MdsABC-Mediated Pathway for Pathogenicity in Salmonella enterica Serovar Typhimurium

Saeme Song,a,b Boeun Lee,a Ji-Hyun Yeom,a Soonhye Hwang,a Ilnam Kang,b Jang-Cheon Cho,b Nam-Chul Ha,c Jeehyeon Bae,d Kangseok Lee,a Yong-Hak Kimb

department of Life Science, Chung-Ang University, Seoul, Republic of Korea; department of Biological Sciences, Inha University, Incheon, Republic of Korea; department of Food and Animal Biotechnology, Department of Agricultural Biotechnology, Seoul National University, Seoul, Republic of Korea; College of Pharmacy, Chung-Ang University, Seoul, Republic of Korea; department of Microbiology, Catholic University of Daegu School of Medicine, Daegu, Republic of Korea

MdsABC is a Salmonella-specific tripartite efflux pump that has been implicated in the virulence of Salmonella enterica serovar Typhimurium; however, little is known about the virulence factors associated with this pump. We observed MdsABC expression-dependent alterations in the degree of resistance to extracellular oxidative stress and macrophage-mediated killing. Thin-layer chromatography and tandem mass spectrometry analyses revealed that overexpression of MdsABC led to increased secretion of 1-palmitoyl-2-stearoyl-phosphatidylserine (PSPS), affecting the ability of the bacteria to invade and survive in host cells. Overexpression of MdsABC and external addition of PSPS similarly rendered the mdsABC deletion strain resistant to diamide. Diagonal gel analysis showed that PSPS treatment reduced the diamide-mediated formation of disulfide bonds, particularly in the membrane fraction of the bacteria. Salmonella infection of macrophages induced the upregulation of MdsABC expression and led to an increase in intracellular bacterial number and host cell death, similar to the effects of MdsABC overexpression and PSPS pretreatment on the mdsABC deletion strain. Our study shows that MdsABC mediates a previously uncharacterized pathway that involves PSPS as a key factor for the survival and virulence of S. Typhimurium in phagocytic cells.

Multidrug efflux pumps are related to the intrinsic resistance of clinically important bacteria to antimicrobial agents (1–3). This mode of resistance is of particular concern for Gram-negative bacteria, which coopt a variety of efflux pumps for the extracellular expulsion of a wide range of antibiotics and toxic compounds. To date, five families of efflux pumps have been classified using sequence homology, including the resistance-nodulation-division (RND), ATP binding cassette (ABC), multidrug and toxic compound exporter (MATE), small multidrug resistance (SMR), and major facilitator superfamily (MFS) classifications (4–7). Among them, RND-type efflux pumps, such as the AcrAB-ToLC system, which are present in a wide range of Gram-negative bacteria, including Escherichia coli, Salmonella enterica, and Vibrio vulnificus, are well known for the export of a variety of small molecules, ranging from solvents and dyes to the majority of amphiphilic and lipophilic antibiotics (8–10). However, there are still many uncharacterized efflux pumps that may affect drug resistance and virulence in bacteria.

S. enterica is a Gram-negative pathogen that causes various diseases worldwide in humans and animals (11). It contains a genus-specific MdsABC (multidrug transporter of Salmonella) efflux pump comprised of an inner membrane RND-type transporter, MdsB, a periplasmic membrane-fusion protein, MdsA, and an outer membrane protein, MdsC. This pump system is known to confer resistance to novobiocin and several toxic chemicals, including crystal violet, acriflavine, and rhodamine 6G, as well as to gold stress (12, 13). Although MdsABC is produced at a basal level significantly lower than that of AcrAB-ToLC, it is required for full virulence to infect mice and colonize host cells (8). Compared with that of ToLC-linked efflux pumps, the overproduction of MdsABC presents a similar resistance to host cell immune responses (14, 15), indicating the involvement of MdsABC in S. enterica pathogenicity.

