Mechanisms of MSMEG_3312-mediated collective antibiotic tolerance to erythromycin in mycobacteria revealed by quantitative proteomic analysis

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Abstract: [Objective] The effects of antibiotics on bacteria are complex, and bacterial response to antibiotics is just beginning to be understood using systems biology. We previously showed that a hemerythrin-like protein, MSMEG_3312, is involved in erythromycin susceptibility. In this study, we explore the mechanisms of collective antibiotic tolerance to erythromycin in mycobacteria through the hemerythrin-like protein MSMEG_3312.

[Methods] We analyzed MSMEG_3312 secondary structure using spectrophotometric and circular dichroism (CD) methods. Tandem mass tag(TMT)-labeled quantitative proteomics was used to compare protein level changes between the wild type strain mc²155 and the knockout strain Δmsmeg_3312, following bioinformatics analysis. Differentially expressed proteins were also verified by qPCR. To confirm our analyses’ conclusions that transporters are involved in MSMEG_3312-related erythromycin susceptibility, we also measured the concentration of mycobacterial erythromycin in vivo in the wild type strain mc²155 and Δmsmeg_3312 using an erythromycin ELISA kit.

[Results] Initially, we confirmed that MSMEG_3312 is a redox-related hemerythrin-like protein using spectrophotometric and CD analysis. Quantitative proteomic analysis revealed that Δmsmeg_3312 has eight up-regulated proteins, including three transporters, and 14 down-regulated proteins, compared with the wild type strain mc²155, while growing in 7H9 medium. In contrast, 448 proteins were identified as being differentially expressed between mc²155 and Δmsmeg_3312, when treated with erythromycin, of which 11 were identified as up-regulated transporter proteins, and 26 were associated with amino acid synthetic pathways. The intracellular erythromycin concentration in Δmsmeg_3312 was also lower than in mc²155.

[Conclusion] We show that MSMEG_3312 mediates erythromycin resistance due to collective antibiotic tolerance arising from antibiotic
titration and high-density populations.

**Keywords:** quantitative proteomics, hemerythrin-like protein, MSMEG_3312, mycobacteria, erythromycin, collective antibiotic tolerance

Antibiotics have saved more than millions of lives, but currently the prevalence of antibiotic-resistant pathogens is a global threat. However, antibiotic effects on bacteria are complex, and bacterial response to antibiotic drug treatment is just beginning to be understood using biological systems approaches[1-3].

Tuberculosis (TB) remains a formidable challenge to global health and is caused by an ancient pathogen, *Mycobacterium tuberculosis*[4]. A recent study comparing genomes to examine *M. tuberculosis* complex (MTBC) evolution indicates that *M. tuberculosis* co-evolved with modern humans[5]. In the pre-antibiotic era, TB caused nearly 20% of all deaths in Europe from the seventeenth through the nineteenth centuries[6]. Millions of lives have subsequently been saved through chemotherapy by the addition of antibiotics to the TB treatment regimen. However, standard anti-tuberculosis therapy is only effective for drug-susceptible TB, not multi-drug-resistant (MDR) and extensively-drug-resistant (XDR) TB. Unfortunately, our knowledge of drug resistance in mycobacteria remains incomplete, presenting obstacles to the development of new anti-tubercular drugs[7]. The basic mechanisms of drug resistance in mycobacteria are complicated, although the techniques of systems biology are beginning to answer questions at the systems level[8], providing new insights into the outcome of drug resistance and for designing control strategies against *M. tuberculosis*.

Many studies have shown antibiotic resistance to be correlated with redox regulation[9]. For example, isoniazid (isonicotinic acid hydrazide, INH) is an important first-line anti-mycobacterial antibiotic that has been widely used in the treatment of active and latent TB[10]. INH is activated by the bacterial catalase-peroxidase KatG, which is encoded by *rv1908c*[11]. KatG-mediated INH activation produces a range of highly reactive oxygen species, including superoxide and hydroxyl radicals[12], and oxidative stress decreases isoniazid resistance in *M. tuberculosis*[10]. Recently, rifampin (RIF), a first-line anti-TB drug, was shown to induce hydroxyl radical formation in *M. tuberculosis*[13-14]. These studies suggest that redox-related proteins influence antibiotic effects in *M. tuberculosis*.

Previous studies have focused on the response of single event in response to antibiotics. However, Collective antibiotic tolerance is not a single event, but a combination of diverse mechanisms, including transporter induction, antibiotic titration, bacterial population, and biofilm formation[15-18]. Few studies have reported collective antibiotic tolerance mechanisms in mycobacterial resistance[15].

Hemerythrin-like proteins are present in all domains of life[19-20]. Several studies have shown that these proteins can function as oxygen sensors, and to store oxygen reserves, as well as to transport oxygen[20]. Hemerythrin-like proteins with oxygen-carrier capabilities might interfere with antibiotic response.

The pathogen *M. tuberculosis* and the soil microorganism *Mycobacterium smegmatis* share many genetic systems. In particular, genes that participate in sensing and responding to stress are conserved between *M. tuberculosis* and *M. smegmatis*[21]. These genes enable survival under environmental stress, including oxidative stress, hypoxia, and exposure to multiple antimicrobial agents[22-23]. *M. smegmatis* has three hemerythrin-like proteins, MSMEG_3312, MSMEG_2415 and
Our previous studies showed that MSMEG_2415 plays an important role in H₂O₂ susceptibility[24], while MSMEG_3312 and MSMEG_6212 modulate erythromycin susceptibility[25]. Additionally, MSMEG_3312 plays a relative dominate role in erythromycin susceptibility, compared with MSMEG_6212[25]. However, the mechanisms of MSMEG_3312 on erythromycin in mycobacteria have not yet been studied.

