Proteomic Analysis of Shigella Virulence Effectors Secreted under Different Conditions

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Introduction

Bacterial pathogens are the major causative agents of most global epidemic diseases by their ability to introduce pathogenic substances into eukaryotic cells via the type three secretion system (T3SS) [1]. Most bacteria with T3SS have the capability to transmit various diseases to all eukaryotes, including plants, animals and humans, although some have a symbiotic relationship with their hosts [2]. The majority of information on bacterial effectors stem from the best-studied pathogenic species of Salmonella, Shigella, and Yersinia [3], all of which contain the T3SS pathogenic substance, and the effector proteins secreted by T3SS have a central importance in causing diseases [4].

Shigella spp. can invade the terminal ileum and colon of human beings and cause shigellosis [5], which infects millions of people and leads to the death of 1.5 million each year [6]. The T3SS in Shigella spp. is composed of approximately 50 proteins. Among these, there is a needle-like protein oligomer (MxiH) that help to stabilize the membrane. The tip of the needle is composed of an IpaB/IpaD complex, which can form a hexamer (5 IpaD molecules and 1 IpaB molecule) that closes the T3SS [8-10]. In bacterial cultures maintained at 37°C, the T3SS is assembled but is not active, and the translocators and some effectors remain secluded by specialized proteins or chaperones [7]. T3SS activity is induced upon contact of the bacteria with epithelial cells or exposure to the dye Congo Red (CR) [11], but is constitutively expressed in strains carrying the ipaB and ipaD mutations [9]. In the early stage of infection, some effectors (IpaA, IpaB, IpaC, IpgB1, IpgB2, IpgD, and VirA) are released into the eukaryotic cells by Shigella via T3SS

A series of novel effector molecules secreted by the type three secretion system (T3SS) of Shigella spp. have been reported in recent years. In this study, a proteomic approach was applied to study T3SS effectors systematically. First, proteins secreted by the S. flexneri wild-type strain after Congo Red induction were separated and identified using two-dimensional electrophoresis to display the relative abundance of all kinds of early effectors for the first time. Then, a gene deletion mutant of known virulence repressor (OspD1) and a gene overexpressed mutant of two known virulence activators (MxiE and IpgC) were constructed and analyzed to discover potential late effectors. Furthermore, the supernatant proteins of gene deletion mutants of two known translocators (IpaB and IpaD), which would constantly secrete effectors, were also analyzed. Among all of the secreted proteins identified in our study, IpaH1.4, IpaH_5, and IpaH_7 have not been reported before. These proteomics data of the secreted effectors will be valuable to understand the pathogenesis of S. flexneri.

Keywords: Shigella flexneri, T3SS, early effectors, late effectors, proteomics
proteins secreted from the T3SS induced by CR. We construct several mutant strains to discover more effectors that are released which allows their interaction and downstream events, including the transcription of late effectors via T3SS activation, IpgC is often found together with IpaB and IpaC, the two molecules MxiE and IpgC are released which allows their interaction and downstream events. The regulation and secretion of T3SS effectors has been a hot-topic field in recent years; however, it is still unclear how many kinds of effector proteins are secreted through the T3SS and what they are. In this study, we attempted to construct several mutant strains to discover more effector proteins secreted from the T3SS induced by CR.

Materials and Methods

Bacterial Strains, Plasmids, and Growth Conditions

Plasmids were constructed in *Escherichia coli* strain DH5a, grown in Luria-Bertani (LB) agar and Brain Heart Infusion (BHI) broth (Difco, USA). Wild-type *S. flexneri* 2a strain 301 was grown in tryptic soy agar (Difco, USA) containing 0.01% CR or in LB broth. Plasmid pKD46 was used to construct gene deletion mutants, and pProEX-HTB was used for overexpressing the target genes. Ampicillin (100 µg/ml), kanamycin (50 µg/ml), or chloramphenicol (30 µg/ml) were added to the cultures when appropriate.

Gene Disruption and Overexpression

Gene deletion mutants were constructed using the lambda red recombination method described previously with slight modifications [14]. Briefly, 500 bps upstream and downstream of the target gene were independently amplified by PCR, and then cloned with the kan antibiotic resistance gene (from pKD4) into the pET22b vector (Novagen, USA). The sequence of cloning was upstream-kan-downstream. Then, dithiothreitol and iodoacetamide were used to equilibrate the strips for 15 min each in the equilibrium buffer (2% SDS, 50 mM Tris-HCl (pH 8.8), 6 M urea, and 30% glycerol). The 12.5% SDS-polyacrylamide gels were used for second-dimensional separation. Protein spots on the Coomassie-stained 2-DE gel were removed with a sharp razor, destained, washed, and then digested for 13 h with sequencing grade modified trypsin (Roche Applied Science, Switzerland). Proteins on the gels were digested to obtain peptides, which were analyzed by matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF/TOF) mass spectrometry (MS).