We aimed to investigate the expression levels of MdsABC in relation to the antimicrobial resistance and virulence of S. Typhimurium. We constructed mdsABC deletion (DT) and overexpression (MT) mutant strains and found that 1-palmitoyl-2-stearoyl-phosphatidylserine (PSPS) was hypersecreted from the MdsABC-overexpressing bacteria, which conferred resistance to some antimicrobial dyes and oxidative stress-inducing agents. Diagonal gel electrophoresis showed alterations in the formation of disulfide bonds in membrane and cytoplasmic proteins obtained from diamide-treated bacteria cultivated with or without the addition of PSPS. Further pathological effects of PSPS treatment on Salmonella infection and host cell death were analyzed by flow cytometry. We report here that the induction of MdsABC expression increased the secretion of PSPS, which led to an increase in the resistance and virulence of S. Typhimurium against phagocytosis.

MATERIALS AND METHODS

Strains and culture conditions. All derivative strains of Salmonella enterica serovar Typhimurium 14028S and plasmids used in this study are shown in Table S1 in the supplemental material. PCR primers used for the

Received 18 May 2015 Returned for modification 17 June 2015 Accepted 11 August 2015


Editor: S. M. Payne

Address correspondence to Kangseok Lee, kangseok@cau.ac.kr, or Yong-Hak Kim, ykim@cu.ac.kr.

Supplemental material for this article may be found at http://dx.doi.org/10.1128/IAI.00653-15.

Copyright © 2015, American Society for Microbiology. All Rights Reserved.
cloning of mdsABC and for the construction of the arcAB tolC and mdsABC deletion constructs (ΔarcAB ΔtolC and ΔmdsABC mutants, respectively) are listed in Table S2 in the supplemental material. The details of the gene deletion and cloning procedures are described elsewhere (13).

The constructed expression plasmid pMdsABC was used to transform the ΔmdsABC and ΔarcAB ΔtolC strains. Bacterial strains were routinely cultivated in Luria-Bertani (LB) broth containing 100 μg ml⁻¹ kanamycin and 0.1% l-araninoic acid at 37°C with shaking at 200 rpm. To test bacterial susceptibility to oxidative stress-inducing agents, exponentially grown cells at an optical density at 600 nm (OD₆₀₀) of 0.8 were serially diluted 10-fold from 10⁻⁰ to 10⁻⁵ with sterile phosphate-buffered saline (PBS), and 10-μl aliquots were spotted on LB agar plates containing 100 μg ml⁻¹ kanamycin, 0.1% l-arabinose, and appropriate concentrations of diamide, H₂O₂, tert-butylhydroperoxide (t-BOOH), NaNO₂, and 1,1-dimethyl-4,4’-bipyridinium dichloride (Parquat).

Antimicrobial susceptibility tests. The MICs of crystal violet, acriflavin, rhodamine 6G, methylene blue, and gentamicin were determined with the S. Typhimurium mutant strains cultivated in Luria-Bertani (LB) broth containing 100 μg ml⁻¹ kanamycin and 0.1% l-araninoic acid, as described previously (13). All experiments were performed with three independent replicates.

Diagonal gel electrophoresis. Diagonal gel analysis was performed with soluble and membrane proteins to identify disulfide-forming proteins. The MOPS minimal (mAOPS) medium-cultured bacteria were washed with ice-cold 1× PBS, and centrifuged cells were treated with 100 mM N-ethylmaleimide (NEM) for 1 h at room temperature to block free cysteine thiol groups. Cells were disrupted by mixing them with 3 volumes of 1× PBS cell lysis buffer containing 1% dodecyl-β-D-glucopyranoside and a protease inhibitor cocktail (Roche Diagnostics, Mannheim, Germany), followed by agitation (1,000 rpm) for 2 h at room temperature and incubation on ice for 2 h to precipitate dodecyl-β-D-glucopyranoside-bound membrane proteins. After centrifugation, the soluble fractions were decanted, and precipitated fractions were washed again with ice-cold 1× PBS. The precipitated membrane proteins were solubilized using 2% octyl-β-D-glucopyranoside and then centrifuged to remove debris. Using soluble or detergent-solubilized proteins, diagonal gel analysis was performed using nondenaturing 4% to 15% gradient SDS-polyacrylamide gels in the first dimension followed by denaturing 15% SDS-polyacrylamide gel conditions in the second, as described previously (16). Disulfide-forming protein spots located above or below the diagonal curves were excised from the gels and analyzed by tandem mass spectrometry.