In this study, we used M. smegmatis, a M. tuberculosis model organism[21], to identify the mechanisms of antibiotic responses. We first characterized MSMEG_3312 as a hemerythrin-like protein, showing that it possesses a typical hemerythrin domain motif H…HxxxE…HxxxH…HxxxxD/E[20]. By combining a quantitative proteomic analysis, the intracellular concentration of erythromycin, and an erythromycin killing curve, we provide a model of collective antibiotic tolerance (CAT) to explain the role of MSMEG_3312 in mediated erythromycin resistance: Constitutively higher levels of transporters in Δmsmeg_3312 compared with mc²155 facilitates lower levels of intracellular erythromycin in Δmsmeg_3312, generating a positive feedback loop that overcomes erythromycin-mediated inhibition of ribosome activity and enables higher population survival. Increasing cell densities lead to an increase in total protein production, thus decreasing erythromycin titers in Δmsmeg_3312, which results in a more resistant phenotype, compared with mc²155.

1 Materials and Methods

1.1 Culture medium and growth conditions

Liquid cultures of M. smegmatis strains were grown in Middlebrook 7H9 medium (Becton Dickinson, Sparks, MD, USA) supplemented with ADS enrichment [Albumin-Dextrose Saline containing 5% (W/V) bovine serum albumin fraction V, 2% (W/V) D-dextrose, 8.1% (W/V) NaCl, 0.5% (W/V) glycerol, and 0.05% (W/V) Tween80]. Antibiotics were used as described: Hygromycin (75 mg/L for M. smegmatis, 150 mg/L for Escherichia coli; Roche) and kanamycin (25 mg/L for M. smegmatis, 50 mg/L for E. coli; AMERCO) were added to the medium as needed. The wild type M. smegmatis strains mc²155, Δmsmeg_3312, and E. coli strains DH5α, BL21(DE3) were stored in our lab. The drug exposure experiments were performed as we previously described[27]. Briefly, cultures were started from glycerol-frozen stocks and grown to log phase (OD₆₀₀ of 0.6–0.8), then mycobacterial cells were diluted approximately 10⁷-fold in fresh medium. After the addition of 31.25 mg/L erythromycin, aliquots were removed at the indicated times and plated on 7H10-ADS. Experiments were performed in triplicate. Standard deviations are indicated by error bars. t-tests were performed using online GraphPad software. ** P<0.01.

1.2 Cloning, expression and purification of MSMEG_3312 in Escherichia coli

The msmeg_3312 coding sequence was amplified from M. smegmatis mc²155 strain genomic DNA and cloned into the expression vector pET23b (+) (Novagen, USA) in-frame, producing a fusion protein with a C-terminal 6×His-tag sequence to generate the plasmid pET23b-3312, which was transformed into E. coli BL21(DE3) (Invitrogen, USA) for expression. Recombinant MSMEG_3312 was induced by incubation with 0.5 mmol/L isopropyl β-D-thiogalactoside (IPTG) at 28 °C for 3 h. Cells were harvested by centrifugation at 10000×g for 5 min, resuspended in lysis buffer (20 mmol/L Tris-HCl pH 8.0; 1 mol/L NaCl, 10% (V/V) glycerol, 20 mmol/L imidazole, 0.1% (V/V) Triton X-100, 1 mmol/L phenylmethylsulfonyl fluoride (PMSF), 1 mg/mL (W/V) lysozyme) and lysed by sonication. Lysates were centrifuged at 12000 r/min for 30 min at 4°C to remove debris before purification. The supernatants were incubated with Ni-NTA agarose (Qiagen, USA) with rotation for 4 h at 4 °C. Beads were then washed three times with washing buffer (20 mmol/L Tris-HCl pH 8.0,
0.5 mol/L NaCl, 10% \((V/V)\) glycerol, 50 mmol/L imidazole, 0.1% \((V/V)\) Triton X-100, 1 mmol/L PMSF, 25 mmol/L MgCl₂. The proteins were eluted with elution buffer [50 mmol/L Tris-HCl pH 7.5, 0.5 mol/L NaCl, 25 mmol/L MgCl₂, 10% \((V/V)\) glycerol, 300 mmol/L imidazole] and protein concentration was measured using the bicinchoninic acid protein assay reagent and a BSA standard. Purified protein was examined using 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis to verify molecular weight and purity.

### 1.3 Spectrophotometric and circular dichroism (CD) analysis of MsmHr

Purified MSMEG_3312 was diluted in 20 mmol/L Tris-HCl buffer (pH 7.5). Deoxy samples were obtained by adding a 10-fold molar excess of Na₂S₂O₄ to MSMEG_3312. UV-Vis spectrophotometric spectra were obtained in 1 mm path length quartz cuvettes on a 2802H UV-Vis spectrophotometer (Unico Shanghai Instruments Co., Ltd., China). Spectra of deoxy-MSMEG_3312 were collected in an anaerobic incubator (Shanghai Yuejin Medical Instruments Co., Ltd., China). CD measurements were performed using a Chirascan Circular Dichroism Spectrometer (Applied Photophysics Ltd. UK). The analysis software provided with the instrument was used for analysis of the results.

