Transcriptional profile of the *Shigella flexneri* response to an alkaloid: berberine

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Abstract
Berberine, a natural isoquinoline alkaloid found in many medicinal herbs, has been shown to be active against a variety of microbial infections. To examine the potential effects of berberine on *Shigella flexneri*, a whole-genome DNA microarray was constructed and a transcriptome analysis of the cellular responses of *S. flexneri* when exposed to berberine chloride (BC) was performed. Our data revealed that BC upregulated a group of genes involved in DNA replication, repair and division. Intriguingly, the expression of many genes related to cell envelope biogenesis was increased. In addition, many genes involved in cell secretion, nucleotide metabolism, translation, fatty acid metabolism and the virulence system were also induced by the drug. However, more genes from the functional classes of carbohydrate metabolism, energy production and conversion as well as amino acid metabolism were significantly repressed than were induced. These results provide a comprehensive view of the changes in gene expression when *S. flexneri* was exposed to BC, and shed light on its complicated effects on this pathogen.

Introduction
*Shigella* is a gram-negative, facultative, intracellular pathogen responsible for endemic shigellosis, which remains a major worldwide health problem, particularly in developing countries. The estimated annual incidence of this disease is 160 million individuals, most of whom are children, and the annual mortality is 1.1 million (Kotloff et al., 1999). Antibacterial agents are used as a mainstay measure to control infection. However, increasing antibiotic resistance is threatening to undermine the effective treatment of shigellosis.

Berberine is a natural isoquinoline alkaloid found in medicinal herbs, such as *Rhizoma coptidis* (Huanglian). Berberine has demonstrated a number of biological activities, including antisecretory, anti-inflammatory, antibacterial, antimalarial, antitumor and anticholesterol activities, and is widely used in the treatment of bacterial diarrhea and intestinal parasite infections in many countries (Yamamoto et al., 1993; Iwasa et al., 1998). It has been shown that berberine has the properties of A–T base-specific DNA partial intercalation and generating singlet oxygen as a functional photosensitizer (Pilch et al., 1997; Brezova et al., 2004). Accumulated evidence suggests several mechanisms that may explain the antimicrobial activity of berberine. It has been reported that berberine interferes with the adherence of streptococci (Sun et al., 1988). It has also been demonstrated that berberine directly inhibits some *Vibrio cholerae* and *Escherichia coli* enterotoxins (Sack & Froehlich, 1982). In *Leishmania donovani*, berberine exhibited an inhibitory action on macromolecular biosynthesis (Ghosh et al., 1985). Although berberine has been used in the treatment of gastrointestinal disorders, especially shigellosis, for some time in China (Chang, 1959), its effect on the causative agent of shigellosis is not yet well understood.

Transcript profiling based on microarray technology enables us to investigate the response of the bacterial genome to antimicrobial agents, which provides useful clues to the mechanism of action of the agents (Fu et al., 2007). In this study, whole-genome DNA microarray was used to examine transcriptional responses elicited by berberine in *Shigella flexneri*. 
Materials and methods

Bacterial strain, medium and minimal inhibitory concentration determination

*Shigella flexneri* 2a strain 301 (Sf301), our sequenced strain, was used in this study (Jin *et al.*, 2002). The bacterium was grown at 37°C with shaking (200 r.p.m.) on cation-adjusted Mueller–Hinton broth (caMHB), a medium recommended by the Clinical and Laboratory Standards Institute (CLSI) for susceptibility testing. Berberine chloride (BC) purchased from Sigma-Aldrich was resolved in dimethyl sulfoxide (DMSO) and diluted with caMHB. The minimal inhibitory concentration (MIC) of BC for Sf301 was determined according to the CLSI broth macrodilution methods for bacteria that grow aerobically (Clinical and Laboratory Standards Institute, 2006).