RNA extraction and RT-PCR. To measure the expression levels of mdsABC, tolC, dnaK, and phosphatidylserine (PS) biosynthesis genes in the wild-type (WT), DT, and MT strains, total RNA was extracted from cells in the mid-exponential phase of growth (OD₆₀₀ of approximately 0.8) without apparent differences in specific growth rates of the three strains. Next, 1 μg RNA was used for cDNA synthesis and PCR using a Prime Script first-strand cDNA synthesis kit (TaKaRa, Seoul, Republic of Korea). For macrophage infection experiments, Salmonella-infected RAW264.7 cells were lysed in 1% Triton X-100 and centrifuged at 3,000 rpm for 10 min to isolate intracellular bacteria. RNA was prepared as described above. Sequences for the reverse transcription-PCR (RT-PCR) primers are given in Table S2 in the supplemental material. The epitope-tagged MdsABC proteins produced by the MT strain were validated by Western blotting with anti-Flag, anti-myc, and antihexahistidine antibodies, as described previously (13).

Lipid analysis. Lipid analysis was performed with WT, DT, and MT strains that were grown to an OD₆₀₀ of 1.0 in MMOPS medium containing 0.1% glucose and 0.1% arabinose. Fatty acid methyl ester (FAME) profiles were analyzed using a Hewlett-Packard 6890 gas chromatograph with Microbial Identification software (MIDI, Newark, DE). Cellular lipids were extracted from cells grown in the presence or absence of arabinose by chloroform-methanol-water-acetic acid (20:10:1:1 by volume), and secreted lipids were extracted with an equal volume of n-butanol added to 0.2-μm membrane filtrates of the acidified culture fluids (10 ml of each spiked with 10 μg of dimyristoyl 1,2-D-glucylalcohol (Sigma, St. Louis, MO) as a reference compound. Cellular and secreted lipid extracts were dried in vacuo and dissolved in methanol for spotting on Polygram Sil G/UV_540 thin-layer chromatography (TLC) plates (Macherey-Nagel, Düren, Germany). Two-dimensional TLC of the cellular lipids was conducted using chloroform–methanol–water–28% aqueous ammonia (130: 70:8.0:5 by volume) in the first dimension, followed by chloroform-acetone-methanol-acetic acid (water) (100:40:20:20:10 by volume) in the second, as described elsewhere (17). One-dimensional TLC of the secreted lipids was performed using the second acidic solvent. Phospholipid spots were sprayed with molybdenum blue staining solution for visualization, and intensities were analyzed using ImageQuant software (GE Healthcare Life Sciences). From silica gels, higher levels of a phospholipid (PL-I) compound were observed in secreted lipid extracts from MdsABC-producing bacteria. This compound was further purified using a reversed-phase C₁₈ column with methanol solvent and analyzed by tandem mass spectrometry.

Tandem mass spectrometry analysis. Phospholipids, which were purified from the used mAOPS culture medium of the S. Typhimurium mutant (MT) strain overexpressing MdsABC, were analyzed by electrospray/collision-induced dissociation mass spectrometry in negative-ion mode using a Thermo Velos Pro Mass instrument (Thermo Scientific). The dried lipids were dissolved in a 50% methanol–0.1% formic acid solution, and a 1-μl sample was directly injected into a Thermo Velos Pro Mass analyzer operated with an exit voltage of −4 kV. A full-scan survey was performed between m/z 150 and 2000 with the delivery of 50% methanol–0.1% formic acid solution by a syringe pump at a flow rate of 3 μl min⁻¹, followed by data-dependent MS² scans of the 3 most intense ions from the preview survey scans. The ion trap mass spectrometer was operated with the following options: isolation width, ±0.8 m/z; collision energy, 35%; and dynamic exclusion duration, 30 s.