### 1.4 MS sample preparation and peptide TMT-labeling

To analyze differences in protein levels between the wild type strain mc²155 and knockout strain Δmsmeg_3312 with or without erythromycin, tandem mass tag(TMT)-labeled proteomic analyses were performed. Briefly, the strains were propagated in three independent 50 mL cultures in 7H9 medium. Fifty-milliliter cultures of the wild-type strain mc²155 or Δmsmeg_3312 strain were harvested by centrifugation when the \(OD_{600}\) of the cultures reached 0.3.

The corresponding cell pellets were washed once and resuspended in phosphate buffer saline (PBS) containing 8 mol/L urea at pH 7.2. Next protein extracts were prepared using a Mini BeadBeater (BioSpec), and the protein concentrations were examined using the bicinchoninic acid protein assay reagent, with a BSA standard. Protein extracts (200 µg) from wild type strain mc²155 or Δmsmeg_3312 strain were reduced with 10 mmol/L dithiothreitol (DTT) and alkylated with 55 mmol/L iodoacetamide.

Sequencing-grade modified trypsin (Promega, Fitchburg, WI, USA) was used for digestion in 50 mmol/L ammonium bicarbonate at 37 °C overnight. After digestion, the peptides were extracted with 50% acetonitrile solution supplemented with 0.1% trifluoroacetic acid for 30 min. The solution was clarified by centrifugation. The resulting peptides were then desalted using Sep-Pak® Vac 3cc (500 mg) certified tC18 Cartridges (Waters Corporation, Waters, MA, USA) followed by TMT labeling. Peptides from mc²155 and Δmsmeg_3312 without erythromycin treatment were labeled with TMT®-127 and TMT®-128, and peptides from mc²155 and Δmsmeg_3312 with erythromycin treatment were labeled with TMT®-129 and TMT®-130, respectively. Next, the desalted samples were fractionated using a 4.6 mm × 250 mm C₁₈ column (Waters Corporation) on a Waters HPLC system (Waters Corporation). Buffer A consisted of water (pH 10) and buffer B consisted of 98% acetonitrile (ACN) (pH 10). 47 fractions were collected during a 70-min gradient of 5% to 95% buffer B. Solvent from each fraction was dried in a Speed Vac, then combined into 10 fractions, and stored at −20 °C for further analysis. All of the proteomic experiments for identification of changes in protein expression between treated and untreated wild-type mc²155 or Δmsmeg_3312 strain were performed in biological triplicate.

### 1.5 Quantitative proteomics analysis and data analysis

For LC-MS/MS analysis, The labeled products were separated using a 60 min gradient elution at a

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flow rate of 0.30 µL/min with an UltiMate 3000 RSLCnano System (Thermo Scientific, USA) directly interfaced to a Thermo Q Exactive benchtop mass spectrometer. The analytical column was a custom fused silica capillary tube (75 µm ID, 150 mm length; Upchurch, Oak Harbor, WA, USA) packed with C-18 resin (300 Å, 5 µm, Varian, Lexington, MA, USA). Mobile phase A consisted of 0.1% formic acid, and mobile phase B consisted of 100% acetonitrile and 0.1% formic acid. The Thermo Q Exactive Orbitrap mass spectrometer was operated in data-dependent acquisition mode using Xcalibur 2.1 software. A single full-scan mass spectrum was acquired in the Orbitrap (300–1800 m/z, 70000 resolution), followed by 10 data-dependent MS/MS scans at 30% normalized collision energy (HCD) and the dynamic exclusion at 20 S.

The MS/MS spectra from each LC-MS/MS run were searched against the selected database (M. smegmatis mc2155) downloaded from UniProt[22] (download date of December 24, 2014; 6647 entries) using Proteome Discoverer (PD) software, v. 1.4 (Thermo Fisher Scientific, USA). We also added 247 common laboratory contaminants (from the Max Plank Institute of Biochemistry) to our M. smegmatis database. The search algorithm SequestHT (in PD software package version 1.4) was used to analyze the data. Standard search parameters were used as follows: One missed cleavage was allowed and fixed post-translational modifications included carbamidomethylation of cysteine residues and TMT sixplexes (K and N-terminal). The oxidation of methionine was set as a variable modification. The precursor ion mass tolerance is set as 10 ppm for all MS data acquired in an Orbitrap mass analyzer. The fragment ion mass tolerance was set at 20 mmu for the MS/MS spectra acquired.

The percolator output provided by PD was used for calculation of the peptide false discovery rate (FDR). The peptide spectrum match (PSM) was considered correct if the q value was smaller than 1%. False discovery was determined based on the PSM when searched against a reverse decoy database. The false discovery rate was set to 0.01 for proteins considered unique, and peptides only mapped to a given protein group were considered unique. Relative protein quantification was performed using PD software (Version 1.4) following the manufacturer’s instructions for the six reporter ion intensities per peptide. Quantification was performed for proteins with at least two uniquely identified peptides. Quantitative precision is expressed as protein ratio variability. When the protein ratio variability was smaller than 30%, the ratios were accepted as the quantitative ratios of proteins. Differentially expressed proteins were further confirmed by qPCR.

