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# Comparative proteomic analysis of outer membrane vesicles from *Shigella flexneri* under different culture conditions

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ABSTRACT

The production of outer membrane vesicles (OMVs) is a common and regulated process of gram-negative bacteria. Nonetheless, the processes of *Shigella flexneri* OMV production still remain unclear. *S. flexneri* is the causative agent of endemic shigellosis in developing countries. The Congo red binding of strains is associated with increased infectivity of *S. flexneri*. Therefore, understanding the modulation pattern of OMV protein expression induced by Congo red will help to elucidate the bacterial pathogenesis.

In the present study, we investigated the proteomic composition of OMVs and the change in OMV protein expression induced by Congo red using mTRAQ-based quantitative comparative proteomics. mTRAQ labelling increased the confidence in protein identification, and 148 total proteins were identified in *S. flexneri*-derived OMVs. These include a variety of important virulence factors, including Ipa proteins, TolC family, murein hydrolases, and members of the serine protease autotransporters of Enterobacteriaceae (SPATEs) family. Among the identified proteins, 28 and five proteins are significantly up- and down-regulated in the Congo red-induced OMV, respectively. Additionally, by comprehensive comparison with previous studies focused on *DH5a*-derived OMV, we identified some key node proteins in the protein–protein interaction network that may be involved in OMV biogenesis and are common to all gram-negative bacteria.



## 1. Introduction

*Shigella* is the main cause of clinical dysentery among children in developing countries [1]. Nonetheless, an effective vaccine against shigellosis has not been developed. Most related research has investigated the characteristics of the bacteria itself. Overall, these studies have led to a better understanding of the genetic and cellular bases of their pathogenesis.

Outer membrane vesicles (OMVs) are generally known to be constitutively secreted by pathogenic and nonpathogenic microbes during growth in liquid culture. With an average diameter of 50–300 nm, OMVs are composed of soluble luminal portions and an insoluble outermost membrane, such as toxins, porins, lipoproteins, periplasmic proteins, cytoplasmic proteins, nucleotide and LPS [2–8]. Membrane vesicle production has been reported in some gram-negative pathogens including *Pseudomonas aeruginosa* [9], *Neisseria gonorrhoeae* [10], *Actinobacillus actinomycetemcomitans* [11], *Bacteroides fragilis* [12], *Haemophilus influenza* [13], and *Escherichia coli* [14]. These spherical bilayered OMVs are typically considered to be virulence factors or serve as novel secretion machinery [4,5,15,16]. Because OMVs are essential to bacterial survival and pathogenesis in the host, the detailed characterization of OMVs is a critical step in the development of alternative vaccination strategies. Many OMV studies have aimed to determine the mechanisms of vesicle biogenesis, OMV composition, conditions that affect OMV production, the design of OMV-based vaccines, and so on. Like other bacteria, *Shigella* spp. secretes membranous vesicles [2,17,18]; however, the composition and potential biological functions of its vesicles are not well defined.

Congo red binding of smooth strains is generally correlated with *Shigella* spp. virulence properties and can be utilized as a reliable alternative to the Sereny test [19–21]. Additionally, Congo red binding is associated with increased infectivity of *Shigella flexneri* in the HeLa cell model. Sankaran et al. found that the proteins regulated in vitro by Congo red were relevant to the pathogenicity of *S. flexneri* 2a in vivo [22,23]. They may mimic the host tissue

*Abbreviations:* OMV, outer membrane vesicle; mTRAQ, mass differential tags for relative and absolute quantification; SPATEs, serine protease autotransporters of Enterobacteriaceae family; T3SS, type III secretion system.

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factor in vitro, providing clues about the potential regulation mechanism of bacteria in contact with the host. Additionally, the study of proteins regulated by Congo red should further our understanding of host-bacterium interactions and contribute to the prevention and treatment of bacterial infections.

With the aim of better understanding the pathophysiological roles of the OMVs of *S. flexneri*, we describe herein a detailed quantitative comparison between the protein content of the purified wild type OMVs and Congo-red induced OMVs. Our results provide a number of indicators that will not only increase our understanding of the mechanism of vesicle biogenesis and OMV functions but also facilitate the development of vaccines and novel antibiotics that are effective against pathogenic strains.