Results and Discussion

Effects of Different In Vitro Induction Conditions on the Activation of *S. flexneri* T3SS

The effector proteins secreted via T3SS play an important role during the invasion process of *S. flexneri*. Once in contact with the intestinal epithelial cells, the secretion of effector proteins will start up. In vitro, when in contact with the CR or EB dyes [16], *S. flexneri* could also secrete those effector proteins. In this study, we first re-analyzed the secretion of effectors under different conditions. The results showed that compared with the non-induced supernatant sample, which has almost no bands in the SDS-PAGE gel (Fig. 1A), the CR-induced supernatant sample had several obvious protein bands appear. When...
detected with western blotting, compared with the non-induced pellet, the induced pellet had almost no signal with anti-IpaB, whereas the strength of the signal of the CR-induced supernatant sample was nearly equal to that of the non-induced pellet sample. This indicated that the early effector molecules like IpaB had been almost completely secreted to the extracellular region from the bacteria cells induced by CR.

It has been reported that another chemical inducer, EB, could also promote *S. flexneri* to secrete effector proteins [16]. In addition, although a high concentration of sodium deoxycholate (DOC) can destroy bacterial cell membranes, resulting in bacterial lysate, a low concentration of DOC might induce bacterial protein secretion [17, 18]. Thus, CR, EB, and DOC were all included for inducing the bacterial secretion (Fig. 1B). The bacteria cultured in BHI and M9 media were both tested to exclude the effect of different nutrition environments. The results showed that the distribution of bands on the lane of sample induced by DOC were different from those of other inducers (Fig. 1B), suggesting that DOC was less efficient in the in vitro secretion induction of *S. flexneri* than CR and EB.

Given that *S. flexneri* is a kind of enteric pathogen that enters the host through the oral route, it should be able to adapt to the acidic environment in the stomach (pH 2.0), and the alkaline environment in the intestines. Therefore, induced secretion by CR (Fig. 1C) and EB (Fig. 1D) under different pH conditions (the range of pH 2.0 to 10.0) was also carried out in this study. As shown in these figures, the bacteria would secrete effectors after induction by both CR and EB when the pH became higher than 4.0, but could not secrete effectors under extreme acidic conditions like that in the stomach (pH < 4.0). This indicated that *S. flexneri* might be in a dormant state in order to survive when they pass through the host stomach, which is an extremely adverse environment for them.

**Early Effector Proteins Secreted by *S. flexneri* after Induction by CR**

The virulent effectors of *S. flexneri* could be divided into
two groups [13], the well-known early effectors and the recently reported late effectors. The main purpose of this study was to illustrate the components of both groups. The early effectors could be easily induced by a chemical inducer in vitro. As the supernatant proteins induced by CR and EB had similar bands in the SDS-PAGE when the bacteria were cultured in BHI, we selected the proteins induced by CR for the following 2-DE. Both of the induced and non-induced supernatant proteins were separated by 2-DE and then identified by mass spectrometry. The information of all proteins separated by the 2-DE gels (Fig. 2) is summarized in Table 1.

Most of these proteins have been reported previously: IpaA could promote the internalization process [19]; IpaC and IpaB can form a transmembrane helix, while inserting into the cell membrane of epithelial cells, and therefore form a tunnel structure to allow protein molecules to pass through [20, 21]; IpgD can dissociate the connection between the cell membrane and actin cytoskeleton [22]; and VirA can bind the α/β-tubulin heterodimer, cause cell microtubules to dissociate, and promote the formation of cell membrane wrinkles [23]. Apart from these well-known early effectors, we also found that OspI might belong to this family. OspI is a glutamine deamidase that facilitates the Q100E conversion in UBC13 [24]. Another interesting finding is that OspC2 is an abundant protein among these early effectors, indicating that this protein may have an important role in the primary stage of invasion. OspC2 has been known to distribute Spa15, its chaperone, among other Osp family proteins [7], and was detected as a strong antigen in western blots using piglet antisera [25]. Additionally, there were three additional high-abundance proteins potentially released from the cell lysate, namely, GadA, GadB, and TufB.