1.6 RNA isolation, RT-PCR and quantitative PCR

Log-phase cultures (OD600 of 0.8–1.0) of the tested strains were diluted 1:50 in 7H9 medium. Bacterial cells were harvested by centrifugation when the OD600 of the culture reached 0.3. The bacterial pellets were resuspended in TRIzol (Invitrogen, USA), and RNA was purified according to the manufacturer’s instructions. cDNA was synthesized using the SuperScript™ III First-Strand Synthesis System (Invitrogen, USA). Quantitative real-time PCR (qRT-PCR) was performed in a Bio-Rad iCycler using 2×SYBR real-time PCR pre-mix (TaKaRa Biotechnology Inc., Japan). Our cycling program follows: 95°C for 90 s, followed by 40 cycles of 95°C for 10 s, 60°C for 10 s, and 72°C for 15 s. DNA-directed RNA polymerase α subunit rpoD was selected to normalize gene expression. To evaluate relative gene expression in different strains and/or different treatments, the 2−ΔΔCt method was used[28]. The primers used are listed in.

1.7 Accumulation with semi-automated fluorometric method

To assess fluorochrome GoodView (Fermentas) accumulation, we followed methods previously described with several changes[29]. Briefly,
mycobacteria strains were grown in 7H9 medium, and the cells were collected at an OD$_{600}$ of 0.3. Cultures were collected using centrifugation at 13000 r/min for 10 min, and the pellets were washed in PBS (pH 7.4). The OD$_{600}$ was adjusted to 0.4 with PBS, and glucose was added to a final concentration of 0.4%. GoodView was added at concentrations of 2 mg/L, and the erythromycin was added as indicated. Fluorescence was measured in a microplate reader (Infinite M200 Pro reader, TECAN), using the 491 nm and 530 nm as the excitation and detection wavelengths, respectively. Fluorescence was acquired every 5 min for 30 min at 37 °C. The experiments were repeated three times.

1.8 Measurement of intracellular erythromycin

To analyze the concentration of erythromycin in vivo in the wild type strain mc$^2$155 and knockout strain Δmsmeg_3312 with erythromycin treatment, a 50 mL culture of mc$^2$155 or Δmsmeg_3312 was harvested by centrifugation when the OD$_{600}$ of the culture reached 0.3 after erythromycin treatment for 1 h. The cells were washed three times with PBSat (pH 7.2) and resuspended with 70% methanol. Then, extracts were prepared using a Mini BeadBeater (BioSpec) of 5 cycles of 1 min homogenization and 1 min cooling on ice. Lysates were clarified by centrifugation, and erythromycin concentrations were measured using an erythromycin ELISA kit (MLBIO CO., Ltd, Shanghai, China) and calculated following the manufacturer’s instruction. This experiment was repeated nine times.

1.9 Statistical analyses

All statistical analyses were performed using the GraphPad Prism 5.0c software. Significant differences in the data were determined by t-tests.

2 Results

2.1 MSMEG_3312 is a bacterial hemerythrin-like protein

Recently studies have shown a correlation between redox-related physiological changes and antibiotic treatment$^{30}$. These studies suggest that redox-related proteins influence antibiotic effects. Hemerythrin-like proteins can carry and reversibly bind oxygen$^{20}$; thus, we reasoned that hemerythrin-like proteins might play important roles in maintaining redox balance in mycobacteria and be involved in drug resistance$^{24}$. MSMEG_3312 is involved in drug resistance$^{26}$, but the relative mechanisms of MSMEG_3312 resistance to erythromycin have not been explored.

Hemerythrin-like proteins are widely distributed in prokaryotes and invertebrate animals. The characteristic hemerythrin-like protein motif is H…HxxxE…HxxxH…HxxxxD/E$^{20}$). Figure 1 shows the secondary structure of MSMEG_3312. Residues H16, H49, E53, H74, H79, H113 and E118 (numbering based on the MSMEG_3312 sequence) match the typical hemerythrin protein motif. The secondary structure predicted by SWISS-MODEL$^{31}$ suggested that MSMEG_3312 has a typical hemerythrin-like structure with four $\alpha$-helices (Figure 1-A).

To confirm the predicted MSMEG_3312 helical structure, we purified MSMEG_3312-His$_6$ protein from E. coli. Circular dichroism (CD) spectra of the E. coli-purified MSMEG_3312 show two minima at 208 nm and 222 nm (Figure 1-B). This $\alpha$-helical protein structure characterization is typical of the CD spectra of previously analyzed bacterial hemerythrins$^{35}$. The UV-visible MSMEG_3312 absorption spectra show peaks at 329 nm and 374 nm, a typical pattern of the di-iron-center of hemerythrin-like proteins$^{36}$. The absorbance peaks disappear after reduction with Na$_2$S$_2$O$_4$, which generates the deoxy form by removing oxygen (Figure 1-C). This spectrophotometric analysis indicates that MSMEG_3312 contains the di-iron oxygen bridge typical of hemerythrin-like proteins. Taken together, these bioinformatics, CD, and spectrophotometric results indicate that MSMEG_3312 is a hemerythrin-like protein.
Figure 1. MSMEG_3312 is a hemerythrin-like protein. A: Characteristic hemerythrin-like domain motifs in MSMEG_3312. H represents histidine. E represents glutamate. The lower panel shows MSMEG_3312 secondary structure. The secondary structure prediction was by SWISS-MODEL. B: Circular dichroism spectra of *E. coli*-purified MSMEG_3312. The clear circle indicates the blank control of 20 mmol/L Tris-Cl, pH 7.5 alone. The black circle indicates 100 mg/L MSMEG_3312 protein in 20 mmol/L Tris-Cl, pH 7.5. The measurements were performed at room temperature. Images represent three independent experiments. C: UV-visible absorption spectra of *E. coli*-purified MSMEG_3312. Solid line strands indicate oxidized MSMEG_3312 protein (oxy). Dotted line strands indicate reduced MSMEG_3312 protein (deoxy). Inset indicates the whole spectra for 100 mg/L MSMEG_3312 protein. Images represent three independent experiments.

