Oxidative Stress Acts on Special Membrane Proteins To Reduce the Viability of Pseudomonas syringae pv tomato

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ABSTRACT: Reactive oxygen species (ROS) play a vital role in reducing the viability of invading pathogens during plant-pathogen interactions. To understand how oxidative stress caused by ROS reduces cell viability, it is important to identify the proteins affected by ROS. In the present study, we investigated the changes in the expression of proteins from the outer and inner membrane fractions in Pseudomonas syringae pv tomato DC3000 under oxidative stress through membrane subproteomics. A total number of 17 differentially expressed proteins from the outer and inner membrane fractions were identified, among which 11 proteins belong to transporters, such as porins and ABC transporters. Their abundance was all decreased under oxidative stress, indicating that transporters are likely to be affected by oxidative stress. The function of two identified transporters was further characterized by constructing their gene mutant and overexpression strains. We found that mutation of one transporter gene PSPTO_1720 rendered Pseudomonas more sensitive to oxidative stress, whereas overexpression of this gene made the strain more resistant. By comparison, the mutant and overexpression strains of another transporter gene PSPTO_2152 exhibited the same sensitivity to oxidative stress compared with the wild-type. Our data suggest that oxidative stress reduces the viability of bacterial cells by acting on special transporters.

KEYWORDS: oxidative stress, cell viability, transporters, membrane subproteomics, Pseudomonas syringae

INTRODUCTION

Oxidative stress caused by reactive oxygen species (ROS), such as superoxide anion, hydrogen peroxide (H₂O₂) and hydroxyl radical, is associated with many physiological and pathological processes. ROS are not only produced as an inescapable byproduct of normal aerobic metabolism, but also often used as a biological weapon to reduce the viability of invading pathogens.1,2 During plant defense against pathogens, ROS are produced, known as “oxidative burst”. A transient, weak and nonspecific oxidative burst (phase I) occurs after inoculation with either a virulent or avirulent pathogen. However, a second and longer-lasting oxidative burst (phase II) is activated only by an avirulent pathogen,3 yielding much higher ROS concentration (in the micromolar to low millimolar range).4 ROS could potentially kill the invading pathogens directly as well as serve as second messengers for activating other plant defense responses.5 Although the critical roles of ROS in the activation of plant defense responses were revealed,5 the antibiotic mechanisms by which ROS reduce the viability of pathogens are poorly understood.

To explore how ROS reduce cell viability, it is vital to identify the biomolecules affected by ROS. ROS can react and damage many macromolecules including DNA, membrane lipids and proteins. In eukaryotes, membrane lipids are a major target of ROS. However, they are not the primary targets of ROS in most bacteria due to the lack of polyunsaturated fatty acids.6 Instead, Fenton-mediated damages to DNA and proteins during oxidative stress are crucial in bacteria.7 It has been proposed that some key proteins, which function in energy metabolism, translation, protein degradation, chaperones, stress resistance, cytoskeleton and so on, are targets of ROS.8 Among them, glyceraldehyde-3-phosphate dehydrogenase is well characterized. ROS may lead to growth arrest of Staphylococcus aureus by targeting this enzyme.9 Recently, it has been reported that ROS target threonyl-tRNA synthetase, an enzyme involved in translational fidelity, to impair growth of Escherichia coli.10 In our previous study, we found that special mitochondrial outer and inner membrane proteins were affected by ROS.11,12 The widely accepted theory that mitochondria arose from bacteria prompts us to hypothesize that ROS may act on cell envelope to reduce the viability of bacterial cells.

Pseudomonas syringae pv tomato DC3000 (hereafter DC3000) is an important agricultural pathogen. This Gram-negative bacterium causes speck disease in tomato and Arabidopsis. Moreover, this pathogen has become a model organism for studying plant-pathogen interactions.13 It has been well documented that massive accumulation of ROS...

Received: May 16, 2012
Published: August 29, 2012
induced by the invasion of plants by DC3000 can activate plant defense responses against this pathogen. However, little information is available for the antibiotic mechanisms by which ROS reduces the viability of this pathogen. As a Gram-negative bacterium, DC3000 contains two membranes: the outer membrane (OM) and the inner membrane (IM). OM proteins, placed between the outmost of the cell and its external environment, are important for the response to environmental changes in osmolarity, temperature, drugs, chemicals, and host defense. IM proteins are involved in a great variety of cellular processes such as cell signaling, material transport and stress response. Considering the essential function of membrane proteins, ROS may reduce the cell viability by acting on specific membrane proteins. Some OM and IM proteins have been reported to be involved in the defense reaction of the bacterial cell against oxidative stress. However, the number of identified OM and IM proteins, functioning in oxidative defense, is limited.

