Differential Secretomics of *Streptococcus pyogenes* Reveals a Novel Peroxide Regulator (PerR)-regulated Extracellular Virulence Factor Mitogen Factor3 (MF3)*

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*Streptococcus pyogenes* is a human pathogen that causes various diseases. Numerous virulence factors secreted by *S. pyogenes* are involved in pathogenesis. The peroxide regulator (PerR) is associated with the peroxide resistance response and pathogenesis, but little is known about the regulation of the secretome involved in virulence. To investigate how PerR regulates the expression of the *S. pyogenes* secretome involved in virulence, a perR deficient mutant was used for comparative secretomic analysis with a wild-type strain. The conditioned medium containing secreted proteins of a wild-type strain and a perR deficient mutant at the stationary phase were collected for two-dimensional gel electrophoresis analysis, where protease inhibitors were applied to avoid the degradation of extracellular proteins. Differentially expressed protein spots were identified by liquid chromatography electrospray ionization tandem MS. More than 330 protein spots were detected on each gel. We identified 25 unique up-regulated proteins and 13 unique down-regulated proteins that were directly or indirectly controlled by the PerR regulator. Among these identified proteins, mitogen factor 3 (MF3), was selected to verify virulence and improve survival (3). There have been many studies of the involvement of virulence factor expression in pathogenesis. During the early exponential phase, the extracellular cysteine protease streptococcal erythrogenic toxin B (SpeB) is expressed at very low levels in *S. pyogenes*. Surface molecules such as M-protein and protein-F were allowed to adhere to the membrane of host cells at the initial stage when *S. pyogenes* shows relatively low SpeB expression. Subsequently, responding to environmental stress such as pH decrease and nutritional lack, speB expression is significantly up-regulated (4). Therefore, *S. pyogenes* can use SpeB to proteolytically inactivate its surface molecules to unfasten *S. pyogenes* from their adhesive binding sites. Simultaneously,
SpeB degrades host cell molecules, assisting *S. pyogenes* dissemination from the site of infection. Previous reports have suggested that *speB* is down-regulated on transitioning to a bacteremic stage, and that *S. pyogenes* triggers the activities of other virulence factors such as M-protein and Sda1 that are beneficial to survival in blood (5). SpeB can be down-regulated in blood, it is clearly produced in situ in human tissue during necrotizing fasciitis, and thus is a critical virulence factor in severe tissue-destructive infections (6–10). Furthermore, Cole et al. (2006) have proposed that altered expression of *speB* in a subpopulation of invading organisms might contribute to *S. pyogenes* tissue pathology (9). Other crucial *S. pyogenes* virulence factors are probably also regulated through similarly complex transcriptional mechanisms (5).

To live in difficult environments, bacterial cells have to develop mechanisms for defending against various stresses, such as reactive oxygen species. Thus, bacteria have developed specific enzymes to detoxify these harmful molecules, for example, catalase, peroxidase, and superoxide dismutase. *S. pyogenes* is a member of the lactic acid family of bacteria that does not produce catalase and oxidoreductase, which are often considered to be essential for resistance to the oxidative stress induced by hydrogen peroxide and growth in aerobic environments. However, *S. pyogenes* can endogenously produce large amounts of peroxide when grown under aerobic conditions and can live successfully in aerobic environments (11). To summarize, data suggests that an adaptation to oxidative stress plays a critical role at some stage in the development of *S. pyogenes* infections. One factor involved in the *S. pyogenes* adaptive responses to oxidative stress is the peroxide stress response regulator (PerR)$^1$, which is one member of the Fur (ferric uptake repressor) super-family of metal binding transcriptional regulators. Previous studies have also demonstrated that PerR is involved in the regulation of iron homeostasis and oxidative stress responses and that it contributes to the virulence of *S. pyogenes* (12–14). Recent research found that although a perR-deficient mutant’s capacity to resist peroxide stress did not directly correlate with its ability to cause disease, the appropriate regulation of the peroxide stress response is critical for virulence (13). This evidence indicates that although the PerR regulon is critical for virulence, its influence might extend beyond adaptation to oxidative stress. However, the mechanism through which PerR contributes to *S. pyogenes* virulence and the specific contribution of PerR-regulated gene expression to bacterial survival at particular host sites during infection is unclear.

The genomic sequences established for *S. pyogenes* have paved the way to high throughput genomics, transcriptomics, and proteomics analyses. The proteomic approach has been successfully used to address a global view of bacterially physiological issues (15–20). Here, we compared *S. pyogenes* secretome samples from the wild-type strain and the perR deficient mutant by using gel-based proteomics analysis for discovery of novel PerR-regulated extracellular virulence factors. The various functions of newly identified PerR-regulated proteins might provide a previously unrecognized role of PerR that might contribute to pathogenesis in *S. pyogenes*.