Tryptic peptides of the disulfide-bonded proteins isolated from the diagonal gels were dissolved in 0.4% acetic acid and analyzed on a Thermo Velos Pro Mass analyzer with a nano-liquid chromatography system and a Magic C18AQ column (75 μm by 75 mm). The chromatographic conditions were a 90-min linear gradient from 5% to 40% acetonitrile in 0.1% formic acid buffer solution, followed by a 20-min column wash with 80% acetonitrile and a 10-min reequilibration to the initial buffer condition at a constant flow rate of 0.3 μl min⁻¹. A full-scan survey was performed between m/z 300 and 2000, followed by data-dependent MS² scans of the 7 most intense ions from the survey scan using the following options: isolation width, ±0.8 m/z; collision energy, 35%; and dynamic exclusion duration, 30 s. The acquired tandem mass spectrometry data were analyzed by the SEQUEST search of Thermo Proteome Discoverer program version 1.3 against the database of S. Typhimurium 14028S proteins (downloaded from GenBank) and the common Repository of Adventitious Proteins (downloaded from URL ftp://ftp.thegpm.org/fasta/cRAP), which are present either by accident or through unavoidable contamination of protein samples. The search options were as follows: average mass (m/z), maximum of 1 miscreavage site of trypsin digestion, precursor mass tolerance of 1.5 Da, fragment mass error of 0.8 Da, and variable modifications such as N-ethylmaleimide modification of free cysteine thiol, idoacetamido modification of dithiothreitol (DTT)-reduced cysteine thiol, and oxidation of methionine and tryptophan. For the identification of peptides and proteins, peptide spectrum matches from the tandem mass spectrum data were filtered with a probability of >0.99 and a target-decoy false-discovery rate (FDR) of <0.01.

Measurements of bacterial infection and cell death. The effects of MdsABC on bacterial invasion and survival in macrophages were examined by a modified gentamicin protection assay (18). Briefly, mouse macrophage macrophage RAW 264.7 cells (Korean Cell Line Bank, Seoul, Republic of Korea) were cultivated in RPMI 1640 medium (Welgene, Daegu, Republic of Korea) supplemented with 10% (vol/vol) fetal bovine serum, 100 U ml⁻¹ penicillin, and 100 μg ml⁻¹ streptomycin. PBS-washed RAW 264.7 cells were infected by S. Typhimurium grown in LB
containing an empty vector (pKAN6B) (Table 1). The rationale for using these agents, including diamide, H2O2, and Paraquat, than in the control strains harboring pKAN6B (designated WT and DT) (Fig. 1). The levels of upregulated MdsABC expression during infection of macrophages with the WT strain were measured by quantitative RT-PCR, and the recombinant MdsABC protein production was confirmed by Western blotting with appropriate anti-Flag, anti-myc, and antihexahistidine antibodies (see Fig. S1 in the supplemental material). These observations suggest that the overexpression of MdsABC may lead to an increase of bacterial resistance to diamide and reactive oxygen species (ROS).

Infection of macrophages by S. Typhimurium induces the expression of the mdsABC operon. Because it was unclear that the induction of mdsABC expression was promoted in wild-type Salmonella-infected cells, the expression levels of the mdsABC genes were analyzed by quantitative RT-PCR with RNA extracts obtained after infection of RAW 264.7 cells with the WT strain. At 5 h postinfection of the RAW 264.7 cells, the mdsABC expression levels were increased by 1.5- to 2.0-fold compared to preinfection levels, while the transcripts of another MdsABC-assembling partner gene, tolC were nearly constant (Fig. 2A to C). The levels of upregulated MdsABC expression during infection of macrophages with the WT strain were calculated to be about 2-fold lower than those observed with the MT strain by producing MdsABC, so their effects on resistance to ROS produced during phagocytosis might be smaller than those observed in vitro. The WT strain overexpressing MdsABC in macrophages had the ability to produce 1.5- to 1.9-fold-increased numbers of intracellular bacteria at 2 h and 15 h postinfection, compared to the DT strain lacking mdsABC (Fig. 2D). This indicated that injection of mac-

### TABLE 1 Susceptibility of Salmonella Typhimurium mutant strains to antimicrobials

<table>
<thead>
<tr>
<th>Relevant characteristics of strain</th>
<th>MIC (µg ml⁻¹)ᵃ</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Crystal violet</td>
</tr>
<tr>
<td>pKAN6B</td>
<td>&gt;64</td>
</tr>
<tr>
<td>ΔmdsABC/pKAN6B</td>
<td>&gt;64</td>
</tr>
<tr>
<td>ΔmdsABC/ΔacrAB/pKAN6B</td>
<td>&gt;64</td>
</tr>
<tr>
<td>ΔacrAB ΔtolC/ΔmdsABC/pKAN6B</td>
<td>4</td>
</tr>
<tr>
<td>ΔacrAB ΔtolC/ΔmdsABC/pMdsABC</td>
<td>16</td>
</tr>
</tbody>
</table>