Proteomic analysis is a powerful tool that, when combined with complementary molecular, cellular and genetic techniques, provides a framework for understanding complex biological processes. It has been proven that a proteomics-based approach is useful to identify the proteins affected by ROS. In the present study, we identified the differentially expressed proteins from the OM and IM fractions of DC3000 cells under oxidative stress by using membrane subproteomics. The representative proteins showing differential expression were selected and their function was further characterized using genetically modified strains during oxidative stress. We found that special protein affected by oxidative stress contributed to the reduced viability of DC3000 cells. To our knowledge, this may be the first report of OM and IM proteomes under oxidative stress in bacteria.

MATERIALS AND METHODS

Bacterial Strains and Media

The strains and plasmids used in this study are listed in Table 1; see also refs 27–31. Escherichia coli strains were grown in LB medium at 37 °C. Pseudomonas syringae pv tomato DC3000 strains were grown in LB medium or AB medium supplemented with 10 mM citrate as the sole carbon source (hereafter ABC medium). Antibiotics were used at the following concentrations (μg mL⁻¹): rifampicin (Rif), 100; kanamycin (Km), 50; chloramphenicol (Cm), 20; tetracycline (Tc), 10.

H₂O₂ Sensitivity Measurements

For the H₂O₂ stress plate assay, DC3000 cells were inoculated into LB medium and cultured for 16 h at 28 °C. Cells were then diluted 1:100 into fresh LB medium and grown to OD₆₀₀ 0.7. Aliquots (10 μL) of the serially diluted cultures were spotted onto the LB solid media containing 2.5 mM H₂O₂ and grown at 28 °C.

For the H₂O₂ stress liquid assay, overnight cultures (16 h) were inoculated into fresh LB medium and grown to OD₆₀₀ 0.7. Five microliters of H₂O₂ solution (concentration 10 times higher than the final concentration) were placed in wells of a 96-well plate (Cell Culture Cluster, Costar, Corning, NY, USA), and then 45 μL of the bacterial culture was added. After 1 h of incubation at 28 °C, 10-fold serial dilutions were made. Aliquots (10 μL) of the diluted cultures were spotted onto the LB solid media and grown at 28 °C.

Table 1. Bacterial Strains and Plasmids

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<tr>
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<td>DC8000 containing pME6032, Rif', Tc'</td>
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For the gene complementarity and overexpression assay, Tc was added into LB liquid medium or solid plates to a final concentration of 10 μg mL⁻¹.

Immunodetection of Carbonylated Proteins

For total protein extraction, DC3000 cells were grown overnight (16 h) and then diluted 1:100 in fresh medium. Bacteria were then grown to OD₆₀₀ 0.7 and treated with H₂O₂ for 1 h. The organisms were harvested by centrifugation at 4000g for 8 min at 4 °C and subsequently washed twice in 50 mM Tris-HCl (pH 7.4). Cells were then disrupted and lysed by adding a buffer consisting of 7 M urea, 2 M thiourea, 4% CHAPS, 50 mM Tris-HCl (pH 7.4), then 1% DTT and 2% carrier ampholytes (CA) (pH 3–10). Protein concentration was determined by the method of Bradford.

Protein carbonylation was measured using an OxyBlot Protein Oxidation Detection Kit (Millipore Corp., Billerica, MA, USA) following the manufacturer’s instructions. Briefly, 20 μg of protein was added to an equal volume of 12% SDS. Then, samples were derivatized to 2, 4-dinitrophenylhydrazine (DNP) by incubation with one additional volume of 2,4-dinitrophenylhydrazine solution for 15 min at room temperature, followed by addition of neutralization solution. Proteins were resolved by 12% SDS-PAGE and transferred to a PVDF membrane (Millipore Corp., Billerica, MA, USA) using a TE 77 semidry transfer unit (GE Healthcare Bio-Sciences AB, Uppsala, Sweden). The carbonylated proteins were detected using anti-DNP antibodies (anti-dinitrophenyl-group antibodies) and visualized by a chemiluminescence detection kit (SuperSignal, Pierce Biotechnology, Rockford, IL, USA). Coomassie Brilliant Blue (CBB) R-250 was used to stain the
proteins in a duplicate gel to monitor the equal loading of samples.