**EXPERIMENTAL PROCEDURES**

**Bacterial Strains, Growth Conditions, and Sample Preparation**—The bacterial strains *S. pyogenes* A-20 (M1 serotype) and *perR* deficient mutant (SW-612) were described previously (21). Two *S. pyogenes* strains were cultured in trypticase soy broth containing 0.5% yeast extract (TSBYE) supplemented with protease inhibitor mixture (PIC) without agitation at 37 °C. After 12 h of culture in protein-free TSBYE, bacteria were centrifuged for 10 min at 3500 × g at 4 °C, and the conditioned medium (CM) were sterilized with a 0.2 μm pore size filter (NalgeNunc, Rochester, N.Y.). Proteins in the CM were concentrated 10-fold with a Millipore Amicon Ultra centrifugal filter with a 3 kDa molecular weight cut-off filter. Culture supernatant proteins were precipitated by 10% w/v trichloroacetic acid in acetone and were centrifuged at 13,000 × g. The pellets were washed with acetone three times and dissolved in isoelectric focusing (IEF) rehydration buffer for two-dimensional gel electrophoresis (2DGE) analysis. Two-dimensional Gel Electrophoresis Analysis—2DGE was performed on six biological replicates in each group using IEF as the first dimension (IPGphor, Amersham Biosciences, Uppsala, Sweden) followed by the second dimensional sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) (Bio-Rad protein II xi cell, Hercules, CA). For the 2DGE experiment, 100 μg of protein pellet was mixed into 350 μl rehydration solution containing 7 μl urea, 2 μl thiourea, 2% 3-[3-cholamidopropyl)dimethylammonio]propanesulfonate, 20 mM dithiothreitol, and 0.5% IPG buffer. IEF was run after a stepwise incremental voltage program: 30 V for 16 h, 500 V for 1 h, 1000 V for 1 h, and 8000 V for 4 h, with a total power of 34 kV·hr. After IEF, the strips were subjected to two-step equilibration in equilibration buffers containing 6 μl urea, 30% glycerol, 2% SDS, and 50 mM Tris-HCl (pH 8.8) with 1% dithiothreitol/w/v for the first step, and 2.5% w/v iodoacetamide for the second step. The strips were then transferred onto the second-dimensional SDS-PAGE, and run on 1.0-mm thick 12% polyacrylamide gels at 15 °C. The gels were fixed in 40% ethanol and 10% acetic acid in water overnight, and then incubated in a buffer solution containing 30% ethanol, 6.8% w/v sodium acetate, with 0.312% w/v sodium thiosulfate for 30 min. After washing three times in water for 5 min each, the gels were stained in 0.25% w/v silver nitrate solution containing 0.02% formaldehyde for 30 min. Development was performed for 10 min in a solution consisting of 2.5% sodium carbonate and 0.01% formaldehyde. Acetic acid solution (5% v/v) was used to stop the development, and the stained gels were then washed three times in water for 5 min each. The stained gels were scanned using an ImageScanner operated by the software LabScan 3.00 (Amersham Biosciences). Intensity calibration was carried out using an intensity step-wedge before gel image capture. Image analysis was carried out using the software ImageMaster 2D (version 2002.1, Amersham Biosciences). Image spots were initially detected, matched, and then edited. Each spot intensity volume was processed by background subtraction, and total spot volume normalization, giving the spot volume percentage (%Vol). Statistical signifi-
Tryptic Digestion, Liquid Chromatography, and Mass Spectrometry—The protein spots were excised manually and transferred to siliconized 0.5 ml Eppendorf tubes. All in-gel digestions of proteins were performed manually with trypsin (Promega, Madison, WI) in a laminar flow hood to reduce the amount of keratin contamination. For the same consideration, disposable gloves were used at all times by the operator who performed the in-gel digestion. The gel pieces were washed twice with 50% acetonitrile and 50% acetonitrile/25 mM ammonium bicarbonate. Then, the gel fragments were kept at 56 °C for 45 min in the dark, in a solution containing 10 mM dithirotretol and 55 mM iodoacetamide in 25 mM ammonium bicarbonate to be reduced and alkylated. Approximately 10 µl of modified trypsin digestion buffer (0.1 µg/µl) in 25 mM ammonium bicarbonate was added to the gel fragments, and the samples were incubated overnight at 37 °C. After the supernatant was transferred to an Eppendorf tube, 20 µl of 50% acetonitrile/5% formic acid was added, and the peptides were further extracted from the gel piece by sonication for 10 min. After centrifuging at 2000 × g, for 10 min, this supernatant was combined with the previous supernatant and then dried down. Twenty microliters of 5% acetonitrile/0.1% formic acid was added to each peptide sample, and the samples were subjected to liquid chromatography-tandem MS analysis for protein identification. Reverse phase-nano-high-performance liquid chromatography-electrospray ionization-tandem mass spectrometry was performed to identify the selected protein spots that had been separated on the 2DGE gel. The peptides obtained from the tryptic in-gel digestion were analyzed using a nano-high-performance liquid chromatography system (LC Packings, The Netherlands) coupled to an Ion Trap mass spectrometer (LCQ DECA XP Plus, ThermoFinnigan, San Jose, CA) equipped with an electrospray ionization source. Eight microliters of peptide solution was loaded into the reverse phase column (C18 PepMap trapping column, 300 mm, id 65 mm, LC Packings) using an autosampler. A linear acetonitrile gradient from 100% buffer A (5% acetonitrile/0.1% formic acid) to 60% buffer B (80% acetonitrile/0.1% formic acid) was used at a flow rate of ~200 nL/min for 70 min. The separation was performed on a C18 microcapillary column (Zorbax 300SB-C18, 3.5 mm, 75 mm id, 150 mm, Agilent, Santa Clara, CA). Peptides eluted from the microcapillary column were electrosprayed into the electrospray ionization-MS/MS with the application of a distal 1.3 kV spraying voltage with a heated capillary temperature of 200 °C. Xcalibur version 1.3 was used to get cycles of one full scan mass spectrum (m/z 450–2000) followed by three data-dependent tandem mass spectra, with the collision energy set at 35%. Data Analysis—Proteomic analysis was performed using the BioWorks 3.3 software (Thermo Electron Corp, San Jose, CA). Each file was searched against the NCBI protein database with taxonomy limited to S. pyogenes (April 22, 2008, 14629 entries) using the Turbo-SEQUEST algorithm. The search used the following parameters: the cleavage rules required fully tryptic cleavage at both ends; only one missed cleavage site was allowed. Variable modifications at cysteine (carboxymethylation) and methionine (oxidation) were allowed. The mass tolerance for the precursor peptide ion was set to 1.5 Da, and that for fragment ion tolerance was set to 1.0 Da. All the accepted results had to be filtered by the following acceptance criteria for protein identification: a DeltaCn (ΔCn) > 0.1, a variable threshold of Xcorr versus charge state (Xcorr = 1.9 for z = 1, Xcorr = 2.2 for z = 2, and Xcorr = 3.75 for z = 3), and number of distinct peptides, 2. Xcorr scores of the data sets of at least 1.9, 2.2, and 3.75, respectively, were considered to give high confidence of protein identification as defined by Washburn et al. (22). The presence of secretory signal peptide cleavage sites was predicted using SignalP version 3.0 (http://www.cbs.dtu.dk/services/SignalP/). All of the identified proteins were searched against this prediction software to look for signal peptide sequences to verify that these identified proteins had been secreted via classic pathways. A sequence prediction based on software SecretomeP version 2.0 (http://www.cbs.dtu.dk/services/SecretomeP) was performed for the prediction of secreted mammalian proteins targeted to nonclassical secretion pathways. The transmembrane domain prediction was performed with TMHMM Server v. 2.0 (http://www.cbs.dtu.dk/services/TMHMM). The identified proteins were annotated as to the presence of a transmembrane helix and discriminated between soluble and membrane proteins.