2.2 Identification of *msmeg_3312*-induced changes in protein expression under normal growth conditions

Using TMT labeling and a mass spectrometric (LC-MS/MS) method, we identified approximately 2800 proteins in each of the samples in three repeated experiments. To explore MSMEG_3312 mechanisms of erythromycin resistance, we compared the protein levels of the wild type mc²155 strain and mutant strain Δ*msmeg_3312* at the early log phase (OD₆₀₀ of 0.3). Based on the TMT ratios (>1.3 or <0.8) in proteins that have two or more unique peptides, eight up-regulated proteins and 14 down-regulated proteins were identified (Table 1). The false-positive rate was estimated to be less than 1%. To analyze the biological relevance of these proteins, the David Bioinformatics Resource 6.7 Analysis Wizard[37–38] was used to cluster functional annotations. Compared with the *M. smegmatis* genome, the differentially expressed proteins were enriched for metal ion binding (P=2.4×10⁻²), oxidoreductase activity (P=3.3×10⁻²), transition metal ion transmembrane transporter activity (P=3.7×10⁻²), and transition metal ion binding (P=5.8×10⁻²) functions.

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Table 1. Selected proteins that were differentially expressed in mc²155 and Δsmeg_3312

<table>
<thead>
<tr>
<th>Gene name</th>
<th>Accession</th>
<th>Protein description</th>
<th>Score</th>
<th>Coverage</th>
<th>Peptides matched</th>
<th>Variability/%</th>
<th>Ratio±SD</th>
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<tbody>
<tr>
<td>MSMEG_6638</td>
<td>A0R6Q9</td>
<td>5-methyltetrahydropteroylglutamate-homocysteine methyltransferase</td>
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<td>12</td>
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<td>MSMEG_4560</td>
<td>A0R0Z3</td>
<td>ABC Fe³⁺-siderophore transporter, periplasmic binding protein</td>
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<td>34</td>
<td>10</td>
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<td>20</td>
<td>4</td>
<td>7.6–18.0</td>
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<td>A0QPV3</td>
<td>Short chain dehydrogenase</td>
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<td>18</td>
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<td>7</td>
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<td>ABC Fe³⁺-siderophore transporter, periplasmic binding protein</td>
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<td>19</td>
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<td>DoxX</td>
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<td>24</td>
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<td>Hemerythrin HHE cation binding domain subfamily protein, putative subfamily protein, putative</td>
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<td>15</td>
<td>2</td>
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</tr>
<tr>
<td>MSMEG_6354</td>
<td>A0R5Y1</td>
<td>Serine esterase, cutinase family protein</td>
<td>17</td>
<td>13</td>
<td>2</td>
<td>4.7–16.7</td>
<td>0.3±0.1</td>
</tr>
</tbody>
</table>

The proteomic analysis suggests that, as an oxygen-related protein, MSMEG_3312 influences the protein levels of several reductases and transporters. Two other hemerythrin-like proteins exhibited down-regulation in Δsmeg_3312. Protein levels of A0QV17 (MSMEG_2415) decreased 0.4±0.1 fold in Δsmeg_3312 compared with that in mc²155 (Table 1). We also identified seven unique MSMEG_2415 peptides covering 34% of the MSMEG_2415 sequence. The MS/MS spectrum from the proteomic analysis matched the sequence “ELDAQELER” in MSMEG_2415 (Figure 2). qPCR analysis indicates that the mRNA level of msme_2415 decreases by a factor of 0.41±0.05 in Δsmeg_3312 compared with that in mc²155 (Figure 3-A). Among the differentially expressed proteins, another predicted hemerythrin-like protein, A0R5J3 (MSMEG_6212), also showed decreased
expression, which was confirmed by qPCR (Table 1 and Figure 3-B). Additionally, two transporters, A0R0Z3 (MSMEG_4560) and A0R0Z4 (MSMEG_4561), were up-regulated and confirmed by qPCR (Table 1 and Figure 3-C, 3-D). Moreover, the mRNA levels of msmeg_6467 (encoding DPS), and msmeg_6242 (encoding alcohol dehydrogenase) were down-regulated and up-regulated in Αmsmeg_3312, respectively, consistent with changes in the expression of the corresponding proteins A0R692 (MSMEG_6467) and A0R5M3 (MSMEG_6242) (Figure 3-E, 3-F). The fact that so few proteins exhibited changed expression levels might explain the lack of conspicuous growth differences between mc^2155 and Δmsmeg_3312 in 7H9 medium. Furthermore, it suggests that MSMEG_3312 is a stress response protein with no growth effects at normal conditions (i.e. 7H9 medium).