**Effector Secretion of the mxiE-Overexpressed Strain and ospD1 Gene Deletion Strain**

Different from the temperature-dependent expression of early effectors, the expression of late effectors had been reported under the positive regulation of a plasmid-encoded transcriptional regulator MxiE, with its co-activator IpgC. IpgC is also the chaperone for IpaB and IpaC. When IpaB and IpaC are secreted, IpgC will bind with MxiE, and then activate the expression of late effectors. In addition, OspD1 has a negative effect on the activity of MxiE [13]. In order to dismiss the transcriptional repression of late effectors of *S. flexneri* and analyze the expression and secretion of these molecules, the MxiE and IpgC co-overexpression strain (301/pPromxiEipgC) and OspD1 deletion mutant (301ΔospD1) were constructed. Furthermore, we also introduced the overexpression plasmid into the OspD1 deletion mutant (301ΔospD1/pPromxiEipgC). Then, the secreted proteins of these strains were recovered for 2-DE separation and
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As shown in Fig. 3, the total amounts of proteins in these three samples were lower than that from the wild type, particularly for the OspD1 mutant. The information of proteins identified is listed in Supplementary Table S1. The results showed that more proteins were identified in the samples of the three overexpression/mutant strains than in the wild-type strain. Among these, there were some high-abundance proteins probably released from the cell lysate, such as OmpA/C/F/X, DnaK, GadB, TufB, etc. Most of the early virulence proteins reported in the wild-type sample above were also identified, such as IpaA/C/D, OspC2, and IpgD. However, only the well-known late effector IpaH7.8 (Spot ID: E26) was identified in the OspD1 mutant sample. These data suggested that the secretion of late effectors is not only constrained by the activation of transcriptional regulators but also by some unknown factors. Secretions of late effectors may be regulated by more complicated mechanisms besides the known secretion regulatory pathways directed by the activator MxiE and the anti-activator OspD1.

T3SS ippA/ippD Mutant Construction and Induced Secretion

Since we could not discover most late effector molecules (such as IpaH9.8, OspE1, OspG) through changing the expression of the regulator mxiE and its chaperone, we intended to construct the T3SS constitutively active mutant to find novel late effectors. IpaB and IpaD had been reported to form hexameric complexes to close T3SS [8–10]. Thus, we constructed ippA and ippD deletion mutants to make the T3SS opening constitutively in our study, so that all the effector molecules could secrete to the supernatant. The information of all proteins separated by the 2-DE gels (Fig. 4) is summarized in Table 2.

After ippA or ippD was knocked out, we identified not only the early effectors (IpaA/C/D, OspC2) but also the well-known late effectors (IpaH9.8, IpaH7.8, OspG, OspE1, OspB) in the supernatant proteins. In addition, we also discovered some potential effector proteins that had not been reported before, such as IpaH1.4, IpaH_5, and IpaH_7. These proteins had not been detected in a thorough proteomics research of whole-cell proteins at the same phase of  S. flexneri, in which more than 700 proteins were