Expression of Recombinant Proteins and Production of Specific Antibodies—Genomic DNA from the strain A-20 was used as a template for amplifying the full-length mf3 gene. The mf3 gene was amplified by PCR with these primers: forward, 5′- CCGGAATTCCGG-CCAGAAAAATTAGAAATTT-3′; and reverse, 5′- CGGTGACGAGC- CGGTTCCAAACTCTT-3′ (restriction sites are in bold). The 465-bp PCR product was digested with EcoRI-XhoI and inserted into pET21b, resulting in plasmid pMW741. Escherichia coli BL21 transformed with plasmid pMW741 was grown to an optical density at 600 nm of 0.6, before induction with 0.5 mM IPTG (isopropyl-β-D-thiogalactopyranoside) for 4 h at 30 °C. For purification, the culture was centrifuged at 10,000 × g for 10 min, resuspended in 20 ml Tris-HCl-100 mM NaCl (pH 7.5), and disrupted by two passages through a French pressure cell. The cell debris was removed by centrifugation at 13,000 × g for 20 min. The supernatant was treated according to the instructions of GE Amershams Biosciences for Ni2+ affinity chromatography. The purified recombinant proteins were dialyzed into 20 mM Tris-HCl-100 mM NaCl-5% glycerol (pH 8.0), and concentrated by ultrafiltration cell (Amicon, Danvers, MA) with a 10-kDa membrane. Female BALB/c mice were immunized subcutaneously with a mixture containing 50 µg of r-MF3 and Freund’s complete adjuvant (1:1, v/v). Mice were boosted with the same protein every 2 weeks. Immune sera were collected 7 days after the second boost and used for subsequent experiments. The sera anti-MF3 titers were measured by enzyme-linked immunosorbent assay as described previously (23).

Real-time RT-PCR of the Selected Genes—RNA was extracted using the Qiagen RNeasy Mini Kit from bacterial lysates obtained by the sonication method. All RNA samples were reverse transcribed for 30 min at 42 °C with a High Capacity cDNA Reverse Transcription Kit according to the standard protocol of the supplier (Applied Biosystems, Foster City, CA). Real-time RT-PCR was performed on an AB PRISM 7300 Sequence Detection System (Applied Biosystems) using the QuantiTect SYBR green RT-PCR kit (Qiagen) as described (23). Expression levels of each test gene were normalized to those of 16 S rRNA, which did not show any change in expression as a consequence of perR mutation. Data were reported as the mean relative expression levels (± standard deviation) in the A-20 strain versus the SW612 strain. Forward and reverse primers used in this study for amplification of target genes are as follows: MF3-F-CGTCGACTTG-GCAAGGT; MF3-R-ATTGCTCAAGATCGCCAAAAA; 16S rRNA-F-GCAAGCTAGAGTGGAAATCT; 16S rRNA-R-CGTCGGAGCA-CCTAGTATTA.