## 2. Materials and methods

### 2.1. Cell culture

Frozen *S. flexneri* 2a str. 301 was kindly provided by the ICDC, China CDC. The bacterial strains were grown in tryptic soy broth (TSB) to the exponential growth phase ($OD_{600}$ = 0.8) at 37 °C and 150 rpm and then harvested by centrifugation for 10 min at 4000*g*. The pelleted cells were washed and, resuspended with TSB (37 °C) and then divided evenly into four parts (Samples A–D). Samples A and B and Samples C and D were incubated at 37 °C and 150 rpm for 1 and 3 h with and without the presence of 0.01% (wt/vol) Congo red, respectively. After 1 or 3 h incubation, the bacteria were removed by centrifugation, and the supernatant was collected and used to isolate OMVs as described below.

### 2.2. Preparation of membrane vesicle samples

OMVs were harvested and purified from the supernatant according to the method of Kadurugamuwa and Beveridge [24]. Purified OMVs were analyzed using a TEM (see Supplemental Methods), or lysed with a buffer containing 4% CHAPS and 100 mM DTT for MS analysis. Protein concentrations were determined by a BCA kit (Pierce). Equal amounts of each of the lysates were digested with trypsin and processed with the FASP method using 10 k filtration units (Microcon YM-10, Millipore) [25]. The resulting peptide mixture was desalted using an Oasis HLB 1 cc cartridge (Waters). mTRAQ labeling protein mixtures were labeled with mTRAQ™ reagent (Applied Biosystems) according to the manufacturer's protocol. Samples were reduced with 50 mM tris(2-carboxyethyl) phosphine (TCEP) for 1 h at 60 °C and treated with 200 mM methylmethane thiosulfonate (MMTS) at room temperature for 10 min. Digestion was performed overnight with proteomics grade trypsin (Roche) at 37 °C. Digestion mixtures were desalted using an Oasis cartridge (Waters) and dried in a Speed-Vac concentrator. The dried samples were reconstituted in 500 mM triethylammonium bicarbonate (TEAB) and incubated with mTRAQ reagent at 25 °C for 1 h. The labeling protocol was performed following the Global Internal Standard (GIS) workflow. A global internal standard was created from a pool of the relevant samples by labeling it with the heavy mTRAQ reagent (Δ8) and then added to each light-labeled individual sample. Individual samples were labeled with mTRAQ reagent Δ0 (Samples B and C) or Δ4 (Samples A and D). Quantitative analysis was carried out by comparing each of the Δ0- and Δ4-labeled samples to the Δ8-labeled GIS mixture.

### 2.3. Mass spectrometry analysis

Labeled sample mixtures were reconstituted in an appropriate amount of 3% ACN and 0.1% formic acid (FA). Nano LC–MS/MS analyses were performed on a nanoACQUITY UPLC platform (Waters) coupled to a LTQ-Orbitrap Velos hybrid mass spectrometer (Thermo Electron) (see Supplemental Methods). A linear gradient of 5–35% solvent B over a 90 min period (solvent A, 0.1% FA, 3% acetonitrile; solvent B, 97% acetonitrile in 0.1% FA) at a flow rate of 400 nL/min was applied. MS data were acquired by a survey scan (*m/z* 300–1,800, *R* = 60,000 at *m/z* 400) in the Orbitrap using lock masses (*m/z* = 445.120025) for internal recalibration in real time [26]. The twenty most intense peaks for fragmentation in the LTQ were selected with a normalized collision energy value of 35%, a dynamic exclusion duration of 30 s, and an ion selection threshold of 500 counts. Raw data were acquired using the Xcalibur software v2.2.6 (Thermo).