Growth curves

Sf301, taken from a 24-h culture in caMHB, was inoculated in the same medium until reaching an OD$_{600}$ nm of about 0.05. The cultures were then allowed to continue growing at 37°C with shaking. When they were grown to early exponential growth phase (an OD$_{600}$ nm of about 0.3), BC was added from the 400× stock dissolved in DMSO into the cultures to give final concentrations of 160, 320, and 640 μg mL$^{-1}$. A control with only DMSO was also included. The final DMSO concentration for all conditions was 1% v/v. At certain time intervals, bacterial densities were determined by measuring the OD$_{600}$ nm.

Drug treatment

When cultures were grown to early exponential growth phase, BC was added to give the final concentration of 160 μg mL$^{-1}$. The final concentration of the solvent (DMSO) was 0.25% v/v. Meanwhile, DMSO solution without BC was added to control cultures at the same final concentration. At 30 min after treatment, samples were collected and washed twice with phosphate-buffered saline at 25°C for subsequent RNA isolation. To prepare biological replicates for RNA isolation, each experiment was performed independently three times.

RNA isolation, preparation of labeled cDNA, hybridizations and microarray data analysis

Total RNA was extracted using SV RNA Isolation System (Promega). Detailed procedures for RNA isolation, preparation of labeled cDNA, hybridizations and microarray data analysis were described previously (Fu *et al.*, 2007).

Quantitative real-time PCR (QRT-PCR) assay

Real-time PCR was performed on the ABI 7000 instrument using Power SYBR Green Universal Master Mix (Applied Biosystems). Gene-specific primers (Supporting Information, Table S1) were designed using the PRIMER PREMIER 5.0 software. Default conditions recommended by the manufacturer were used for real-time PCR. Abundance of each gene was measured relative to a standard transcript, 16S rRNA gene, and each cDNA was assayed in triplicate PCR reactions.

Results and discussion

Antimicrobial activity of BC

BC showed an antimicrobial effect on *S. flexneri*, and the MIC of BC was 640 μg mL$^{-1}$. The growth curve of *S. flexneri* is shown in Fig. 1. As measured by OD$_{600}$ nm, the growth rate of *S. flexneri* was not affected appreciably by the treatment with 160 μg mL$^{-1}$ of BC; however, growth was inhibited to different extents by higher concentrations. As growth inhibition may confound the specific effect of a drug on the transcriptional profile, and it has been revealed that the best results were obtained at concentrations that are just low enough not to affect the growth of the organism (Hutter *et al.*, 2004), in our study, 160 μg mL$^{-1}$ and a short incubation period (30 min) were selected to perform subsequent microarray experiments.

Overview of transcriptional profiles

Triplicate datasets were normalized and analyzed as described in Materials and methods. A total of 397 genes were found to be responsive to BC, including 164 upregulated...
genes and 233 downregulated genes. The differentially expressed genes were grouped by functional category according to the COG database of Sf301 and the influences of BC on the expression of genes from various functional groups are shown in Fig. 2. We found that most of the responsive genes, which are from functional categories of cell division and chromosome partitioning, lipid metabolism, translation apparatus, DNA replication and repair as well as cell envelope biogenesis, were induced by BC. However, a great many of the responsive genes from the functional classes of carbohydrate metabolism, energy production and conversion as well as amino acid metabolism were significantly downregulated. Generally, the majority of the repressed genes in the transcriptional profile are likely not to be drug-specific, but may have resulted from a general decline in cell metabolism (Shaw et al., 2003). Our discussion therefore mainly focuses on the induced genes in our dataset. A complete list of all genes differentially expressed by BC treatment can be found in Table S2.

Validation of microarray data by QRT-PCR

To validate the microarray data, QRT-PCR assays were performed with the same cDNA preparations used in array hybridizations. Overall, nine genes of interest were selected. A strong positive correlation (Pearson’s correlation coefficient $R = 0.97$) between the microarray and QRT-PCR data was observed (Fig. 3), indicating that the microarray data obtained from the present work were reliable.