Protein Immunodetection—Western blotting was used to measure the levels of MF3 and PerR in the CM or cell extracts. Both of the cytosolic proteins and the secreted proteins were separated using a 4–12% NuPAGE Bis-Tris gel (Invitrogen, Carlsbad, CA). The electrophoretically separated proteins were transferred onto polyvinylidene difluoride membranes and blocked with 5% nonfat milk in Tris buffer saline/Tween-20, containing 20 mM Tris-HCl (pH 7.5), 0.5 mM NaCl, and 0.5% Tween-20. The membranes were then blotted with mouse anti-PerR antibody, mouse anti-MF3 antibody, and goat anti-mouse immunoglobulin (Chemicon, Temecula, CA). The blots were then de-
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veloped with the Enhanced Chemiluminescent Substrates (PerkinElmer Life Science, Boston, MA) and the relative intensities of the bands were measured using an image analysis system (Amersham Biosciences Uppsala, Sweden).

Construction of mf3 Deficient Mutant—A S. pyogenes A-20 mutant with an internal 761 bp deletion in the mf3 gene was constructed by the reverse PCR procedure. First, oligonucleotide primers (forward, 5’-CGGAATTCGAAATATGAAAAAATCCTCCC-3; reverse, 5’-AAGTGCAAGCTGACCTTTGGCCGAATAT-3’) (restriction sites in bold) were used for amplification of the mf3 region. The 2.4 kb PCR product was digested by EcoRI and PstI and cloned into the plasmid pSF152, subsequently designated pMW721. Plasmid pMW721, with an internal 761 bp deletion in the mf3 gene was amplified using primers: forward, 5’-GTTAACGGTAAACAACTGAAACCTTGCCAAGT-3; reverse, 5’-GTTACGGTAAACAGAATTTGAGAACCGAAAG-3’. A chloramphenicol resistance cassette (Cm) was cut from a plasmid, vector 78, with HincII and blunt-end ligated into the deletion region, which was filled in by Klenow enzyme. The resulting construction was designated Plasmid 6625. Electroporation of this plasmid into the strain A-20 was conducted by the standard protocol described previously (21).

Mouse Infection Assays—S. pyogenes strains A-20, SW612, and SW917 were grown in TSBYE until the OD600 reached 0.4. The cells were washed once in sterile phosphate-buffered saline (pH = 7.4), and diluted with phosphate-buffered saline to 1 x 10^9 CFU/ml. Viability counts were performed to verify the accuracy of the dilutions. Eight-week-old female BALB/c mice were challenged with the air sac skin infection model. Each mouse received subcutaneous injections of 900 µl of air together with 200 µl of the bacterial suspension. Two sets of experiments were performed. In the first set, two groups of mice (n = 9) were inoculated with 2 x 10^9 CFU of either the wild-type strain A-20 or the perR deficient mutant SW-612. In the second set, two groups of mice (n = 10) were inoculated with 2 x 10^8 CFU of either the wild-type strain A-20 or the mf3 deficient mutant SW-917. Mice were checked at regular intervals four times per day. Data from both experiments were expressed as percentages of mice surviving over time. For the animal data, statistical significance (p < 0.05) was calculated with both the Fisher exact test and the Mann-Whitney U test by using SPSS 12.0 statistical software (SPSS Science, Apache Software Foundation, Chicago, IL).

Electrophoretic Mobility Shift Assays (EMSA)—Recombinant PerR (r-PerR) binding to promoter DNA containing a putative PerR-binding sequence was performed as described, with modification (14). Promoter DNA (428 bp) of the mf3 gene was amplified by PCR using the wild-type strain A-20 as a template with these primers: forward, 5’-CGAAGTCGACACCTTCAACTTCTTTC-3; reverse, 5’-GGGTA