### 2.4. MS data processing and analysis

The raw mass spectrometric data were processed using the MaxQuant software v1.2.2.5 [27] applying default settings unless otherwise noted. The initial peptide mass tolerance was set to

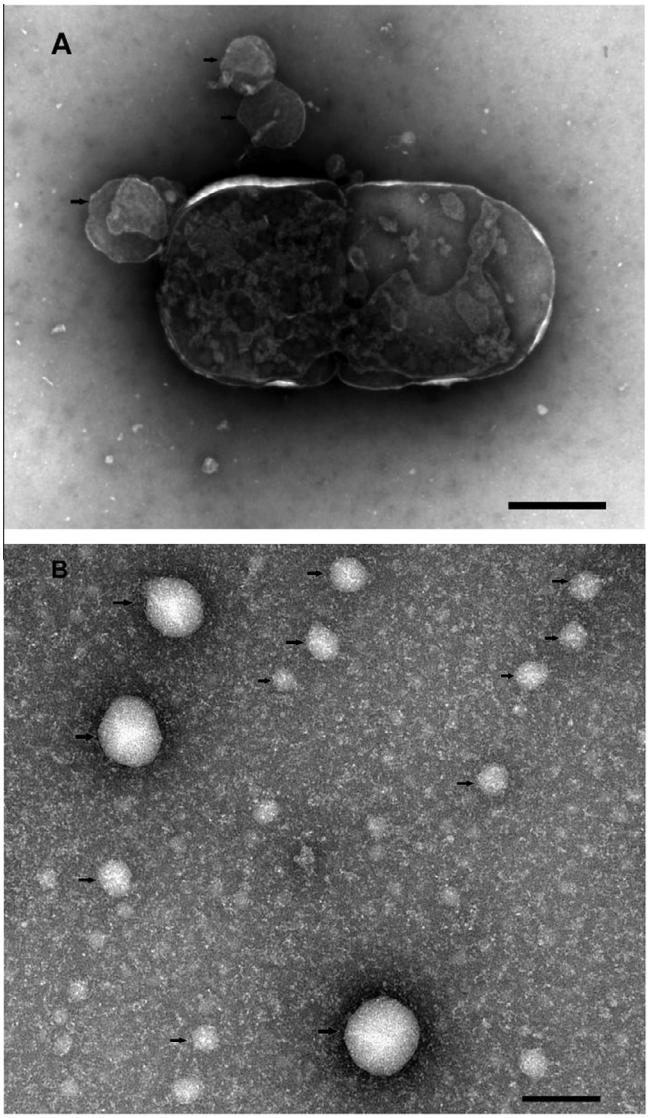


**Fig. 1.** Electron micrographs of negatively stained *S. flexneri* and isolated outer membrane vesicles. (A) Arrows indicate the OMVs present in the spaces as well as blebbing from the surface of *S. flexneri*. Bars, 200 nm. (B) Negative staining TEM of OMVs enriched following a modified OMV isolation protocol. Arrows indicate some of the OMVs present. Bars, 200 nm.

6 ppm, and the fragment mass tolerance was set to 0.5 Da for CID MS/MS spectra and 20 ppm for HCD MS/MS spectra. Full tryptic specificity was required, and up to two missed cleavages were allowed; the minimal length required for a peptide was set to six amino acids. Variable modification of methionine oxidation and fixed modification of cysteine alkylation with MMTS were assigned. MS/MS spectra were searched against the *S. flexneri* 2a str. 301 database (4437 entries) combined with 247 common contaminants by the Andromeda search engine. To pass statistical evaluation, the maximum peptide and protein false discovery rates (FDR) were set to 0.01, and the maximal posterior error probability (PEP), which is the probability of each peptide being a false hit considering the identification score and peptide length, must be equal to or below 0.01. Two unique peptides were required for high-confidence protein identification. The MS/MS data from the three biological replicates were analyzed and filtered with identical settings, and only the overlapped proteins were accepted and analyzed further. For quantification, default parameters were used. Only protein groups identified with at least two quantitation events were taken for data analysis. Moreover, if the ratio's relative standard deviation (RSD) was greater than 1, the quantitation event was excluded from further analysis. The coefficient of variation (CV) was determined by comparing protein ratios among the replicates. Finally, average normalized ratio was calculated as the protein ratio.