**Preparation of OM and IM Fractions**

OM and IM fractions were prepared using methods described previously. DC3000 cells were inoculated into LB medium and grown overnight. The cultures were then diluted 1:100 into fresh LB medium and grown to OD$_{600}$ 0.7. H$_2$O$_2$ was added at the final concentration of 5 mM. After 1 h of incubation, cells were harvested by centrifugation at 4000g for 8 min at 4 °C and washed twice with 50 mM Tris-HCl (pH 7.4). The pellet was resuspended in sonication buffer (50 mM Tris-HCl, pH 7.4, 1 mM PMSF) before being disrupted by sonication. Unbroken cells and cellular debris were removed by centrifugation at 4000g for 8 min at 4 °C. The supernatant was collected and diluted with ice cold 0.1 M sodium carbonate (pH 11.5) and stirred slowly on ice for 1 h. The carbonate treated membranes were collected by ultracentrifugation at 125000g for 40 min at 4 °C. The membrane pellet was washed with 50 mM Tris-HCl (pH 7.4) and then resuspended in 2% (w/v) sodium lauryl sarcosinate (Sigma-Aldrich) in 50 mM Tris-HCl (pH 7.4). The sodium lauryl sarcosinate suspension was incubated at room temperature for 40 min and then pelleted by ultracentrifugation at 125000g for 40 min at 4 °C. The supernatant containing the IM fraction was saved, and the resulting pellet representing the OM fraction was washed with 50 mM Tris-HCl (pH 7.4) and stored at −80 °C until use. The IM fraction was further pelleted in cold acetone (1:4) at −20 °C for 4 h and pelleted by centrifugation at 25000g for 15 min at 4 °C. The pellet representing the IM fraction was washed with cold acetone, air-dried and stored at −80 °C until use.

**Two-Dimensional Gel Electrophoresis and Image Analysis**

The proteins from the OM and IM fractions were solubilized in IEF buffer containing 7 M urea, 2 M thiourea, 1% (w/v) ASB-14, 40 mM Tris, 2 mM tributyl phosphine (TBP) and 0.5% (v/v) CA (pH 3–10) and IEF buffer containing 7 M urea, 2 M thiourea, 1% (w/v) ASB-14, 1% (w/v) β-DM, 4% (w/v) CHAPS, 50 mM DTT and 1% (v/v) CA (pH 3–10), respectively. Protein concentrations were determined by the method of Bradford using bovine serum albumin as a standard. Aliquots of 150 μg of proteins from the OM fraction or 300 μg of proteins from the IM fraction resolved in 250 μL respective IEF buffer plus 0.001% (w/v) bromphenol blue were applied to rehydrate immobilized pH gradient gel strips (13 cm, pH 3–10 nonlinear; GE Healthcare Bio-Sciences AB, Uppsala, Sweden) for 16 h. The first-dimensional IEF was performed at 20 °C for a total of 20 kVh on an Ettan IPGphor unit (GE Healthcare Bio-Sciences AB, Uppsala, Sweden) following the manufacturer’s instruction. The IPG strips were then reduced and alkylated in equilibration buffer containing 150 mM Tris-HCl (pH 6.8), 8 M urea, 20% (v/v) glycerol, 2% (w/v) SDS and 1% (w/v) DTT for reduction or 4% (w/v) iodoacetamide for alkylation for 15 min, respectively. Following the two step equilibration, the second-dimension separation was carried out using 5% stacking gels and 15% resolving gels at a constant 30 mA per gel. Proteins in the gel were stained with CBB R-250 solution containing 50% (v/v) methanol, 15% (v/v) acetic acid and 0.1% (w/v) CBB R-250.

Gel images were captured using a flatbed scanner (GE Healthcare Bio-Sciences AB, Uppsala, Sweden). Image analyses were performed using Image Master 2D Elite software (GE Healthcare Bio-Sciences AB, Uppsala, Sweden). To account for experimental variation, at least three biological repeats resulting from independent experiments were used for each sample. Spot quantities were normalized as a percentage of the total volume of all spots on the gel to compensate for nonexpression-related variations between gels. The minimum requirement for spot quantification was spot presence in at least three replicate 2D gels obtained from independent protein extraction. The normalized intensity of spots on three replicate 2D gels was averaged and independent-sample t-test was conducted to determine whether the relative change was statistically significant between samples using SPSS software (SPSS Inc., Chicago, IL, USA). Protein spots from OM fraction whose expression levels changed significantly (containing <1.5-fold) were excised for protein identification because multiply outer membrane protein spots can correspond to one single protein. Protein spots from IM fraction whose expression levels changed significantly by more than 1.5-fold were excised for protein identification.

**In-Gel Digestion, Mass Spectrometry, and Database Searching**

In-gel digestion was performed according to a procedure described previously with some modifications. Protein spots were excised from the gels and destained with 50 mM NH$_4$HCO$_3$ in 50% (v/v) methanol for 1 h at 40 °C until the blue color of CBB was removed. After they were completely dried in a vacuum centrifuge, the gel pieces were digested at 37 °C for 16 h with 10 ng μL$^{-1}$ trypsin. Digested peptides were extracted by three changes of 0.1% trifluoroacetic acid (TFA) in 50% acetonitrile, lyophilized and analyzed by MALDI-TOF-TOF tandem mass spectrometry.