Validation of Characteristics of the perR Deficient Mutant—Previous studies have reported that the perR deficient mutant was highly attenuated in virulence in a murine air sac model of infection (12–14). However, the mechanism by which PerR contributes to S. pyogenes virulence and the specific contribution of PerR-regulated protein expression to bacterial survival at particular host sites during infection remains unknown. The construction of the strain SW-612 has been previously described in detail (21). The expression levels of the perR gene products in strain SW-612 were also determined for virulence evaluation in the present study. The transcriptional level of the perR gene was determined by RT-PCR analysis in the strain A-20 and the strain SW-612. The RNA bands were clearly detected in the A-20 strain from 2 to 14 h, whereas there was no signal in strain SW-612 (Fig. 1A). This result demonstrated that the perR deficient mutant cannot transcribe any perR gene product. In addition, the translational level of PerR protein was measured by using Western blotting analysis in strain A-20 and strain SW-612. The level of PerR protein expression in strain A-20 was gradually increased from 2 to 14 h. The highest level of PerR expression was detected at 12 h in strain A-20. In contrast to strain SW-612, there was no PerR protein signal detected from 2 to 14 h (Fig. 1B). These data demonstrated that the perR deficient mutant does not express any perR gene products, and the PerR protein is expressed at the highest level after 12 h in wild-type S. pyogenes. After we had confirmed that the perR deficient mutant cannot express the perR gene products, the air sac skin infection model was used to determine the virulence of the perR-deficient mutant in comparison to the wild-type strain. We tested the virulence of the wild-type A-20 strain and the perR deficient mutant SW-612 strain in a BALB/c mice infection model. The skin air sacs were inoculated with 2 x 10^8 CFU of bacteria, and mouse survival was monitored for 8 days. When using 2 x 10^8 CFU, 0% of the mice infected with the strain A-20 were alive within 2 days, compared with 66.7% of the group inoculated with strain SW-612 (p < 0.05) (Fig. 1C). This confirmed that the virulence of the perR deficient mutant was attenuated in the animal model in accordance with previous reports (12–14). To summarize, these data demonstrated that the perR deficient mutant is useful for discovery of PerR-regulated virulence factors in S. pyogenes.

Growth Features of the perR Deficient Mutant of S. pyogenes—S. pyogenes secretes an exceptionally large amount of extracellular proteases, which can cause degradation of...
many extracellular proteins and can adversely affect the reproducibility of 2DGE analysis (4). To avoid this problem, we added a protease inhibitor mixture (PIC) to the TSBYE broth to culture the wild-type strain A-20 and the perR deficient mutant SW-612. To determine whether or not the PIC affected bacterial growth in vitro, the growth of the strain A-20 and the strain SW-612 in the TSBYE broth or in the TSBYE broth supplemented with PIC were monitored by total bacterial count. The optical density at 600 nm was measured at given intervals of 1 h for a total 12 h. No obvious difference between the wild-type strain and the perR deficient mutant was observed (Fig. 2). We concluded that the PIC does not affect the growth of the wild-type strain or the perR deficient mutant. Moreover, there was no difference between strain A-20 and strain SW-612 in TSBYE culture (Fig. 2). The data revealed that deletion of the perR gene in S. pyogenes also makes no difference in bacterial growth. We believe that the TSBYE supplemented with PIC is better for collecting the secretome of S. pyogenes for 2DGE analysis, as it should help avoid protein degradation. In addition, we also determined that hydrogen peroxide levels in CM of the wild-type strain and the perR deficient mutant increased from 8 to 12 h in early stationary phase (supplementary Fig. S1).

Proteomics Analysis—To gain insight into the protein secretion controlled by the PerR regulator in the wild-type strain A-20 and to identify candidate proteins contributing to virulence of S. pyogenes, we compared the secretome of the strain A-20s and SW-612. According to the results of our...
previous PerR protein expression studies, PerR regulator is expressed at the highest level at 12 h by the wild-type strain A-20. Thus, it was of interest to characterize extracellular proteins that were regulated by PerR at 12 h. The A-20 and SW-612 strains were grown in protein-free TSBYE broth supplemented with PIC for 12 h for CM collection. Subsequently, the concentrated secretome samples collected from CM were analyzed by 2DGE analysis and were stained by a silver staining method. More than 330 spots were detected on each gel using the image analysis software. Analysis of the 2DGE gels identified 45 spots (25 unique proteins) that were down-regulated in the mutant compared with the wild-type strain, whereas in the perR-deficient mutant, and 21 spots (13 unique proteins) that were up-regulated compared with the wild-type strain (Fig. 3). These differentially expressed proteins identified by liquid chromatography-tandem MS analysis are summarized in Table I and supplemental Table 1. Among the 25 identified proteins up-regulated by PerR, five proteins were predicted as possible secreted proteins using SignalP3.0, TMHMM2.0, and SecretomeP2.0, whereas eight of 13 proteins down-regulated by PerR were possible secreted proteins. After this computational prediction, we hypothesize a novel role for PerR in regulating protein secretion in S. pyogenes. In previous investigations, the PerR regulator was shown to be involved in oxidative stress responses and virulence, and there was no evidence to show that PerR is involved in protein secretion (12–14). In the present study, we discovered 13 PerR-regulated secreted proteins, which demonstrates that PerR can control the protein secretion directly or indirectly to influence the virulence of S. pyogenes. Of the 13 predicted secreted proteins, six proteins have never been reported to be associated with the pathogenesis of S. pyogenes (4, 25–29). These proteomics results not only provide a global view of the expression of PerR-dependent proteins, but also provide evidence for novel roles for PerR in pathogenesis.