ᵃThe MICs of antimicrobials to each strain were determined with various concentrations of crystal violet and acriflavine (0, 1, 2, 4, 8, 16, 32, and 64 µg ml⁻¹), rhodamine 6G (0, 4, 8, 16, 32, 64, 128, and 256 µg ml⁻¹), and gentamicin (0, 0.25, 0.5, 1, 2, 4, 8, 16, and 32 µg ml⁻¹).
rophages with wild-type S. Typhimurium led to upregulation of MdsABC, but the lack of MdsABC led to a marked decrease in invasion and proliferation in phagocytic cells. This confirmed the previous suggestion that MdsABC is required for the full virulence of S. Typhimurium (8).

MdsABC causes hypersecretion of phosphatidylserine, conferring bacterial resistance to oxidative stress. To find an MdsABC-associated factor responsible for bacterial resistance and virulence during macrophage infection, TLC analyses of secreted and cellular lipid components were performed with the strains used for Fig. 1. In this experiment, we found that compared to the MdsABC-deficient bacteria, the MdsABC-expressing bacteria were able to secrete a higher level of a phospholipid compound (PL-1), whereas they had similar levels of cellular phospholipids (Fig. 3A; see S4 in the supplemental material). When the optical density of cells at 600 nm (OD600) was measured each hour during arabinose-induced batch cultures, the MT strain had significantly decreased growth rates in the late exponential phase and transition phase between 3 h and 9 h, compared to the WT and DT strains, which showed no significant difference in the growth curve (Fig. 3B). Despite the differences in growth rate before entry into stationary phase, the three strains reached similar maximum densities in the stationary phase. The altered growth rate of the MT strain might be due to the overproduction of MdsABC, which was not toxic but rather was responsible for the adaptation process with a compositional change in the cytoplasmic and/or outer membrane of the Gram-negative bacteria, since it led to an increased secretion of PL-1, which can affect various membrane functions by changes not only in phospholipid composition or in membrane properties but also nonmembranous cellular events (20).

The secreted PL-1 was purified by reversed-phase column chromatography and was identified as 1-palmitoyl-2-stearoyl-phosphatidylserine (PSPS) by tandem mass spectrometry (Fig. 3C; see S4 in the supplemental material). When fatty acid methyl esters were analyzed by gas chromatography, two peaks were clearly present, representing palmitate (C16:0) and stearate (C18:0) methyl esters (see Fig. S5 in the supplemental material). The MT strain resulted in a higher level of C16:0 than the WT and DT strains (see Table S3 in the supplemental material). It was unclear whether MdsABC promoted phosphatidylserine (PS) biosynthesis in the bacteria. To assess this possibility, the expression levels of the genes encoding CDP-diacylglycerol synthase (CdsA) and CDP-diacylglycerol:serine O-phosphatidyltransferase (PssA), the enzymatic activities of which are considered to largely contribute to the rate-determining steps of PS biosynthesis in S. Typhimurium (KEGG map00564), were assessed by quantitative RT-PCR with RNA extracts from the WT, DT, and MT strains. The results showed no significant differences in the levels of cdsA and pssA transcripts among the three strains (Fig. 4A), which indicated that the expression of MdsABC did not alter the rate of cellular PS biosynthesis per se.

Although there was a strong relationship between the expression of MdsABC and the secretion of PSPS, it was still unclear whether PSPS played a role in the bacterial defense against oxidative stress. To examine this effect, spot dilution assays were performed after the exposure of the WT, DT, and MT strains to 0.7 mM diamide with or without the addition of purified PSPS at 50 \mu M, a dose similar to endogenous levels (determined by two-dimensional TLC). Without PSPS treatment, the MdsABC-overexpressing MT strain was more resistant to diamide than the DT and WT strains (Fig. 4B). In the presence of 50 \mu M PSPS, how-
ever, all of the strains were more resistant to diamide than in the absence of PSPS. These results suggest that the hypersecretion of PSPS from the MdsABC-overexpressing bacteria confers resistance to oxidative stress.