MALDI-MS/MS was carried out using a MALDI-TOF-TOF mass spectrometer (4800 Proteomics Analyzer, Applied Biosystems, Framingham, MA, USA), which was operated in the positive reflection mode. Prior to sample analysis, the instrument was externally calibrated using the tryptic peptide mixtures of myoglobin (Sigma-Aldrich). The peptides were resuspended with 5 mg mL$^{-1}$ matrix solution (α-cyano-4-hydroxycinnamic acid in 50% acetonitrile containing 0.1% TFA) and spotted onto the MALDI target plates. MS spectra were obtained with 1600 laser shots per spectrum, whereas MS/MS spectra were generated using 2500 laser shots per fragmentation spectrum. The 10 strongest peaks of each MS spectra were selected as precursor ions, excluding trypsin autolytic peptides and other known background ions, to acquire the MS/MS fragmentation spectra. Spectra analyses and generation of peak list file were performed using the 4000 Series Explorer software (Applied Biosystems), with parameters of a signal-to-noise threshold of 10 and a minimum area of 100. The generated peak lists were searched against NCBI nr protein databases (version 20120212; 17 258 491 sequences and 5 919 220 959 residues) with Mascot MS/MS Ions Search program (version 2.1) on the Matrix Science (London, U.K.) public Web site (http://www.matrixscience.com). Search parameters were set as taxonomy, bacteria (eubacteria); enzyme: trypsin; mass missed cleavages: 1; fixed modifications: carbamidomethyl (C); variable modifications: oxidation (M) and phospho (ST); peptide mass tolerance: ± 0.2 Da; fragment mass tolerance: ± 0.3 Da. In addition, peptide charge of +1 and monoisotopic mass were chosen, and the instrument type was set to MALDI-TOF-TOF. A total of 10 129 714 sequences in the database were actually searched. Mascot uses a probability-based “Mowse Score” to evaluate data obtained from tandem mass spectra. Mowse scores were
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Figure 1. Viability and total protein carbonylation of DC3000 cells under H$_2$O$_2$ stress. (A) Viability of DC3000 cells upon exposure to H$_2$O$_2$. The cell viability was determined by the H$_2$O$_2$ stress liquid assay. (B) Total protein carbonylation of DC3000 cells treated with H$_2$O$_2$. The carbonylated proteins were separated by SDS-PAGE, blotted, and detected using anti-dinitrophenyl-group antibodies (right panel). The arrowheads indicate highly carbonylated protein bands under H$_2$O$_2$ stress. To monitor equal loading of samples, Coomassie Brilliant Blue R-250 staining was used (left panel). Error bars represent standard deviation of three independent experiments.

RESULTS

Viability and Total Carbonylated Protein Levels of DC3000 Cells under H$_2$O$_2$ Stress

The viability of DC3000 cells under H$_2$O$_2$ stress was assessed in liquid LB medium. As shown in Figure 1A, H$_2$O$_2$ caused a significant concentration-dependent loss of viability. The cell viability decreased gradually with increasing concentration of H$_2$O$_2$. Exposure of cells to 3 and 5 mM H$_2$O$_2$ for 1 h led to 28% and 46% losses in viability, respectively. In addition, total protein carbonylation was determined because it is a widespread indicator of severe oxidative damage. The intensity of immunostaining of several bands of total proteins increased significantly after 5 mM H$_2$O$_2$ exposure, whereas H$_2$O$_2$ at 3 mM had no significant effect (Figure 1B). The concentration of 5 mM H$_2$O$_2$ was chosen in the subsequent studies. To understand the mechanisms by which H$_2$O$_2$ reduced the viability of DC3000 cells, we conducted comparative
subproteomics analysis of proteins from the OM and IM fractions under H₂O₂ stress.

**Analysis of OM Proteome**

Analysis of OM proteome upon exposure to 5 mM H₂O₂ was conducted using two-dimensional (2D) gel electrophoresis. Approximately 190 protein spots were reproducibly detected by Image Master 2D Elite software on CBB-stained gels after ignoring very faint spots and spots with undefined shapes and areas (Figure 2A and B). Quantitative image analysis revealed a total of 15 protein spots that changed their intensities significantly (p < 0.05) (containing <1.5-fold) (Supplemental Table S3). Of them, 14 spots were identified with Mascot scores significantly higher than the threshold (p < 0.05). The abundance of all 14 identified protein spots was decreased under H₂O₂ stress (Figure 2C). These identified protein spots were categorized into 3 groups, including transporters (12 spots), metabolic enzymes (1 spot) and lipoproteins (1 spot) (Figure 2D; Table 2).