### 2.5. Bioinformatics analysis of proteomic data

The subcellular locations of the identified and quantified proteins were analyzed using SignalP 4.0 server [15], LipoP 1.0 server [28], and TMHMM server v.2.0 [29]. The functional annotation of the identified proteins were categorized according to the COG annotation terms [16]. Protein–protein interaction information among the identified proteins was derived from the STRING database version 9.0 via its web portal (http://string-db.org) [30].

## 3. Results

### 3.1. Isolation of OMV

All Gram negative bacteria could shed off OMVs, which were spherical, bilayer membranous structures ranging in size from 50 to 300 nm. As shown in Fig. 1A, native OMVs were clearly visible on the cell surface by TEM. To isolate OMVs, we followed the well-established protocol with some modifications (see Supplemental Methods). The identification of purified OMVs by negative staining TEM showed the presence of round vesicles ranging from 50 to 200 nm in diameter (Fig. 1B). As reported in previous studies, the production and composition of OMVs were regulated and depended on physiological circumstances and species, including in biofilms [31], in liquid broth or on agar plates [32,33], within an animal host [28,34], and with other external stresses [35]. In addition to environmental conditions, treatment with some membrane active antibiotics such as gentamicin could also enhance OMV formation [24].

### 3.2. Protein profile of OMV

To obtain the detailed composition of the OMV, we performed a shotgun proteomic analysis of three independent OMV preparations obtained from GIS labeled with mTRAQ reagent Δ8 (see Section 2). Consistent with a previous study, mTRAQ labeling increased the confidence in protein identification [36]. After analysis with MaxQuant against the target-decoy *S. flexneri* database, 179, 180, and 180 proteins were assigned in the three replicates (see Fig. 2). Finally, 148 overlapped proteins were selected for

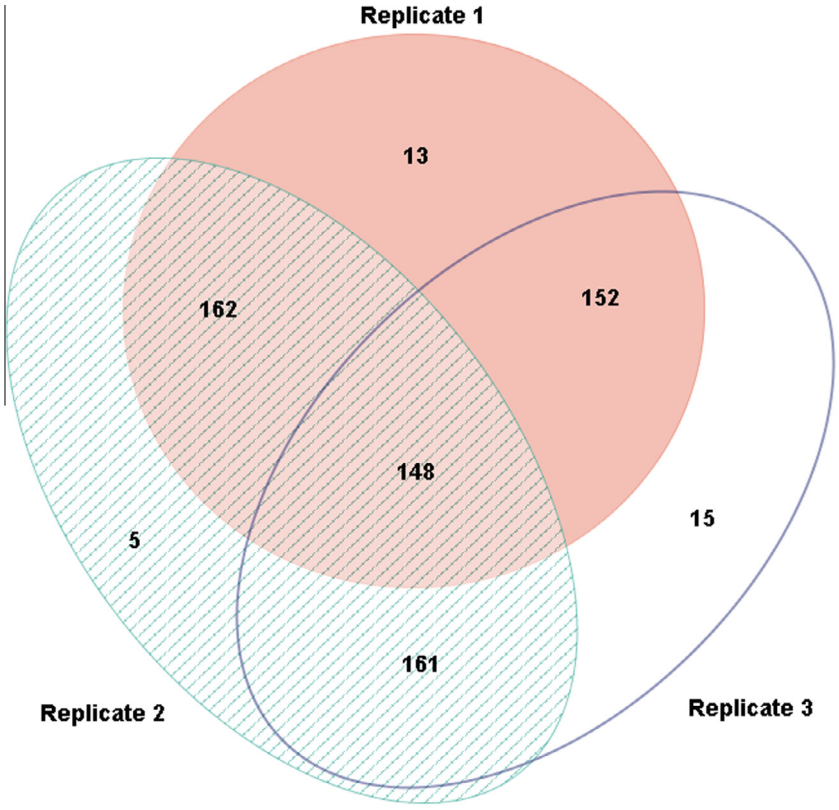


**Fig. 2.** Comparison between the independent proteomic experiments. The Venn diagram shows the number of unique and shared OMV proteins identified in three independent biological replicates. Replicates 1, 2, and 3 allowed the identification of 179, 180 and 180 OMV proteins of *S. flexneri*, respectively. The overlaps show the number of assignments that are shared between replicates.

