The results of this proteomic survey will increase our understanding of host–pathogen interactions.

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Conflict of interest: The authors declare that they have no conflict of interest.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.ijid.2015.09.014.

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