![Two-dimensional patterns of proteins from the OM fraction under H₂O₂ stress and classification of identified proteins.](image)

**Table 2. Identification of Proteins from the OM Fraction under H₂O₂ Stress Using MALDI-TOF-TOF**

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<th>expt. M&lt;sub&gt;r&lt;/sub&gt; (kDa)/pI&lt;sup&gt;b&lt;/sup&gt;</th>
<th>NP&lt;sup&gt;c&lt;/sup&gt;</th>
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<sup>a</sup> theor. M<sub>r</sub> (kDa)/pI, theoretical molecular mass and isoelectric point based on amino acid sequence of the identified protein. 
<sup>b</sup> expt. M<sub>r</sub> (kDa)/pI, experimental molecular mass and isoelectric point estimated from the 2D gels. 
<sup>c</sup> NP, the number of matched peptides, corresponding to peptides assigned above the 95% confidence level. 
<sup>d</sup> SC, amino acid sequence coverage for the identified protein. 
<sup>e</sup> Fold changes, average fold change of protein expression levels in H₂O₂-treated DC3000 cells versus control from three replicate 2D gels obtained from independent protein extraction. The sign + represents overexpressed proteins, and − represents down-expressed proteins.
It is notable that the abundance of 12 spots corresponding to 4 unique proteins belonging to transporters was significantly decreased under H2O2 stress. Many of the OM proteins were reported to be present in multiply charged isoforms, meaning that multiply protein spots can correspond to one single protein. Of these proteins, OprD family outer membrane protein (spots O2, O3 and O4), OprB (spots O5 and O6) and OprF (spots O13 and O14) are known as porins. The remaining 5 protein spots (spots O7, O8, O10, O11 and O12) were identified as the same protein, outer membrane protein (hereafter PSPTO_1720). This protein is highly conserved in strains of Pseudomonas with sequenced genomes, showing 31% sequence identity to PaFadL of Pseudomonas aeruginosa PAO1 based on sequence alignment with BLASTP. In DC3000, it is the sole homologue of the E. coli FadL. PaFadL and FadL are involved in the transport of long-chain fatty acids (LCFAs).

Besides, the expression of the GDSL family of autotransporting lipase (spot O1), which is associated with fatty acid...
metabolism, was reduced under H$_2$O$_2$ stress. It is notable that several proteins (O10, O11, O12, and O13) appeared to be a breakdown product rather than the intact proteins under oxidative stress.

**Analysis of IM Proteome**

Analysis of IM proteome upon exposure to 5 mM H$_2$O$_2$ was also performed using 2D gel electrophoresis. Approximately 750 protein spots could be detected on 2D gels (Figure 3A and B). Quantitative image analysis revealed 15 protein spots were differentially expressed upon exposure to H$_2$O$_2$ by more than 1.5-fold ($p < 0.05$) (Supplemental Table S3). Among them, 12 spots were successfully identified with Mowse scores greater than the threshold ($p < 0.05$) (Figure 3C). The identified proteins were grouped into particular biochemical groups, including transporters (7 spots), metabolic enzymes (3 spots) and protein synthesis (2 spots) (Figure 3D; Table 3).

The abundance of 7 proteins grouped into transporters was reduced significantly under H$_2$O$_2$ stress. Among them, 4 proteins (spot I3, I9, I10 and I11) were the subunits of ABC transporters. Three proteins (spot I1, I2, and I5) were TonB-dependent iron-siderophore transporters. The abundance of spot I2 (hereafter PSPTO_2152) was decreased by 8.5-fold under H$_2$O$_2$ stress. Spot I5 was shown to be a breakdown product of larger protein during H$_2$O$_2$ stress. TonB-dependent iron-siderophore transporters are known as OM proteins, but they were identified in the IM fraction, which might be caused by the interaction between these proteins and the IM protein TonB.

In addition, 2 proteins were identified as metabolic enzymes. The flavoprotein subunit of succinate dehydrogenase (spot I4) was involved in the tricarboxylic acid cycle and electron-transfer chain. The abundance of this protein was increased under H$_2$O$_2$ stress. Two spots (I7 and I8) were identified as type I glyceraldehyde 3-phosphate dehydrogenase, which is involved in glycolysis. H$_2$O$_2$ stress induced the shift of this protein to a more acid pI, because the abundance of the high-pI form of this protein (spot I8) was decreased, whereas that of the low-pI form (spot I7) was increased under H$_2$O$_2$ stress.