Interestingly, approximately half of the PerR-regulated proteins in the A-20 strain are involved in sugar metabolism and stress response. This result suggests a role for PerR in regulating sugar uptake and use by S. pyogenes. Peroxide stress response regulator activation of sugar use pathways might reflect the increased energy use required to counteract cell toxicity inflicted on the bacteria by the inflammatory response. By using various sugar sources during infection, it might be possible to increase bacterial survival and this could be an essential contribution of PerR-regulated gene expression to S. pyogenes virulence. The dramatically attenuated virulence of the perR deficient mutant in the animal skin infection model could thus be the result of poor uptake or use of available sugar sources in the host together with reduced resistance to stress. Recent evidence shows that S. pyogenes use of maltodextrins is specifically up-regulated in human saliva, an event that has been proposed to contribute to S. pyogenes survival in saliva and to efficient colonization of the mouse oropharynx (30). In this study, we found that the PerR protein up-regulated a putative cyclomaltodextrin glucanotransferase, which would allow S. pyogenes to use maltodextrins as a nutrient source. Thus, the present data demonstrates that PerR is also involved in use of maltodextrins for bacterial survival. Based on these results, we be-
## Summary of differentially expressed protein spots on two-dimensional gel

| Spot no. | Protein name | Fold change | Xcorr score | MW/p | Functional category |
|----------|--------------|-------------|-------------|-----|---------------------|
| A9       | Sec         | 3.67        | 90.27       | 57466 | Translation          |
| A10      | Elongation factor EF-2 | 16.2  | 118.30      | 76480 | Response to stress  |
| A11      | Elongation factor EF-2 | 16.2  | 118.30      | 76480 | Response to stress  |
| A12      | Elongation factor EF-2 | 16.2  | 118.30      | 76480 | Response to stress  |
| A13      | Elongation factor EF-2 | 16.2  | 118.30      | 76480 | Response to stress  |
| A14      | Elongation factor EF-2 | 16.2  | 118.30      | 76480 | Response to stress  |
| A15      | Elongation factor EF-2 | 16.2  | 118.30      | 76480 | Response to stress  |
| A16      | Elongation factor EF-2 | 16.2  | 118.30      | 76480 | Response to stress  |
| A17      | Elongation factor EF-2 | 16.2  | 118.30      | 76480 | Response to stress  |
| A18      | Elongation factor EF-2 | 16.2  | 118.30      | 76480 | Response to stress  |
| A19      | Elongation factor EF-2 | 16.2  | 118.30      | 76480 | Response to stress  |
| A20      | Elongation factor EF-2 | 16.2  | 118.30      | 76480 | Response to stress  |
| A21      | Elongation factor EF-2 | 16.2  | 118.30      | 76480 | Response to stress  |
| A22      | Elongation factor EF-2 | 16.2  | 118.30      | 76480 | Response to stress  |
| A23      | Elongation factor EF-2 | 16.2  | 118.30      | 76480 | Response to stress  |
| A24      | Elongation factor EF-2 | 16.2  | 118.30      | 76480 | Response to stress  |
| A25      | Elongation factor EF-2 | 16.2  | 118.30      | 76480 | Response to stress  |
| A26      | Elongation factor EF-2 | 16.2  | 118.30      | 76480 | Response to stress  |
| A27      | Elongation factor EF-2 | 16.2  | 118.30      | 76480 | Response to stress  |
| A28      | Elongation factor EF-2 | 16.2  | 118.30      | 76480 | Response to stress  |
| A29      | Elongation factor EF-2 | 16.2  | 118.30      | 76480 | Response to stress  |
| A30      | Elongation factor EF-2 | 16.2  | 118.30      | 76480 | Response to stress  |
| A31      | Elongation factor EF-2 | 16.2  | 118.30      | 76480 | Response to stress  |
| A32      | Elongation factor EF-2 | 16.2  | 118.30      | 76480 | Response to stress  |
| A33      | Elongation factor EF-2 | 16.2  | 118.30      | 76480 | Response to stress  |
| A34      | Elongation factor EF-2 | 16.2  | 118.30      | 76480 | Response to stress  |
| A35      | Elongation factor EF-2 | 16.2  | 118.30      | 76480 | Response to stress  |
| A36      | Elongation factor EF-2 | 16.2  | 118.30      | 76480 | Response to stress  |
| A37      | Elongation factor EF-2 | 16.2  | 118.30      | 76480 | Response to stress  |
| A38      | Elongation factor EF-2 | 16.2  | 118.30      | 76480 | Response to stress  |
| A39      | Elongation factor EF-2 | 16.2  | 118.30      | 76480 | Response to stress  |
| A40      | Elongation factor EF-2 | 16.2  | 118.30      | 76480 | Response to stress  |
| A41      | Elongation factor EF-2 | 16.2  | 118.30      | 76480 | Response to stress  |
| A42      | Elongation factor EF-2 | 16.2  | 118.30      | 76480 | Response to stress  |
| A43      | Elongation factor EF-2 | 16.2  | 118.30      | 76480 | Response to stress  |
| A44      | Elongation factor EF-2 | 16.2  | 118.30      | 76480 | Response to stress  |
| A45      | Elongation factor EF-2 | 16.2  | 118.30      | 76480 | Response to stress  |
| A46      | Elongation factor EF-2 | 16.2  | 118.30      | 76480 | Response to stress  |
| A47      | Elongation factor EF-2 | 16.2  | 118.30      | 76480 | Response to stress  |
| A48      | Elongation factor EF-2 | 16.2  | 118.30      | 76480 | Response to stress  |
| A49      | Elongation factor EF-2 | 16.2  | 118.30      | 76480 | Response to stress  |
| A50      | Elongation factor EF-2 | 16.2  | 118.30      | 76480 | Response to stress  |