2.3 Expression profiles of mc^2155 and Δmsmeg_3312 in response to erythromycin

The previous comparisons showed no large differences (22 differentially expressed proteins) in protein expression levels between mc^2155 and Δmsmeg_3312, which is consistent with the observation that both strains grow similarly when cultured in 7H9 medium\[26\]. We then examined protein expression response when treated with 3.125 mg/L erythromycin in wild-type mc^2155 versus Δmsmeg_3312. Fewer differentially expressed proteins were identified in mc^2155 (proteins were considered to be significantly differentially expressed if the change was >1.3 or <0.8). Upon exposure to 3.125 mg/L erythromycin, only 21 proteins were differentially expressed in wild type mc^2155. The differentially expressed proteins detected by MS were assigned using the David Bioinformatics Resource 6.7 Analysis Wizard[37–38]. The 21 proteins are involved in protein disulfide oxidoreductase activity (\(P=1.3\times10^{-2}\), response to stress (\(P=4.8\times10^{-2}\), and cell redox homeostasis (\(P=6.3\times10^{-2}\)). Strikingly, a highly induction of A0QTT1 (WhiB7) was observed, with an increase of 2.80±0.74 fold, and the qPCR results show a 98.5±6.5 fold up-regulation with erythromycin treatment, compared with wild type without antibiotic treatment (Figure 3-G).

Figure 2. MSMEG_3312 affects the expression of MSMEG_2415 at protein levels. Quantitative comparison of MSMEG_2415 expression using MS/MS.

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In contrast, a total of 324 proteins were differentially expressed when Δmsmeg_3312 was treated with erythromycin. To determine the biological relevance of these proteins, we clustered the differentially expressed proteins based on functional annotation using the DAVID Bioinformatics Resource 6.7 Analysis Wizard. Against the whole genome, the differentially expressed proteins assort into 22 categories, including translation ($P=4.0\times10^{-7}$), cellular
homeostasis ($P=4.8 \times 10^{-3}$), cellular chemical homeostasis ($P=1.8 \times 10^{-2}$), and others. Similar to the wild type mc²155 strain in response to erythromycin, transcriptional regulator A0QTT1 (WhiB7) was also up-regulated 3.3±0.3-fold at the protein level in Δmsmeg_3312, and the qPCR results indicate an increase of 101.6±7.8 fold in response to erythromycin treatment (Figure 3-H). WhiB7 is a Fe-S-dependent transcription factor, and is required for the activation of drug resistance genes. Our results indicate that WhiB7 is an erythromycin induced transcriptional regulator. Moreover, these results suggest that WhiB7 response to erythromycin is independent of MSMEG_3312.

Those differentially expressed proteins between mc²155 and Δmsmeg_3312 with erythromycin treatment were also compared, and 448 proteins were identified. Twenty-three categories were enriched, including translation ($P=7.1 \times 10^{-6}$), nitrogen compound biosynthetic process ($P=1.0 \times 10^{-5}$), homeostatic process ($P=5.3 \times 10^{-3}$), and others (Figure 4). Among the differentially expressed proteins, the levels of two Ag85C protein, A0QY95 (MSMEG_3580) and A0R624 (MSMEG_6399), were increased 1.9±0.1 fold and 1.4±0.1 fold respectively, in the mutant strain Δmsmeg_3312, compared with wild type mc²155 (Table 2). qPCR

![Functional classification of proteins differentially expressed in mc²155 and Δmsmeg_3312 under erythromycin treatment using the DAVID Analysis Wizard.](image)

- Translation
- Nitrogen compound biosynthetic process
- Carboxylic acid biosynthetic process
- Organic acid biosynthetic process
- Pyridoxal phosphate-dependent enzyme, beta subunit
- Amine biosynthetic process
- Sulfur amino acid biosynthetic process
- Cysteine and methionine metabolism
- Methionine biosynthetic process
- Dufur metabolic process
- Cell division and chromosome partitioning
- Aspartate family amino acid metabolic process
- Homeostatic process
- Lipid metabolism
- Purine biosynthesis
- Coenzyme metabolic process
- Aromatic compound biosynthetic process
- Butanoate metabolism
- Threonine metabolic process
- Glycine, serine and threonine metabolism
- Oxygen and tractive oxygen species metabolic process
- Macromolecule cleavage metabolic process
- Nucleobase, nucleoside and nucleotide biosynthetic process
- Else

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Table 2. Selected proteins that were differentially expressed in mc\(^2\)155 and Δmsmeg\(_{3312}\) under erythromycin treatment