Moreover, 2 identified proteins were related to protein synthesis including translation elongation factor Tu (spot I6) and 50 S ribosomal protein L2 (spot I12), whose abundance was decreased upon exposure to H$_2$O$_2$. It has been reported that metabolic enzymes and protein synthesis components are present in some membrane protein complexes. The occurrence of translation-related proteins indicates the strong association of these proteins with the membrane during the localization of the membrane proteins, cotranslationally, into the IM.

**Functional Characterization of Candidate Proteins from the OM and IM Fractions**

By analyzing changes in OM and IM proteome under H$_2$O$_2$ stress, we found that marked decreases in abundance were recorded in transporters. Since some transporters have been shown to be closely related to bacterial viability, our results indicated that H$_2$O$_2$ may reduce the viability of DC3000 cells by acting on special transporters. One transporter PSPTO_1720 (spot O7, O8, O10, O11, and O12) was chosen for further investigation because this protein had multiply charged isoforms, indicating complicated posttranslational modifications. PSPTO_1720 is associated with the transport of LCFAs which are involved in multiple cellular processes.

To probe the function of PSPTO_1720 under H$_2$O$_2$ stress, we generated a PSPTO_1720 in-frame deletion mutant. We first tested the sensitivity of the PSPTO_1720 mutant to H$_2$O$_2$ by using stress plate assay as described in Materials and Methods. In this assay, the PSPTO_1720 mutant was more sensitive to H$_2$O$_2$ (Figure 4A). Moreover, the PSPTO_1720 mutant exhibited an increased sensitivity to H$_2$O$_2$ in liquid LB medium (Figure 4B). Furthermore, to verify the function of the deleted gene, we constructed PSPTO_1720 complemented and overexpression strains. The wild-type and PSPTO_1720 mutant harboring either the control plasmid pME6032 or the complementing plasmid p1720 were analyzed for their susceptibilities to H$_2$O$_2$ using stress plate assay. The PSPTO_1720 mutant containing the control vector pME6032 was more sensitive to H$_2$O$_2$ than the wild-type containing the control vector pME6032. Furthermore, the wild-type or PSPTO_1720 mutant containing complementing plasmid p1720 exhibited an increased resistance to H$_2$O$_2$ than cells containing the control vector pME6032 (Figure 5A). The susceptibilities to H$_2$O$_2$ of the wild-type and PSPTO_1720 mutant harboring either the control plasmid pME6032 or the complementing plasmid p1720 were also compared in liquid
LB medium. The results are similar to the previous H2O2 stress plate assay (Figure 5C). Our data suggest that PSPTO_1720 was sensitive to H2O2 and played an important role in response to H2O2 stress.

Since iron metabolism is very important during oxidative stress and the abundance of TonB-dependent iron-siderophore transporters was noticeably reduced. PSPTO_2152 was chosen to characterize its function during H2O2 stress. This protein is encoded by PSPTO_2152. To investigate the function of PSPTO_2152 under H2O2 stress, we generated a PSPTO_2152 in-frame deletion mutant. Interestingly, the colony color of the PSPTO_2152 mutant appeared to be yellow. Using H2O2 stress plate assay, we found that the PSPTO_2152 mutant displayed the same sensitivity to H2O2 as the wild-type (Figure 4A). The colony color of the PSPTO_2152 mutant could be restored by a plasmid containing a wild-type copy of the PSPTO_2152 gene (Figure 5B). The PSPTO_2152 mutant containing the control vector pME6032 showed the same sensitivity to H2O2 when compared with the wild-type containing the control vector pME6032. Overexpression of PSPTO_2152 in wild-type did not alter the sensitivity to H2O2 (Figure 5B). Taken together, our results indicate that PSPTO_2152 has no effect in response to H2O2 stress.

**DISCUSSION**

Upon recognition of a microbial pathogen, a plant or an animal host starts to release high levels of ROS, which can reduce the viability of the pathogen. In the present study, we show that H2O2 exposure caused a concentration-dependent loss of viability in the phytopathogenic bacterium DC3000 in vitro (Figure 1A). To investigate the mechanisms whereby H2O2 reduced the viability of the pathogen, we first detected the level of total protein carbonylation, a widespread indicator of severe oxidative damage. We found that H2O2 at 5 mM induced an increased level of total protein carbonylation as compared to the control (Figure 1B). This indicates that the bacterial cells suffer severe oxidative damage under H2O2 stress and that the change of protein function may be involved in the reduction of...
cell viability. Proteomics has been used to study the differential expression of bacterial proteins in response to oxidative stress, but these studies focused on the whole cellular proteins rather than proteins from an enriched cell membrane preparation. The role of membrane proteins in reduced cell viability induced by oxidative stress is largely unknown.