further bioinformatics analysis (see Fig. S1 and Table S1); 69% of the proteins were identified by all three replicates, and 85% of the proteins were identified two of the three replicates, implying a very high analytical reproducibility. Of the 148 proteins identified in this study, the major ones are involved in translation processes (33.1%), envelope biogenesis (17.6%) and energy production and conversion (6.8%), while the functions of 19 proteins (12.9%) are poorly characterized (see Fig. 3). The presence of many ribosomal proteins, tRNA synthetases and elongation factors in the OMVs indicated that the translation of certain OMV proteins may occur simultaneously with the blebbing of OMVs.

### 3.3. Quantitative proteomics of OMV derived from Congo red-stimulated and WT bacteria

An mTRAQ-based quantitative proteomic approach was applied to quantitatively compare proteins between Congo red-induced and native OMV samples. For 1- and 3-h cultures, 124, 125, 126 and 122, 125, 126 proteins were, respectively, quantified in three replicate experiments with an FDR below 1% and a PEP equal to or below 0.01. To select an appropriate cutoff for differentially expressed proteins, protein ratios with a CV greater than 10 were excluded from the analysis. For proteins quantified more than twice, the average normalized ratio was used as the protein ratio, and a twofold cutoff value was chosen as the threshold for screening the significantly changed proteins. Table 1 shows the proteins that were determined to have a significant change, including 18 and 25 proteins up- or down-regulated across 1 and 3 h Congo red-induced culture, respectively. Moreover, the CR-3 treatment yielded the largest number of significant changes among the treatment groups; a total of 33 proteins were found to be differentially expressed, with 28 up-regulated and five down-regulated in the Congo red-induced OMV. In addition, Table S2 contains the normalized ratio changes for individual proteins. The range of log