Genes involved in cell division, DNA replication and repair

In the present study, most of the key elements involved in replication initiation, such as DnaA, subunits of DNA polymerase III, DnaG and DnaC, were induced by BC. Of these proteins, DnaA (encoded by $dnaA$) binds to the origin of replication, oriC, resulting in the initiation of chromosome replication. Evidence suggests that transcription from the promoters of gid operon and mioC are involved in the initiation control of the replication of the whole E. coli chromosome when oriC is under suboptimal conditions (Ogawa & Okazaki, 1991; Bates et al., 1997). As a result, the induction of gidAB, mioC and dnaA in our study strongly suggests that berberine may influence $dnaA$ and $oriC$ function. It has been established that berberine binds strongly to DNA predominantly by intercalation with a preference for the AT sequence (Pilch et al., 1997). Generally, replication origins contain AT-rich sequences. Sf301 has an E. coli-like origin with an AT-content of 59% (Sugimoto et al., 1979), which is much higher than the average value (49%) of Sf301 whole genome. Therefore, it is likely that BC may be able to inhibit the initiation of replication through interaction with the origin region of Sf301.

MreB has been shown to be necessary for the segregation of origin-proximal chromosome. During a state associated with inhibition of cell division, the expression of mreB was generally upregulated (Chiu et al., 2008). We found that $mreB$ was induced by BC, which was further confirmed by QRT-PCR. It has been reported that excessive copies of the $mreB$ gene led to filamentous cells, a reflection of cell division inhibition (Wachi & Matsuhashi, 1989). In the present study, microscopic examination of Sf301 treated
with 160 μg mL⁻¹ of BC for 30 min revealed an increase in the percentage of elongated cells (Fig. S1). These results indicate that chromosome segregation may be inhibited. It has been shown that inactivation of DnaA dramatically inhibited segregation of the oriC region in E. coli (Kruse et al., 2006). Therefore, it is likely that the drug may inhibit cell segregation through its influence on dnaA and oriC function. Additionally, a set of DNA repair genes — hepA, recJ, xseA, recQ, nfo, lig, SF2540, nei and dead — were also induced by BC, indicating that the drug may cause certain DNA damages. In Staphylococcus aureus, it has been found that a group of genes involved in DNA replication and repair were also upregulated by a subinhibitory concentration of BC, which therefore further supports our suggestion that BC may be able to interfere with DNA replication and cause DNA damages (Wang et al., 2008).

**Genes associated with the outer membrane (OM) and virulence**

The major component in the outer monolayer of the OM in gram-negative bacteria is lipopolysaccharide. Lipopolysaccharide is a complex glycolipid composed of lipid A, core oligosaccharide, and the O-specific polysaccharide chain. We observed in this study that many genes required for the biosynthesis (lpxDAB, lpxC, lpxH, and msbB2), transportation (crcA) and modification (msbA) of lipid A were significantly upregulated. Among these genes, msbB2 and crcA are known to be induced by a lack of Mg²⁺ (Guo et al., 1998; Goldman et al., 2008). Therefore, it is likely that Mg²⁺ in the envelope may be limited due to BC treatment. Divalent cations such as Mg²⁺ are absolutely required for the activity of MdoB, which aids in generating a net negative charge in membrane-derived oligosaccharides to maintain periplasmic osmolarity (Jackson & Kennedy, 1983). We found that the gene encoding the MdoB protein was induced, indicating that MdoB activity may be inhibited due to the lack of divalent cations, which may in turn disturb the periplasmic osmolarity. In accordance with this suggestion, some high-osmolarity-inducible OM genes were downregulated by the drug, including blc, bolA, yehZ and osmB. The induction of lpxC and repression of blc, bolA and yehZ were also monitored in the QRT-PCR assay.