Phosphatidylserine reduces the formation of disulfide bonds in membrane-bound proteins with attenuation of superoxide dismutases. Because diamide promotes the formation of disulfide-bonded proteins via a nonspecific reaction (21), diagonal gel electrophoresis was performed to compare the patterns of disulfide bonds in cytoplasmic and membrane proteins of the MdsABC-deficient DT strain treated or not treated with 50 μM PSPS (Fig. 5A). PSPS treatment markedly reduced the diamide-mediated formation of disulfide-bonded proteins in the membrane fraction compared to the untreated control. In addition, one spot, which formed an intermolecular disulfide bond in alcohol dehydrogenase (AdhE), was found in the cytoplasmic fraction of the bacteria exposed to PSPS. From the 20 spots isolated from the diagonal gels, 19 proteins were identified with high confidence by tandem mass spectrometry (see Table S4 in the supplemental material). From the data for the 19 identified proteins, 8 proteins contained tandem mass spectra that matched the iodoactamide modification (carbamidomethylation) of DTT-reduced cysteine thiol groups at Cys215 of CodA, Cys206 of GlpQ, Cys167 and Cys239 of TalB, Cys88 of SpeB, Cys213 of α-dehydro-β-deoxy-β-D-glucarate aldolase, Cys70, Cys111, and Cys201 of DeoD, Cys88 of Ppa, and Cys93 of YoaB. The tandem mass spectra of these peptides are shown in Fig. S6 in the supplemental material. Because they did not involve N-ethylmaleimide modification, these cysteine residues, which were detected by DTT reduction followed by iodoacetamide modification, were suggested to primarily form disulfide bonds in membrane-bound proteins under oxidative stress conditions. The other 11 proteins, although they did not have a matched peptide modified by carbamidomethylation, involve reactive cysteine residues near or in catalytic or transient metal-binding sites susceptible to regulation by oxidative modifications. The overall results indicate that PSPS is effective in reducing the formation of disulfide bonds in membrane-bound proteins.

Notably, PSPS treatment resulted in a marked decrease of the disulfide-bonded superoxide dismutase (SOD) SodB in the membrane fraction. With respect to defense against oxidative stress, SodB is generally known as a primary enzyme catalyzing the de-
composition of superoxide anion to \( \text{H}_2\text{O}_2 \) in the cytoplasm. In this study, however, Western blots showed that the DT strain treated with diamide and/or PSPS had significantly decreased SodB in the membrane fraction compared to the untreated strain (Fig. 5B). Moreover, the PSPS-treated DT strain markedly decreased extracellular SOD activity, to a level similar to that observed with forced overproduction of MdsABC, and increased secretion of PSPS from the MT strain (Fig. 5C). These patterns demonstrated that the hypersecretion of PSPS could protect bacteria from oxidative damage through alterations in the bacterial cell envelope, leading to a decrease in extracellular SODs.

**Phosphatidylserine enhances the infection, but not killing, of macrophages.** To characterize the pathogenic effects of MdsABC and PSPS, RAW 264.7 cells were infected with the DT, WT, and MT strains in the presence or absence of 50 \( \mu \text{M} \) PSPS. As shown Fig. 2D, in the absence of PSPS, the DT strain lacking mdsABC had low invasive potential measured at 2 h postinfection and low growth measured at 15 h postinfection, compared to the wild-type strain overexpressing the mdsABC operon during phagocytosis. In contrast, all of the PSPS-treated bacteria increased invasion and proliferation, by up to 1.7- to 2.1-fold relative to the untreated bacteria (Fig. 6A). The addition of purified PSPS had a relatively lesser effect on the growth of the MT strain, because the hypersecreted PSPS might be sufficient to increase infection of macrophages by the preadapted bacteria.