<table>
<thead>
<tr>
<th>Gene name</th>
<th>Accession</th>
<th>Protein description</th>
<th>Score</th>
<th>Coverage</th>
<th>Peptides matched</th>
<th>Variability/%</th>
<th>Ratio±SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>MSMEG(_{1520})</td>
<td>A0QSL4</td>
<td>50S ribosomal protein L36</td>
<td>14</td>
<td>59</td>
<td>3</td>
<td>0.2–7.4</td>
<td>1.9±0.2</td>
</tr>
<tr>
<td>MSMEG(_{3580})</td>
<td>A0QY95</td>
<td>Antigen 85-C</td>
<td>24</td>
<td>13</td>
<td>5</td>
<td>4.0–20.2</td>
<td>1.9±0.1</td>
</tr>
<tr>
<td>MSMEG(_{4560})</td>
<td>A0R0Z3</td>
<td>ABC Fe(^{3+})-siderophores transporter, periplasmic binding protein</td>
<td>62</td>
<td>27</td>
<td>12</td>
<td>2.2–7.4</td>
<td>1.9±0.1</td>
</tr>
<tr>
<td>MSMEG(_{4557})</td>
<td>A0R0Z1</td>
<td>ABC transporter, ATP-binding protein</td>
<td>10</td>
<td>13</td>
<td>3</td>
<td>0.9–5.5</td>
<td>1.7±0.1</td>
</tr>
<tr>
<td>MSMEG(_{1437})</td>
<td>A0QSD2</td>
<td>50S ribosomal protein L4</td>
<td>98</td>
<td>53</td>
<td>11</td>
<td>9.0–17.6</td>
<td>1.6±0.3</td>
</tr>
<tr>
<td>MSMEG(_{5684})</td>
<td>A0R429</td>
<td>Phosphoserine aminotransferase</td>
<td>41</td>
<td>24</td>
<td>11</td>
<td>4.7–12.5</td>
<td>1.6±0.1</td>
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<tr>
<td>MSMEG(_{3507})</td>
<td>A0QY23</td>
<td>Fructose-bisphosphate aldolase</td>
<td>186</td>
<td>75</td>
<td>16</td>
<td>2.8–7.8</td>
<td>1.5±0.2</td>
</tr>
<tr>
<td>MSMEG(_{4743})</td>
<td>A0QSG7</td>
<td>50S ribosomal protein L30</td>
<td>27</td>
<td>48</td>
<td>2</td>
<td>9.5–18.8</td>
<td>1.5±0.1</td>
</tr>
<tr>
<td>MSMEG(_{4956})</td>
<td>A0R624</td>
<td>Threonine synthase</td>
<td>200</td>
<td>75</td>
<td>20</td>
<td>2.5–12.3</td>
<td>1.4±0.1</td>
</tr>
<tr>
<td>MSMEG(_{4561})</td>
<td>A0R0Z4</td>
<td>ABC Fe(^{3+})-siderophores transporter, periplasmic binding protein</td>
<td>37</td>
<td>26</td>
<td>5</td>
<td>1.7–9.9</td>
<td>1.4±0.1</td>
</tr>
</tbody>
</table>

Analysis indicates that the mRNA levels of msmeg\(_{3580}\) (encoding Ag85C) and msmeg\(_{6399}\) (encoding Ag85C) were increased by a factor of 3.8±0.5 and 2.1±0.2 respectively, in mutant strain Δmsmeg\(_{3312}\) compared with wild type mc\(^2\)155 with erythromycin treatment (Figure 3-K, 3-L). Ag85C has been shown to have an effect on the cell envelope biogenesis in mycobacteria\(^{[39–40]}\) and thus influences cell wall composition. Moreover, 11 transporters were also identified among the differentially expressed proteins. Interestingly, 24 proteins are involved in amino acid synthesis pathways, according to KEGG-User Data Mapping (Figure 4).

### 2.4 Decreased accumulation of erythromycin in Δmsmeg\(_{3312}\)

Quantitative mass spectrometry results showed that the knockout strain Δmsmeg\(_{3312}\) exhibits an increased expression of certain transporters both under normal growth conditions and with treatment by erythromycin. We reasoned that an increasing expression of transporters might efflux erythromycin, thus lowering intracellular erythromycin levels compared with mc\(^2\)155, resulting in better survival. We measured the intracellular erythromycin concentration of both Δmsmeg\(_{3312}\) and mc\(^2\)155 post-treatment with erythromycin for 1 hour: The concentration of intracellular erythromycin in Δmsmeg\(_{3312}\) was 19.8±1.9 μg/mL, which was statistically lower than in mc\(^2\)155 (22.8±1.8 μg/mL) (Figure 5-A). Lower levels of erythromycin were detected in the knockout strain Δmsmeg\(_{3312}\) than in mc\(^2\)155, which might be due to a higher rate of drug export. Furthermore, to rule out the possibility of low drug uptake in the mutant strain, we compared the accumulation of intracellular fluorometric GoodView in both Δmsmeg\(_{3312}\) and mc\(^2\)155. As shown in Figure 5-B, no differences were observed between the mycobacteria strains with or without erythromycin treatment.

### 3 Discussion

Recent studies have shown that antibiotic treatment...
Figure 5. Decreased accumulation of erythromycin in Δmsmeg_3312 and benefit-growth compared to mc^2155. A: Intracellular erythromycin concentration in wild type mc^2155 and knockout-strain Δmsmeg_3312 (Δ3312) under erythromycin treatment. The experiment was repeated for three times. ** P<0.01. B: Accumulation of semi-automated fluorometric curve. Strains were grown in 7H9 medium with the addition of GoodView (2 mg/L) and erythromycin as indicated. The experiment was repeated three times. C: Erythromycin killing curve. Standard deviations are indicated by error bars. t-tests were performed using online GraphPad software. The experiment was repeated three times. ** P < 0.01.

Erythromycin belongs to the macrolides family; it binds to the 50S subunit of the bacterial ribosomal complex and inhibits protein synthesis[43]. M. tuberculosis WhiB7 is a well-identified transcriptional regulator involved in ribosomal-targeting antibiotic resistance, including erythromycin, in mycobacteria[9,44-45]. The characterized erythromycin resistance mechanisms in mycobacteria are complex, including an impermeable mycolic acid-containing cell wall, drug efflux, and antibiotic modification[9,44,46].