Many studies have shown that cationic peptides have high affinities for divalent cation-binding sites in the lipopolysaccharide, and they therefore easily displace the cations, which are known to be essential for maintaining OM integrity (Hancock & Lehrer, 1998). Berberine alkaloids are amphiphatic cations. We therefore propose that BC may competitively displace divalent cations of the lipopolysaccharide, resulting in the limitation of Mg²⁺. The increased synthesis and transportation of lipid A may represent an adaptive response of Shigella to OM stress caused by BC. In Salmonella typhimurium and E. coli, the PhoQ–PhoP system confers resistance to cationic peptides by lowering the overall negative charge of lipopolysaccharide (Groisman et al., 1997). The expression of several PhoP-activated genes such as crcA (also known as pagP) and yihv was increased, indicating that the PhoQ–PhoP system was weakly induced at concentrations well below the MIC of BC. Consistent with our results, a recent study has shown that a suprainhibitory concentration (10 × MIC) of berberine can not only increase the transcription of some genes required for the biosynthesis of lipopolysaccharide but also enhance the level of phoQ and Mg²⁺ transport protein encoded by msbC (Zhang et al., 2009).

Notably, the expression of some effector proteins encoded by the type III secretion system, such as IpaH cognates, OspB and OspC1, was upregulated by BC. These effector proteins are known to be stimulated primarily within the intracellular environment, but not during growth in liquid culture (Kane et al., 2002). The mechanism triggering the expression of these effectors proteins when Shigella reaches the eukaryotic cytosol is still unknown. However, some studies have indicated that a low Mg²⁺ concentration is a signal of an intracellular environment (Groisman, 1998). According to our results, we speculate that Mg²⁺ may be the unique signal that induces the expression of these virulence-associated genes in S. flexneri.

**Genes involved in fatty acid metabolism and cell secretion**

We observed an increase in the expression of two genes (dxs and lytB) involved in the nonmevalonate pathway of isoprenoid biosynthesis. dxs gene is responsible for the generation of D-1-deoxyxylulose 5-phosphate (DXP), which is an intermediate component of the pathway (Kuzuyama, 2002). In E. coli, DXP is also a precursor for the biosynthesis of thiamine and pyridoxol (Lois et al., 1998). We noted that the transcription of some genes responsible for the biosynthesis of thiamine (thiC, thiE, thiF, thiG, and thiH) and pyridoxol (pdxI) was repressed by the drug. Thus, the biosynthesis of thiamine and pyridoxol may be reduced, which enables more DXP to be diverted to the isoprenoid synthesis pathway. The terminal step of the isoprenoid synthesis is catalyzed by the product of lytB. Isoprenoids in bacteria act as carriers in the biosynthesis and transportation of exopolysaccharides that are needed in the synthesis of the O antigen of bacterial lipopolysaccharide (Sutherland, 2001; Hood et al., 2004). As we found that lipopolysaccharide synthesis was enhanced by the drug treatment, it is unsurprising that more isoprenoid lipid is produced and participates in the synthetic process.

Under low temperatures, membrane fluidity is decreased, leading to enhanced synthesis of unsaturated fatty acids (UFAs) to overcome such variation (Aguilar & de Mendoza, 2006). Cold shock also induces palmitoleoyl transferase (encoded by ddg) to maintain the optimal OM fluidity of...
the bacterium. We observed that both UFA biosynthesis (fabA and fabB) and the transcription of dag were increased. The induction of fabA was also confirmed by a QRT-PCR assay. As a result, BC may have a similar influence to that of cold shock on the membranes. In other words, the envelope fluidity may be decreased. SecG is dispensable for protein translocation at 37 °C, whereas its function is critical at low temperatures or in the absence of membrane potential [proton motive force (PMF)] even at 37 °C (Hanada et al., 1996). Therefore, based on the discussion above, the induction of SecG after BC treatment (as validated by the QRT-PCR assay) may have resulted from the change in membrane fluidity. However, this does not exclude other possibilities, as it has been found that cation peptides cause partial collapse of PMF at concentrations well below their MICs (Hancock, 1997).