**Notes:**
- The designation "A" is used for those spots overrepresented in the strain A-20, and "P" is used for those spots overrepresented in the pefR deficient mutant SW-612.
- Theoretical MW and pl calculated from the sequence.
- Theoretical results of SignalP 3.0, TMHMM2.0, and SecretomeP2.0, y = yes, n = no.
believes that PerR plays an important role in the use of multiple sugar sources for *S. pyogenes* pathogenesis. In addition, we also found that PerR down-regulated M protein and fibrinogen-binding protein F, which means that PerR can increase *S. pyogenes* resistance of phagocytic killing. To summarize, we suggest that PerR controls expression of a diverse group of proteins that enhances *S. pyogenes* resistance to phagocytic killing and allows use of multiple sugar sources for survival.

Validation of RNA Expression and Protein Expression of the Virulent Candidates MF3—Based on the functions of the identified proteins, we believe that MF3 is a potential virulence factor involved in host infection. This protein was first found in the CM of *S. pyogenes* by Hasegawa et al. (2002). Previous studies have reported that the mf3 gene was shown to be present in most clinically isolated strains of *S. pyogenes*, and the MF3 protein in clinically isolated *S. pyogenes* strains was shown to have DNase activity (31). However, research has not determined whether or not production of MF3 by *S. pyogenes* enhances virulence. According to the result of 2DGE analysis in the present study, the expression of MF3 was measured to be 4.3 times as high in the wild-type strain A-20 as in the perR deficient mutant SW-612 (p < 0.05). This data suggest that the PerR regulator positively regulates the expression of the MF3 protein in *S. pyogenes* to enhance virulence. To confirm these 2DGE results, we performed immunoblotting analysis to determine the levels of MF3 protein in the CM and cytoplasm of the A-20 strain and the SW-612 strain. The expression levels of MF3 in the CM and cytoplasm were found to be up-regulated from 8 to 12 h in early stationary phase (Fig. 4A, 4B; p < 0.05). These data indicate that protein levels measured by 2DGE analysis correlate well with protein levels determined by Western blotting analysis. Moreover, the data from real-time RT-PCR show that mRNA expression levels of mf3 gene in the SW-612 strain are clearly lower than those in the A-20 strain from 8 to 12 h (Fig. 4C). The transcription level of mf3 in the perR-deficient mutant is lower than that of the wild-type strain. We therefore found that MF3 is up-regulated by PerR at the early stationary phase. When *S. pyogenes* grows during the early stationary phase, bacteria start to face nutrient-limiting conditions. To find additional available sources of nutrients, we suggest that MF3 can play an important role in degradation of DNA and use of DNA as a source of carbon, nitrogen, and phosphate. However, the positive regulator of PerR to MF3 was culture under the TSBYE broth containing a protease inhibitor mixture condition. Whether a protease inhibitor mixture has any effect to PerR regulations remain to be studied.

**PerR Binds to the mf3 Promoter Region**—To determine whether the PerR protein can directly regulate the expression of mf3 gene, an EMSA was performed. The recombinant His6-tagged PerR was expressed and purified under native conditions. The estimated size of r-PerR with tag was 19 kDa (21). A 428-bp upstream region containing the Per-like box (TTTTATTAATTTTAA) of the mf3 gene was amplified by PCR. When the mf3 promoter fragment was incubated with different amounts of r-PerR in the binding assay, band shifts were observed resulting from formation of an r-PerR-DNA complex (Fig. 5, lanes1–5). The r-PerR was able to bind the promoter region of mf3 gene in a concentration-dependent manner. This demonstrates that PerR can directly regulate MF3 expression in *S. pyogenes*. It is possible that the other regulators can also affect MF3 expression in *S. pyogenes* under special conditions. From this study, we suggest that PerR can control MF3 expression in response to nutrient stress.

**Construction of mf3 Deficient Mutants for Virulence Test**—To examine the virulence of MF3 in *S. pyogenes*, the mf3 deficient mutant SW-917 was created from the A-20 strain by gene replacement with a chloramphenicol resistance cassette. The mutant, SW-917, was constructed and confirmed by Southern blotting (supplementary Fig. S2). The mutant DNA and the wild-type DNA were digested with HindIII, and the blots were hybridized to the 0.8 kb chloramphenicol cassette or the 0.8 kb mf3 gene. One 5.4-kb band was detected in the wild-type strain when DNA was hybridized to mf3 probe, whereas no band was detected at 5.4 kb in the mf3-deficient mutant (supplementary Fig. S3A; lanes 2, 3). Another one 5.4-kb band was detected in the SW-917 strain when the DNA was hybridized with the chloramphenicol cassette probe, and none of the bands were detected in the wild-type strain. (supplementary Fig. S3B; lanes 2, 3). The results of the Southern blotting analysis demonstrated that the strain SW-917 completely deleted the mf3 gene from the wild-type strain.