The pathological effects of PSPS on macrophages were evalu-
ated using flow cytometry by staining cells with FITC-annexin V (Ann V) and propidium iodide (PI). During an incubation time period of 15 h, the uninfected RAW 264.7 cells treated with 50 \( \mu \)M PSPS displayed no significant change in the distribution of labeled cells compared to the untreated control. PSPS (50 \( \mu \)M) induced neither apoptosis nor necrosis of the uninfected cells only (data not shown). Both MT and WT strains that overexpressed MdsABC pre- and postinfection similarly showed a marked increase in cell death of the infected macrophages, but they had no significant changes in the apoptosis ratio compared to the uninfected cells only (data not shown). Both MT and WT strains that overexpressed MdsABC pre- and postinfection similarly showed a marked increase in cell death of the infected macrophages, but they had no significant changes in the apoptosis ratio compared to the uninfected macrophages (Fig. 6B and C). In contrast, the MdsABC-deficient DT strain producing less PSPS than the WT and MT strains induced a moderate effect on cell death of the infected macrophages without any significant change of the apoptosis rate. Remarkably, when treated with 50 \( \mu \)M PSPS, the DT strain markedly increased cell death of the infected macrophages up to \( \sim 19\% \) of the total cells at 15 h postinfection, but there was no significant effect on apoptosis of the infected cells. This is similar to the case with the MT and WT strains overexpressing MdsABC, but they are clearly different from etoposide-treated cells that readily undergo apoptosis.

**DISCUSSION**

We demonstrated that MdsABC plays a role in the secretion of phosphatidylserine from the cell membranes of *S. Typhimurium* to the culture medium as well as the export of antimicrobial dyes. In this study, artificially induced overexpression of MdsABC caused an increased secretion of PSPS with a significant temporal decrease in growth rate of the bacteria during late exponential phase and transition phase, which influences changes in the membrane composition as well as numerous cellular events that are known to occur at the end of exponential growth to promote adaptation and proliferation in host cells (22). However, there was no clear relationship between MdsABC expression and growth rate of wild-type *S. Typhimurium* in vitro in culture medium (data not shown). We found that wild-type *S. Typhimurium* overexpressed MdsABC during infection of macrophages, consistent with a previous study which suggested that this efflux pump plays a physiological role inside host cells (1). The overproduced MdsABC can complement the *acrAB* or *tolC* mutant to a certain degree, which is consistent with previous studies that showed that MdsAB assembles with TolC and confers bacterial resistance to antimicrobial dyes, such as acriflavine, crystal violet, methylene blue, and rhodamine 6G (13). It assists in exuding a wide range of extracellular and intracellular compounds to maintain bacterial homeostasis in concert with TolC-linked tripartite efflux pumps (7, 23, 24). Bacterial efflux pumps have a role in exporting not only abiotics such as antibiotics, detergents, and dyes but also host-derived antimicrobial agents. Furthermore, some transporters are involved in pathogenicity and are suspected to export bacterium-derived toxins, degradative enzymes, and other substrates are suspected to establish infection in the host and to evade host defenses (7).

*S. Typhimurium* is able to promote invasion and proliferation within the host by increasing resistance to host innate immune responses through the secretion of virulence factors and alterations in the bacterial cell envelope (25). Macrophage-infecting bacteria exploit multiple pathways to cope with host innate im-
Among these pathways, it is most important for intracellular bacteria to induce a protective factor against ROS-induced oxidative stress because phagocytes kill bacteria by stimulating the generation of ROS, causing oxidative damages in the bacteria and potentiating the host antimicrobial defense (27). A recent study argued that disparate impact of oxidative host defenses determines the fate of Salmonella during systemic infection in mice (28). While Salmonella is largely killed through and NADPH oxidase-dependent mechanism by generating excess H$_2$O$_2$ in the phagosomes of neutrophils and monocytes, most live Salmonella organisms can reside in the phagosomes of macrophages, with apparently little impact of NADPH oxidase on the generation of H$_2$O$_2$ (28). In addition to the disparate effects of host cells on ROS production, bacterial resistance to oxidative stress allows intracellular bacteria to grow and survive in host cells (29–31). The present study shows that the overexpression of MdsABC leads to a small change in the phenotype of Salmonella by increasing secretion of PSPS but confers
some resistance to ROS-producing phagocytosis. Using diagonal gel analysis, we showed that external PSPS played an important role in the ability of the macrophage-infecting bacteria to moderate oxidative damage in membrane-bound proteins with the attenuation of extracellular SODs. This mechanism is intimately linked to the defense mechanism of *S.* Typhimurium against phagocytosis (32). It is likely that the overexpression of MdsABC during infection of macrophages also alters the relative abundance of membrane efflux proteins which cooperate in the export of a variety of antimicrobial agents and host antimicrobial peptides (19, 33, 34, 35). A previous study showed that the induction of MdsABC transcription is controlled by a MerR-like GoIS regulator in response to gold stress (12). The *Salmonella*-specific MdsABC operon controlled by a divergent MerR regulator may be involved in various mechanisms of pathogenesis as well as in gold resistance.