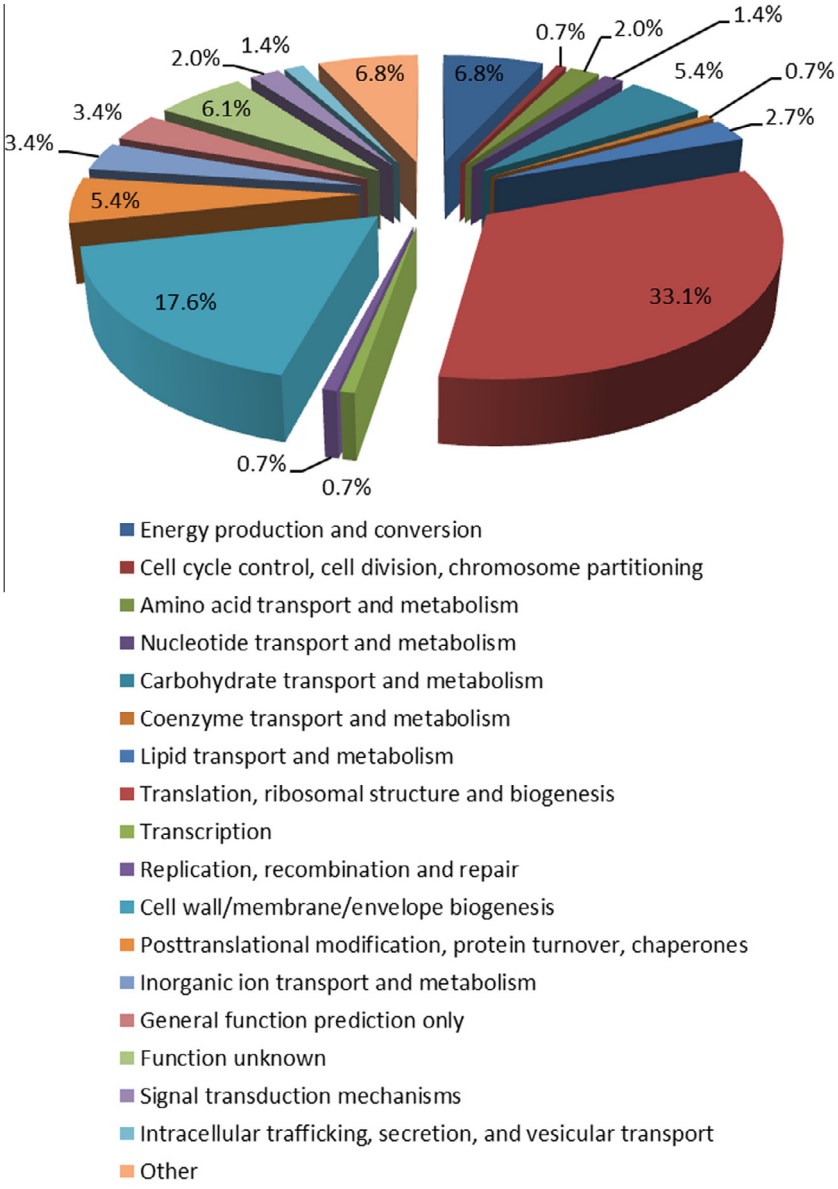


**Fig. 3.** Distribution of COG category classification of 148 OMV proteins. Identified proteins are classified into functional categories based on the COG biological process annotations. The pie chart is color-coded to show the frequency of standard COG functions. The size of each slice represents the proportion of total COG assignments associated with the term. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

**Table 1**
List of the 33 proteins differentially expressed across the 1 or 3 h Congo red-induced OMVs. For proteins quantified more than twice, average normalized ratios were calculated as the ratios of the target proteins. The coefficient of variation (CV) of target protein ratios was less than 10.