<table>
<thead>
<tr>
<th>ID</th>
<th>Score</th>
<th>Gi</th>
<th>Gene</th>
<th>Product</th>
<th>Localization</th>
<th>Relative abundance (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>W02 144</td>
<td>gi</td>
<td>18462560 mxiC</td>
<td>MxiC, secreted by a putative component of the Mxi-Spa secretion machinery</td>
<td>Extracellular</td>
<td>4.957 ± 0.177</td>
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</tr>
<tr>
<td>W03 573</td>
<td>gi</td>
<td>18462581 ipaA</td>
<td>IpaA, secreted by the Mxi-Spa machinery, modulates entry of bacteria into epithelial cells</td>
<td>Extracellular</td>
<td>13.885 ± 2.46</td>
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<tr>
<td>W04 342</td>
<td>gi</td>
<td>18462548 ospC2</td>
<td>OspC2, probably secreted by the Mxi-Spa secretion machinery, function unknown</td>
<td>Cytoplasmic</td>
<td>12.791 ± 0.434</td>
<td></td>
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<tr>
<td>W05/17 578</td>
<td>gi</td>
<td>56383093 ipaC</td>
<td>IpaC, secreted by the Mxi-Spa secretion machinery, required for entry into epithelial cells</td>
<td>Extracellular</td>
<td>30.748 ± 0.789</td>
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<tr>
<td>W06 482</td>
<td>gi</td>
<td>56383102 virA</td>
<td>VirA, secreted by the Mxi-Spa secretion machinery, function unknown</td>
<td>Extracellular</td>
<td>2.958 ± 0.682</td>
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<tr>
<td>W07/08 408/560</td>
<td>gi</td>
<td>18462578 ipaD</td>
<td>IpaD, secreted by the Mxi-Spa machinery, required for entry into epithelial cells</td>
<td>Extracellular</td>
<td>13.604 ± 2.99</td>
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<tr>
<td>W09 205</td>
<td>gi</td>
<td>18462546 ospD1</td>
<td>OspD1, secreted by the Mxi-Spa secretion machinery, function unknown</td>
<td>Unknown</td>
<td>1.616 ± 0.116</td>
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<tr>
<td>W10 367</td>
<td>gi</td>
<td>18462567 ipgD</td>
<td>IpgD, secreted by the Mxi-Spa machinery, modulates entry of bacteria into epithelial cells</td>
<td>Extracellular</td>
<td>7.219 ± 5.804</td>
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<tr>
<td>W12 92</td>
<td>gi</td>
<td>18462570 ipgB1</td>
<td>IpgB1, secreted by the Mxi-Spa machinery, function unknown</td>
<td>Extracellular</td>
<td>2.618 ± 1.501</td>
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<tr>
<td>W13 83</td>
<td>gi</td>
<td>18462657 ospl</td>
<td>Glutamine deaminase</td>
<td>Extracellular</td>
<td>0.341 ± 0.173</td>
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<tr>
<td>W14/18 293/573</td>
<td>gi</td>
<td>56383927 gadA</td>
<td>Glutamate decarboxylase isozyme</td>
<td>Periplasmic</td>
<td>0.405 ± 0.235</td>
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<tr>
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<td>gi</td>
<td>56383506 gadB</td>
<td>Glutamate decarboxylase isozyme</td>
<td>Periplasmic</td>
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</tr>
<tr>
<td>W16/20 166/815</td>
<td>gi</td>
<td>30042997 tufB</td>
<td>Protein chain elongation factor EF-Tu</td>
<td>Cytoplasmic</td>
<td>1.237 ± 0.082</td>
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</tr>
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identified [15]. The location of all the proteins is reported in the PSORTdb database. Several proteins such as OspC2, OspB, and IpgC detected in this study, and considered as effectors, were predicted to be located within the cytoplasmic rather than the extracellular region. The reason may be that these three proteins are synthetized within the cytoplasmic region at the beginning, and then secreted to the extracellular region when the T3SS opens. Among the three newly discovered late effectors, the abundance of protein IpaH_5 was especially higher (3.33% and 2.70% in the gels of \( \text{iapB} \) and \( \text{ipaD} \) mutants, respectively) than the others. The function of IpaH_5 requires further study. All

Fig. 3. Two-dimensional electrophoretic profiles of 301/\( \text{pPromxiEipgC} \), 301\( \Delta \text{ospD1} \), and 301\( \Delta \text{ospD1}/\text{pPromxiEipgC} \) supernatant proteins induced by Congo Red. (A) 301/\( \text{pPromxiEipgC} \); (B) 301\( \Delta \text{ospD1} \); (C) 301\( \Delta \text{ospD1}/\text{pPromxiEipgC} \). Identified proteins are indicated with arrows.

Fig. 4. Two-dimensional electrophoretic profiles of supernatant proteins of \( \text{ipaB} \) and \( \text{ipaD} \) mutants. (A) \( \text{ipaB} \) mutant; (B) \( \text{ipaD} \) mutant. Identified proteins are indicated with arrows.
three newly discovered effectors have an NEL domain, or C-terminal novel E3 ligase, which is found at the C-terminus of bacterial virulence factors. The known late effector IpaH9.8 also has this E3 ligase domain [26].

Finally, we carried out experiments of exogenous expression and CR-induced secretion of these three effector molecules, with known late effector IpaH9.8 as the positive control. All of these four molecules, IpaH1.4/_5/_7/9.8 could be expressed and detected by SDS-PAGE and western blotting in the cytoplasm, but they could not be secreted after being induced by CR in our experimental conditions (Fig. S1). This is in accordance with our results demonstrated in section “Effector secretion of the mxiE overexpressed strain and ospD1 gene deletion strain” of this manuscript. The precise secretion signal for late effectors should be elucidated in the future.

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