**MF3 is Involved in Virulence of S. pyogenes**—To determine whether MF3 is a potential virulence factor in *S. pyogenes*, a mouse air sac infection model was performed to test the virulence of the wild-type strain A-20 and the mf3 deficient mutant SW-917. A skin air sac was injected with 2 × 10⁸ CFU of bacteria in each mouse, and the survival rate of infected mice were measured for 5 days. All mice infected with the wild-type strain when DNA was hybridized to mf3 probe, whereas no band was detected at 5.4 kb in the mf3-deficient mutant (supplementary Fig. S3A; lanes 2, 3). Another one 5.4-kb band was detected in the SW-917 strain when DNA was hybridized with the chloramphenicol cassette probe, and none of the bands were detected in the wild-type strain. (supplementary Fig. S3B; lanes 2, 3). The results of the Southern blotting analysis demonstrated that the strain SW-917 completely deleted the mf3 gene from the wild-type strain.

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the activity might contribute to virulence (4). However, the classification of these DNases is still not complete and their specific roles in the pathogenicity of *S. pyogenes* are not yet fully understood. One interpretation of these results is that DNases (1) protect the bacterial cell against potentially mutagenic heterologous DNA (32), (2) contribute to the escape of the host immune response by degrading neutrophil extracellular traps (NETs) (33, 34), and (3) are required for use of DNA as a nutrient source (24). However, in the absence of evidence for natural DNA transformation, it seems unlikely that MF3 is necessary to protect *S. pyogenes* from heterologous DNA, because the cell wall is an efficient barrier against DNA entry. The skin infection model revealed that MF3 can improve bacterial dissemination and influence lesion size. DNA is present in pus, and extracellular MF3 can decrease the viscosity of pus and facilitate bacterial dissemination. We also found that the wild-type strain (A-20) showed higher growth rate in the chemically defined BM2 medium supplemented with DNA than the mf3-deficient mutant (SW-917) did (Fig. 7). This result indicated that the mf3 gene might be involved in using DNA as a nutrient for growth. Besides, we constructed the mf3 complementary strain to be a control for proving the role of MF3 in scavenging nutrients from DNA. We also found that the mf3 complementary strain reverted to the growth rate of the wild-type strain in the chemically defined BM2 medium supplemented with DNA (Fig. 7). On the basis of these results, we suggest that *S. pyogenes* can use DNA as nutrient source by secreting MF3 to digest exogenous DNA. However, the role of MF3 in promoting bacterial growth still needs further investigation. To summarize, MF3 helps *S. pyogenes* dissemination

**Fig. 4.** Validation of expression level of *mf3* gene product in the wild-type strain (A-20) and the *perR* deficient mutant (SW-612). **A,** Analysis of MF3 expression by Western blotting. Total proteins (10 μg/lane) from the CM of *S. pyogenes* strains were extracted and probed by anti-MF3 mouse antibody. Silver staining of the gel is shown in the supplemental Fig. 5 as a protein loading control. **B,** Analysis of MF3 expression by Western blotting. Total proteins (10 μg/lane) from the lysates of the A-20 strain and the SW-612 strain were extracted and probed by an anti-MF3 mouse antibody. **C,** Relative gene expression of *mf3* in the wild-type strain (A-20) and the *perR* deficient mutant (SW-612) measured by real time RT-PCR analysis. The fold change of *mf3* gene expression was calculated by dividing the expression value of the SW-612 strain by the expression value of the A-20 strain. Bars represent mean values (three biological replicates) of fold changes ± standard deviation (S.D.).
CONCLUSION

In conclusion, our comparative secretomics data provides crucial information on PerR-regulated protein expression in the pathogenesis of *S. pyogenes*. Moreover, the data from extracellular protein prediction suggests a novel regulatory function of PerR that can directly or indirectly regulate protein secretion to influence virulence of *S. pyogenes*. The various functions of these newly identified PerR-regulated proteins suggest a previously unrecognized role of PerR that might contribute to increase bacterial fitness in the host and help evade phagocytic killing. Further investigations into one potential secreted virulence factor (MF3) have demonstrated that PerR regulator can directly regulate MF3 protein expression. In addition, we believe that MF3 is involved in the virulence of *S. pyogenes* and is required for use of DNA as a nutrient source. The analysis of PerR-regulated secretome has provided information for the elucidation of disease mechanisms and might lead to the development of chemotherapeutic strategies to prevent or treat severe streptococcal diseases.

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**Fig. 5.** Confirmation of PerR binding to the mf3 promoter region. The mf3 promoter and positive control dpr promoter were incubated with different concentrations of r-PerR (lanes 1–5 and lanes 6–7) for 15 min at room temperature. The mixtures were separated by native PAGE, and the mobility shift of the promoter was visualized by ethidium bromide staining.

**Fig. 6.** Virulence of *S. pyogenes* wild-type and mf3 deficient mutant strains. Skin air sacs of BALB/c mice (*n* = 10 per group) were injected with 2 × 10⁸ CFU of the strain A-20 and the strain SW-917. Animals were monitored four times per day. The results are expressed as percentages of survival over time.

* and is required for use of DNA as a nutrient source in pathogenesis.
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