During the infection of macrophages, expression of the *mdsABC* operon is upregulated in *S.* Typhimurium, whereas expression of the *tolC* transcript remains constant. This implies that the *mdsABC* operon is regulated independent of TolC. In *S.* en-
terica, TolC is highly linked to the expression of chemotaxis genes, motility genes, and SPI-1-encoded type III secretion system genes under the PhoP regulon (35). In another study, we found that the induction of mdsABC expression was strongly related to an increase in the secretion of SPI-1 proteins and cellular stress response proteins in the cytoplasm, but there was no significant alteration in the expression levels of SPI-1 genes and the global regulator phoPQ genes (unpublished data). The induction of mdsABC expression during macrophage infection provides a previously uncharacterized pathway for bacterial resistance and pathogenicity in addition to the AcrAB-ToLC and type III secretion systems, although the mechanism is unknown. Our study shows that the upregulation of MdsABC increases secretion of PSPS to promote infection and host cell death as well as resistance to some antimicrobial dyes and defense against diadime-mediated formation of protein disulfide bonds in the membrane fraction. PSPS is a key factor contributing to the MdsABC-mediated pathway for bacterial survival and virulence in phagocytic cells, because the external addition of PSPS enhances the ability of S. Typhimurium to infect and reproduce in macrophages and increases nonapoptotic cell death (necrosis) of the infected cells. It is possible that externalized PSPS on the bacterial surface acts as a ligand for annexin V on macrophages to stimulate phagocytosis without triggering apoptotic signaling (36–38). It may also be helpful for macrophage-infesting bacteria to escape from pathogen-associated, molecular pattern-induced immune responses because phospholipids derived from the inner membrane of bacteria can inhibit the binding of lipopolysaccharide (LPS) to the LPS-binding protein of the host cells (39). The secreted PSPS has the potential to increase the pathogenicity of S. Typhimurium throughout the population; however, it remains unclear whether this potential is shared by other bacteria that utilize similar indole signaling pathways in E. coli and S. Typhimurium (40, 41). Further studies are needed to understand the physiopathological role of PSPS and related compounds in pathogenic or commensal bacteria in host animal environments.

In summary, we find that MdsABC not only plays a certain role in the export of antimicrobial agents but also influences the phenotype of S. Typhimurium by changing the growth rate and membrane composition in phagocytic cells. Overexpression of MdsABC increases secretion of PSPS, enhances infection, and leads to an increase of intracellular bacteria and cell death of the infected macrophages. Macrophage infection experiments with the mdsABC deletion and overexpression mutant strains showed that artifically induced overexpression of MdsABC and external addition of PSPS have a similar potential to promote bacterial infection and mortality of macrophages, as seen during infection with wild-type S. Typhimurium. The PSPS treatment was particularly effective in reducing the formation of protein disulfide bonds in the membrane fraction of S. Typhimurium with the attenuation of the extracellular SOD activities. These findings suggest that PSPS is a potential new virulence factor that may affect microbial defense mechanisms against ROS produced during phagocytosis, and they will provide new insights necessary to understand the potential role of PSPS in host-microbe interactions.

ACKNOWLEDGMENTS

We thank Hanyoung J. for assistance with FACS analysis. This work was supported by the Basic Science Research Program through the National Research Foundation of Korea funded by the Ministry of Education (2013R1A1A2061369 and 2014R1A2A2A09052791) and by the Strategic Initiative for Microbiomes in Agriculture and Food (914010-04-1-HD020), Ministry of Agriculture, Food, and Rural Affairs, Republic of Korea.

REFERENCES


Downloaded from http://iai.asm.org/ on August 29, 2017 by guest