Genes related to translation

Several genes encoding proteins involved in translation (rpsU, infA, serS, asnS, tyrS, argS) and processing of rRNA and tRNA genes (rph, rumB and ygiO) exhibited a 2.0–3.3-fold induction following BC treatment. The expression increase for asnS determined by QRT-PCR was also above twofold (Fig. 3). Of these genes, serS, asnS, tyrS and argS encode seryl-, asparaginyl-, tyrosyl- and arginyl-tRNA synthetase, respectively. Intriguingly, tRNAs synthesized by all of them except seryl-tRNA synthetase needed to be modified with queuosine. The level of queA that is involved in queuosine synthesis was also increased. In addition to queuosine modification, modified nucleoside 2-thiocytidine (s2C) has so far been found in position 32 of Ser- and Arg-queuosine modification, modified nucleoside 2-thiocytidine in queuosine synthesis was also increased. In addition to and tRNA genes (Jager et al., 1997) and suggest that BC may inhibit the initiation of replication and chromosome segregation, perhaps through interaction with oriC, which may be involved in the antibacterial mechanism of BC. Furthermore, many genes related to OM, fatty acid metabolism and cell secretion were upregulated by BC. These findings are consistent with a mechanism of action where BC competitively displaces divalent cations of lipopolysaccharide, thus influencing membrane fluidity. It is well known that divalent cations are essential for maintaining the structural integrity of the bacteria. Therefore, the substitution of these cations by BC may also result in antimicrobial effects. In gram-negative species, some multidrug resistance pumps (MDRs) extrude amphipathic compounds across the OM (Tegos et al., 2002). We found that two kinds of MDRs encoded by acrA and emrA were induced by BC (Table S2), indicating that they are both involved in the development of drug resistance. In general, these results not only provide further insights into the molecular mechanisms of BC against S. flexneri, but enhance our understanding of the physiology of the bacterium in response to the perturbation in replication initiation and surface stress.

Conclusion

In this study, we defined the expression profiles of S. flexneri in response to BC.

Approximately 9% of the genes from the functional class of DNA replication and repair and 11% of the genes from the class of cell division and chromosome partitioning were significantly induced by BC. These results further support the previous findings that berberine is able to interact with DNA (Pitch et al., 1997) and suggest that BC may inhibit the initiation of replication and chromosome segregation, perhaps through interaction with oriC, which may be involved in the antibacterial mechanism of BC. Furthermore, many genes related to OM, fatty acid metabolism and cell secretion were upregulated by BC. These findings are consistent with a mechanism of action where BC competitively displaces divalent cations of lipopolysaccharide, thus influencing membrane fluidity. It is well known that divalent cations are essential for maintaining the structural integrity of the bacteria. Therefore, the substitution of these cations by BC may also result in antimicrobial effects. In gram-negative species, some multidrug resistance pumps (MDRs) extrude amphipathic compounds across the OM (Tegos et al., 2002). We found that two kinds of MDRs encoded by acrA and emrA were induced by BC (Table S2), indicating that they are both involved in the development of drug resistance. In general, these results not only provide further insights into the molecular mechanisms of BC against S. flexneri, but enhance our understanding of the physiology of the bacterium in response to the perturbation in replication initiation and surface stress.

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Genes associated with adaptations to oxidative stress

Alkaloids are photosensitive molecules that can induce the production of superoxide and singlet oxygen upon irradiation in the UVA region (Hudson & Towers, 1991; Brezova et al., 2004). Although artificial light sources emitting UVA were not used in our experiment, we found that a variety of superoxide-inducible genes were upregulated by BC (Table S2). In addition, both microarray and QRT-PCR assays revealed a significant increase in the expression of mutT, which is involved in preventing guanines from oxidization by singlet oxygen. Consequently, we suppose that the photochemical behavior of berberine may be initiated during certain experimental process, possibly during the preparation of drug solutions under exposure to the natural UVA from ambient sunlight. However, further study is necessary to confirm this hypothesis.
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References


Supporting Information

Additional Supporting Information may be found in the online version of this article:

Fig. S1. Shape of Shigella flexneri after the addition of BC.

Table S1. Gene-specific primers for quantitative real-time RT-PCR.

Table S2. Expression ratios for total genes that were differentially regulated by BC.

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