We present here for the first time a comparative analysis of the OM and IM subproteomes of a bacterial pathogen to seek the proteins affected by H2O2. A set of 17 proteins from OM and IM fractions that appeared to change significantly in abundance under H2O2 stress were identified by MALDI-MS/MS (Table 2 and 3). Notably, a total of 11 proteins were transporters, whose expression was all significantly decreased, suggesting that transporters are likely to be affected by H2O2 stress. Transporters, as a large and extremely important class of membrane proteins, mediate passive and active transport of small solutes across membranes such as amino acids, peptides, sugars, inorganic ions, vitamins, drugs, and so on. The down-regulated expression of transporters indicates that material transport through the cell membrane might be attenuated under H2O2 stress.

Two representative transporters, PSPTO_1720 and PSPTO_2152, were selected for further investigation of their function in response to H2O2 stress. PSPTO_1720 is most likely involved in the transport of LCFAs which represent important sources of metabolic energy. Beta oxidation of LCFAs can directly generate reducing equivalents (NADH and FADH2) or produce acetyl-CoA that can then enter the tricarboxylic acid cycle to indirectly produce reducing equivalents (NADH and FADH2). The generated reducing equivalents are subsequently used in the electron transport chain to produce ATP. LCFAs can be synthesized de novo, but exogenous LCFAs are important sources of metabolic energy and carbon, and need to be taken up efficiently. Besides, LCFAs or their derivatives are involved in a wide variety of cellular processes including phospholipid biosynthesis, protein export and modification, enzyme activation or deactivation, membrane permeability, cell signaling, transcriptional control and bacterial pathogenesis. The OM of Gram-negative bacteria provides an efficient barrier for the passage of LCFAs owing to the polar lipopolysaccharide that comprises the outer leaflet of the OM. To date, the only protein family known to be involved in the uptake of LCFAs is the FadL family, named after the archetypal long-chain fatty acid transporter FadL of E. coli. FadL has been proven to be related to antibiotic resistance and cell survival under extreme pH conditions in E. coli. It has been reported that the fadL mutant conferred resistance to a novel antibacterial compound A-344583 in Haemophilus influenzae. However, the role of FadL homologue in oxidative stress is unknown. In DC3000, PSPTO_1720 (Figure 2; Table 2), indicating that PSPTO_1720 plays an important role in response to H2O2 stress. Supposing that PSPTO_1720 is involved in the defense against ROS released by plant cells during DC3000 infection, a decrease in virulence of the PSPTO_1720 mutant should be observed. However, the PSPTO_1720 mutant showed the same pathogenesis as the wild-type (Supplemental Figure S2). This may be explained by the relatively low concentration of H2O2 in vivo. Based on our proteome analysis, we also found that the abundance of the GDSL family of autotransporting lipase (spot O1) was decreased under H2O2 stress. The GDSL family of autotransporting lipase hydrolyzes ester substrates to produce fatty acids. If the fatty acid products are LCFAs, they will possibly become the substrates of long chain fatty acid transporter, i.e. PSPTO_1720. The down-regulation of GDSL family of autotransporting lipase and PSPTO_1720 may combine to impair the supply of LCFAs to DC3000, eventually leading to the loss of cell viability under H2O2 stress.

Another transporter, PSPTO_2152, belongs to a family of TonB-dependent iron-siderophore transporters, which are involved in high affinity iron uptake. Iron is an essential element for microorganisms because it acts as a cofactor of many metabolic enzymes. On the other hand, iron also promotes the formation of hydroxyl radicals via Fenton reaction, which indiscriminately damage all cellular components. In this study, the decreased abundance of these TonB-dependent iron-siderophore transporters under H2O2 stress may lead to limited iron availability to cells. In addition, this may represent the initial response to H2O2 stress to reduce oxidative cell damage. To further elaborate the role of this protein under H2O2 stress, we constructed the PSPTO_2152 deleted, complemented and overexpression strains. We found that these strains showed the same sensitivity to H2O2 (Figures 4A and 5B), indicating that PSPTO_2152 individually was not important in response to H2O2 stress. However, it should be noticed that the colony color of the PSPTO_2152 mutant was yellow (Figure 4A). Given the fact that PSPTO_2152, PSPTO_2151 (another gene encoding TonB-dependent siderophore receptor) and pyoverdine synthesis genes are located in a single pyoverdine cluster, and pyoverdine, the primary iron acquisition system in Pseudomonas, is described as a yellow-green pigment, we suppose that mutation of PSPTO_2152 is complemented by other genes. The PSPTO_2152 mutant had the same pathogenesis as the wild-type (Supplemental Figure S2).