| Protein_descriptions | CR-1 | | | CR-3 | | |
|---|---|---|---|---|---|---|
| | Mean (log2) | STDEP | CV | Mean (log2) | STDEP | CV |
| gi\|24111558\|ref\|NP_706068.1\| pyruvate dehydrogenase subunit E1 | −1.44 | 0.00 | 0.74 | −2.28 | 0.01 | 4.95 |
| gi\|24111559\|ref\|NP_706069.1\| dihydrolipoamide acetyltransferase | | | | −1.11 | 0.00 | 1.02 |
| gi\|24112311\|ref\|NP_706821.1\| formate acetyltransferase 1 | | | | 1.49 | 0.03 | 1.21 |
| gi\|24112687\|ref\|NP_707197.1\| enoyl-(acyl carrier protein) reductase | | | | 3.59 | 0.88 | 7.27 |
| gi\|24112888\|ref\|NP_707398.1\| 50S ribosomal protein L20 | 2.96 | 0.28 | 3.65 | 2.22 | 0.36 | 7.69 |
| gi\|24113033\|ref\|NP_707543.1\| putative outer membrane protein | | | | −1.32 | 0.03 | 6.75 |
| gi\|24113066\|ref\|NP_707576.1\| murein lipoprotein | 1.10 | 0.11 | 5.09 | | | |
| gi\|24113610\|ref\|NP_708120.1\| DNA gyrase subunit A | | | | 1.42 | 0.27 | 9.94 |
| gi\|24113669\|ref\|NP_708179.1\| phosphate acetyltransferase | 2.49 | 0.08 | 1.44 | 2.13 | 0.25 | 5.80 |
| gi\|24114572\|ref\|NP_709082.1\| 50S ribosomal protein L17 | 2.98 | 0.52 | 6.62 | | | |
| gi\|24114573\|ref\|NP_709083.1\| DNA-directed RNA polymerase subunit alpha | | | | 3.53 | 0.82 | 7.15 |
| gi\|24114576\|ref\|NP_709086.1\| 30S ribosomal protein S13 | | | | 2.02 | 0.19 | 4.73 |
| gi\|24114583\|ref\|NP_709093.1\| 50S ribosomal protein L6 | | | | 2.81 | 0.23 | 3.32 |
| gi\|24114585\|ref\|NP_709095.1\| 30S ribosomal protein S14 | 1.75 | 0.15 | 4.33 | | | |
| gi\|24114586\|ref\|NP_709096.1\| 50S ribosomal protein L5 | 2.69 | 0.20 | 3.13 | | | |
| gi\|24114589\|ref\|NP_709099.1\| 30S ribosomal protein S17 | | | | 1.80 | 0.17 | 4.91 |
| gi\|24114590\|ref\|NP_709100.1\| 50S ribosomal protein L29 | −1.26 | 0.00 | 0.86 | 1.67 | 0.09 | 2.73 |
| gi\|24114591\|ref\|NP_709101.1\| 50S ribosomal protein L16 | | | | 2.06 | 0.35 | 8.43 |
| gi\|24114599\|ref\|NP_709109.1\| 30S ribosomal protein S10 | 1.21 | 0.02 | 1.03 | | | |
| gi\|24114604\|ref\|NP_709114.1\| GTP-binding protein chain elongation factor EF-G | 3.71 | 0.84 | 6.40 | | | |
| gi\|24114906\|ref\|NP_709416.1\| 50S ribosomal protein L28 | | | | 2.01 | 0.36 | 9.02 |
| gi\|24115225\|ref\|NP_709735.1\| ATP-dependent protease ATP-binding subunit HslU | | | | 2.09 | 0.30 | 7.08 |
| gi\|24115268\|ref\|NP_709778.1\| 50S ribosomal protein L11 | 3.96 | 1.41 | 9.03 | | | |
| gi\|24115270\|ref\|NP_709780.1\| 50S ribosomal protein L10 | 1.04 | 0.15 | 7.34 | 1.55 | 0.17 | 5.76 |
| gi\|24115273\|ref\|NP_709783.1\| DNA-directed RNA polymerase subunit beta | | | | 1.71 | 0.11 | 3.40 |
| gi\|31983588\|ref\|NP_858261.1\| IpaB, secreted by the Mxi-Spa secretion machinery, required for entry into epithelial cells | 2.70 | 0.56 | 8.62 | 1.53 | 0.11 | 3.68 |
| gi\|56404015\|ref\|NP_858260.2\| IpaC, secreted by the Mxi-Spa secretion machinery, required for entry into epithelial cells | 3.39 | 0.75 | 7.17 | 4.13 | 0.96 | 5.51 |
| gi\|56479605\|ref\|NP_706070.2\| dihydrolipoamide dehydrogenase | | | | −3.77 | 0.00 | 2.50 |
| gi\|56479617\|ref\|NP_706114.2\| 30S ribosomal protein S2 | | | | −1.31 | 0.01 | 3.45 |
| gi\|56479896\|ref\|NP_707335.2\| glyceraldehyde-3-phosphate dehydrogenase | 1.72 | 0.29 | 8.76 | 2.46 | 0.14 | 2.54 |
| gi\|56480292\|ref\|NP_709028.2\| 50S ribosomal protein L13 | 1.74 | 0.19 | 5.68 | | | |
| gi\|56480313\|ref\|NP_709113.2\| elongation factor Tu | 1.41 | 0.06 | 2.22 | 1.57 | 0.07 | 2.26 |
| gi\|56480500\|ref\|NP_709782.2\| DNA-directed RNA polymerase subunit beta | 1.46 | 0.14 | 5.03 | 2.14 | 0.22 | 4.95 |

(twofold change) observed for significant proteins ranged from 1.04 to 3.96 and 1.11 to 4.13 in 1 and 3 h Congo red-induced OMV samples, respectively.

## 4. Discussion

A previous study found that the presence of Congo-red could change the *S. flexneri* 2a membrane protein profiles during its culture, resembling the regulatory effect during the invasion of epithelial cells by *S. flexneri.* Congo red mimics the effects of certain host tissue factors in vitro [22]. We wanted to know if these Congo red-induced membrane vesicles differed from normal ones both in terms of quantity and quality.