Proteomics is a powerful approach for understanding the network of protein changes that respond to drug treatment, facilitating a broad view of antibiotic resistance. This is particularly true when a key gene is found to be involved in drug influences bacterial protein response and induces bacterial reprogramming in response to these drugs[41]. Antibiotics have complex mechanisms and targets, thus systems biological approaches, such as proteomics, are beginning to allow us to understand the complicated and coordinated system underlying response to drug treatment[42]. In this study, we compared two strains of mycobacteria using quantitative mass spectrometry, one wild-type strain, mc^2155, and one msme_3312 knockout strain, Δmsmeg_3312, to determine which is more resistant to erythromycin. We observed few differences in protein expression between the strains under baseline growth conditions in 7H9 medium, but 448 proteins were differentially expressed upon treatment with erythromycin. Interestingly, these changes in protein expression included increases in transporters and amino acid synthetic proteins in Δmsmeg_3312. Furthermore, our analyses suggest that the knockout strain Δmsmeg_3312 increases transporter and ribosomal protein expression, and exhibits collective antibiotic tolerance, including antibiotic titration and high population density (Figure 6). To our knowledge, MSMEG_3312 is the first hemerythrin-like protein identified to be involved in antibiotic resistance in mycobacteria.
resistance, which can lead to the development of novel antibacterial therapies\textsuperscript{[42]}. To understand the mechanisms of MSMEG\textsubscript{3312}-mediated erythromycin resistance, we compared the differences in mc\textsuperscript{2}155 and MSMEG\textsubscript{3312} protein expression levels using TMT-labeled quantitative proteomics. Consistent with previous studies, WhiB7 (A0QTT1) was highly induced at both protein and mRNA levels in mc\textsuperscript{2}155 (Figure 3-G). WhiB7 was also highly up-regulated in response to erythromycin in MSMEG\textsubscript{3312} (Figure 3-H). The induction of WhiB7 expression in both mc\textsuperscript{2}155 and MSMEG\textsubscript{3312} indicates that the WhiB7-mediated erythromycin resistance pathway is distinct from the MSMEG\textsubscript{3312}-mediated erythromycin resistance pathway. Moreover, none of the differentially expressed proteins identified are involved in WhiB7 dependent pathways, such as A0R5B1 (MSMEG\textsubscript{6129}), A0R2P5 (TetV) and A0R2G3 (ABC transporter)\textsuperscript{[47]}, which suggests that the MSMEG\textsubscript{3312}-involved erythromycin response is via a WhiB7 independent pathway.

Recent studies have shown that efflux pump induction is an important mechanism of drug resistance in many bacteria\textsuperscript{[46,48–49]}. In mycobacteria, the evolution of efflux pumps were an early step in the development of antibiotic resistance\textsuperscript{[15,50]}. Our results show that knocking out msmeg\textsubscript{3312} increases the protein levels of A0R0Z3 (ABC Fe\textsuperscript{3+} siderophore transporter), A0R0Z1 (ATP-binding ABC transporter), and A0R0Z4 (ABC Fe\textsuperscript{3+} siderophore transporter), compared with the wild type strain mc\textsuperscript{2}155 in the 7H9 medium (Table 1). We reasoned that the transporters with a higher level of constitutive expression, compared with that in wild type mc\textsuperscript{2}155, might show a relative decrease in intracellular erythromycin concentration. Our results show that the concentration of intracellular erythromycin in MSMEG\textsubscript{3312} is significantly lower than in mc\textsuperscript{2}155, as we predicted (Figure 5-A). Moreover, no difference in the accumulation GoodView is found between the mycobacteria strains, which suggests an equal drug uptake in both the resistant strain MSMEG\textsubscript{3312} and wild type mc\textsuperscript{2}155 (Figure 5-B). This also explains why we only observed a low-level of drug resistance in knockout msmeg\textsubscript{3312} (increment 4×MIC of the wild type strain mc\textsuperscript{2}155)\textsuperscript{[26]}. Based on our results, we provide a model of MSMEG\textsubscript{3312} involvement in erythromycin resistance as part of collective antibiotic tolerance via a combination of diverse

Figure 6. Model of MSMEG\textsubscript{3312} function in erythromycin resistance. More erythromycin was transported out of MSMEG\textsubscript{3312} with high-level expression transporters than from mc\textsuperscript{2}155 during the initial drug treatment.
mechanisms, including antibiotic titration and high-density population (Figure 6). More erythromycin is transported out of Δmsmeg_3312 with high-level expression transporters than from mc^2155 during our initial drug treatments. Furthermore, higher cell survival populations result in relatively lower intracellular concentrations in Δmsmeg_3312, than so in mc^2155, and Δmsmeg_3312 exhibits an advantage over mc^2155 in the presence of erythromycin (Figure 5-C).

We conclude that MSMEG_3312 is a hemerythrin-like protein, and that the Δmsmeg_3312 strain exhibits increased erythromycin resistance. On the basis of our proteomics results and erythromycin-killing assay, we show that the resistance to erythromycin in strain Δmsmeg_3312 was not caused by a single event, but by collective antibiotic tolerance, including the transport of erythromycin upon early drug treatment, and a relative concentration decrease via antibiotic titration, followed by the achievement of a high-density population.


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定量蛋白质组分析蚯蚓血红蛋白样蛋白 MSMEG_3312 介导的分枝杆菌耐红霉素机制

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摘要:

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