Of the remaining transporters, 3 belong to porins, including OprF, OprB and OprD family protein. OprF, a major, multifunctional, and general porin, functions in OM stability and cell shape determination in Pseudomonas. OprB is a carbohydrate-selective specific porin, which acts as a central component of carbohydrate transport. OprD family outer membrane proteins are involved in the selective transport of diverse molecules such as amino acids, dipeptides as well as structurally diverse carboxylic acids. The decreased expression of the general and specific porins may cause nutrient uptake limitation, and thus contributed to the reduced cell viability.
under H$_2$O$_2$ stress. Besides, 4 of identified transporters belong to the components of ABC transporters, suggesting that ABC transporters are likely to be affected by H$_2$O$_2$. ABC transporters play an important role in bacteria, including nutrient uptake and toxic substance export. They import almost every class of substrate imaginable, including carbohydrates, amino acids, peptides, inorganic ions, and so on.\textsuperscript{51,62} Notably, we found that the abundance of three amino acid ABC transporters, which are involved in the transport of amino acids, was significantly decreased under H$_2$O$_2$ stress. Amino acids can be used for protein synthesis and are key intermediates in both carbon and nitrogen metabolism of bacteria.\textsuperscript{63} One of the identified amino acid ABC transporters is cysteine ABC transporter, which has been reported to be involved in oxidative defense in \textit{Lactobacillus fermentum}.\textsuperscript{64,65} In addition, we identified two proteins related to protein synthesis. 50 S ribosomal protein L2 is absolutely required for the association of 30 and 50 S subunits and is involved in tRNA binding to both A and P sites and peptidyl transfer.\textsuperscript{66} Translation elongation factor Tu is responsible for the selection and binding of the cognate aminoacyl-tRNA to the A site of the ribosome. In this study, the decreased abundance of amino acid ABC transporters and protein translation-related proteins during H$_2$O$_2$ stress might together result in impaired protein synthesis, and thus contributed to the reduced cell viability.

Moreover, we identified two metabolic enzymes whose abundance changed under H$_2$O$_2$ stress. The abundance of the flavoprotein subunit of succinate dehydrogenase SdhA (spot 14) was significantly increased under H$_2$O$_2$ stress, which may represent the initial response to H$_2$O$_2$ in cells. Succinate dehydrogenase is an enzyme of the tricarboxylic acid cycle and is involved in the electron-transfer chain. It can oxidize succinate to fumarate to deliver reducing equivalent FADH$_2$, which can be directly transferred to the respiratory chain. The increased expression of SdhA may be used in maintaining the proton motive force and consequently ATP synthesis under H$_2$O$_2$ stress. Differentially expressed glyceraldehyde 3-phosphate dehydrogenase (Gap; spots I7 and I8), the key enzyme of the glycolytic pathway, was also identified. Soluble metabolic enzymes have been reported to be present in the bacterial membrane proteome.\textsuperscript{2,4} Gap was also found to be located in the plasma membrane in some animal and plant cells.\textsuperscript{67−69} It has been shown that H$_2$O$_2$ induced the acidic pH shift of Gap, resulting from the oxidation of cysteine, and led to Gap inactivation and growth arrest in \textit{Staphylococcus aureus}.\textsuperscript{9} In the present study, the shift of Gap to acidic pH was also observed upon exposure to H$_2$O$_2$ in DC3000, indicating that Gap may be partially inactivated.

In conclusion, we found that the expression of membrane transporters in \textit{P. syringae pv tomato} DC3000 were affected by H$_2$O$_2$ stress. By constructing genetically modified strains, we found that one of these transporters PSPTO$_{1720}$ played an important role in response to H$_2$O$_2$ stress, whereas another transporter PSPTO$_{2152}$ individually was not important in response to H$_2$O$_2$ stress. Our data suggest that special bacterial membrane transporters play an important role in response to H$_2$O$_2$ stress.

**ASSOCIATED CONTENT**

\* Supporting Information

Supplemental figures and tables. Supplemental Figure S1, annotated spectra for identifications based on single peptides. Supplemental Figure S2, disease symptoms and growth in \textit{planta} of the wild-type, PSPTO$_{1720}$, and PSPTO$_{2152}$ mutants after inoculation by syringe. Supplemental Table S1, scores and matched peptides of the identified proteins based on tandem mass spectrometry. Supplemental Table S2, primers used in this study. Supplemental Table S3, intensities for differentially expressed proteins from the OM and IM fractions under H$_2$O$_2$ stress. This material is available free of charge via the Internet at http://pubs.acs.org.

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**Notes**

The authors declare no competing financial interest.

**ACKNOWLEDGMENTS**

This study was supported by the National Natural Science Foundation of China (31030051, 30972069), by National High Technology Research (863) Program of China (2012AA101607), and by the Chinese Academy of Sciences (KSCX2-EW-G-6).

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