Dutta reported that the vesicles released from *S. dysenteriae* type 1 were composed of only outer membranes [18]. In contrast, our *S. flexneri*-derived OMVs contained some cytoplasmic proteins including 36 ribosomal proteins, tRNA synthetases (AlaS, AspS, IleS, PheS, ProS and SerS), and elongation factors (FusA, Tsf and Efp) associated with the outer membrane. This finding was consistent with the previous study on *DH5α* native OMV [8]. For illustration purposes, all 61 identified proteins exhibiting overlap between *DH5α*- and *S. flexneri*-derived OMV were searched against the STRING database version 9.0 for protein–protein interactions. The STRING "confidence score," a metric used to quantify interaction confidence, was $\geqslant 0.4$ (medium confidence). The resulting interactome had 46 nodes and 302 interactions (see Fig. S2). Among the 61 overlapping proteins in the two data sets, some are previously known vesicular marker proteins such as FusA, OmpW and OmpF. These observations implied that a special sorting mechanism of particular proteins into OMVs may exist. Although the mechanism of OMV formation has not yet been elucidated, several vesicular proteins identified by both proteomic analysis including OmpX, Lpp, Tol-Pal, and YbgF may contribute to this process and facilitate the liberation of OMVs from the bacterial cell surface by initiating faster expansion of the outer membrane [37]. Surely, further study of this mechanism is needed. Other key node proteins, such as the TolC family and multidrug-resistance efflux pumps may contribute to bacterial survival by reducing the levels of toxic compounds such as antibiotics [38]. Both OMVs also contained a number of proteins involved in the transport of nucleosides (Tsx), fatty acids (FadL), and ferric iron (FhuA), leading us to consider the hypothesis that OMVs act as an alternative secretion system. Gram-negative bacteria including *E. coli* and *P. aeruginosa* could kill other bacteria via their murein hydrolases contained in their OMV [39,40]. Several murein hydrolases including MltA, MipA and SLP were identified in the overlapping vesicular proteins; these may be involved in the antimicrobial activity.

Type III secretion systems (T3SS) are present in many pathogenic Gram-negative bacteria and mediate the interaction of bacteria with host cells. In *S. flexneri*, the invasion of epithelial cells was mediated by a set of translocated bacterial invasins, the Ipa proteins, and its dedicated Mxi-Spa T3SS system, which were illustrated as tightly connected network clusters, as obtained from STRING (see Fig. S1).

The growth of the wild-type strain in the presence of Congo red is shown to induce Ipa protein secretion through the Mxi-Spa translocon [41], and these effectors may be delivered proportionally via

OMV vehicles. The IpaB and IpaC proteins in OMVs exhibited a significant change across 1 or 3-h Congo red-induced cultures. This characteristic unique to OMV secretion allowed effectors to be delivered at high concentrations over greater distances. Furthermore, OMVs could simultaneously co-transport factors to a remote site. Due to the combination of these characteristics, OMV secretion was both effective and energy consuming (see Fig. 3). Thus, the ability of OMVs to adhere to and invade host cells via adhesins and invasins such as the identified IpaA, IpaB, IpaD, IpgC and IpgB would play an important role in initiating vesicle-mediated pathogenesis.

Two proteins belonging to the serine protease autotransporters of Enterobacteriaceae (SPATEs) family were identified in this study: SepA and Pic, which contribute to enterotoxin activity. SepA is non-T3SS effector encoded on the virulence plasmid. Pic is a mucinase that is encoded on the DNA strand directly opposite of the ShET1 genes and targets a broad range of human leukocyte glycoproteins [42]. Other proteins involved in pathogenicity such as the outer membrane IcsA/IcsP are also present in the OMVs. Shigella cell-to-cell spreading requires the expression and polar surface exposition of IcsA, which is found to be exclusively exposed at the old bacterial pole [43,44]. These findings implied the presence of certain mechanisms for sorting specific proteins into OMVs and suggested that OMV may pave the way for bacteria to more effectively invade the host cells. In addition, the host-pathogen balance may be influenced by the active microbial modulation of OMV formation and/or contents in response to different environmental conditions such as Congo red-induced culture.

In this study, we have utilized mTRAQ-based quantification to study protein changes in Congo red-induced OMVs. To the best of our knowledge, this work represents the first application of quantitative comparative analyses of OMVs shed from *S. flexneri*. We have successfully identified a number of protein changes specific to Congo red inducement with different culture times. Our study provides new insights into the infection and invasion processes of *Shigella* spp.

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## 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.bbrc.2014.09.142.

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