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Two amino acids missing of MtrA resulted in increased erythromycin level and altered phenotypes in *Saccharopolyspora erythraea*

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Abstract

The MtrA-MtrB two-component regulatory system is highly conserved in *Actinobacteria* and plays crucial roles in cell cycle progression, cell morphology, antibiotic resistance, and osmoprotection. Previously, we revealed that the MtrA protein of *Saccharopolyspora erythraea* E3 strain (a high erythromycin-producing strain) had a two amino acid (H197 and V198) deletion in the DNA recognition helices of the C-terminal domain compared to the wild type *S. erythraea* strain NRRL2338. Here, we identified *mepA* (encoding a membrane protein related to metalloendopeptidases) as an MtrA target gene, and found that deleting the two amino acids in MtrA (MtrA^{del}) resulted in the loss of its DNA-binding activity for the *mepA* gene. The mutant MtrA^{del} lost its regulatory activity and affected various physiological functions consistent with *mtrA* deletion, including increased erythromycin biosynthesis, enhanced antibiotic resistance, deregulated osmoprotection, and improved transport of substances. The introduction of the wild type *mtrA* gene into the *S. erythraea* E3 strain with the *mtrA*^{del} gene decreased the erythromycin yield by approximately 50%, confirming that MtrA repressed erythromycin production. These findings demonstrate that MtrA is an important pleiotropic regulator of erythromycin biosynthesis, antibiotic resistance, osmoprotection, and substance transport in *S. erythraea* and provide new insights for improving erythromycin production. Future studies linking the molecular effects of MtrA to these phenotypes will improve our understanding of the MtrA-MtrB two-component regulatory system in *Actinobacteria*.

Keywords MtrA-MtrB · Two-component regulatory system · Erythromycin biosynthesis · Antibiotic resistance

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Introduction

Sensing and responding to the environment is essential for the survival of all living organisms (Papandreou et al. 2005). Bacteria have developed beneficial two-component regulatory systems which respond to various environmental stimuli, including nutrients, pH, temperature, and antibiotics (Stock et al. 2000). In the classical two-component system, a sensor histidine kinase is autophosphorylated by sensing a specific signal, then transfers its phosphoryl group to its corresponding response regulator which regulates the expression of relevant genes and affects physiological function (Laub and Goulian 2007). Multiple important actinobacterial two-component systems have been widely researched, including VanRS, AbsA1/2, SenRS, σ^E-CseABC, and MtrAB (Anderson et al. 2001; Hutchings 2007; Hutchings et al. 2006; Lucana et al. 2005; Paget et al. 1999). Among these, MtrAB has received the most attention as it is conserved in all actinobacterial genomes and thus plays a significant role in survival (Hutchings 2007).

In the past few decades, MtrAB has been extensively studied in some microorganisms, such as *Mycobacterium tuberculosis* (Li et al. 2010; Nguyen et al. 2010; Via et al. 1996; Zahrt and Deretic 2000), *Corynebacterium glutamicum* (Brocker and Bott 2006; Hoskisson and Hutchings 2006; Moker et al. 2004; Moker et al. 2007), and *Streptomyces coelicolo* (Zhang et al. 2017). These studies revealed that

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MtrAB is involved in many different cellular functions. The *dnaA* gene, which regulates DNA replication, was the first direct target gene of MtrA to be confirmed in *M. tuberculosis* (Fol et al. 2006), suggesting that MtrAB plays an essential role in cell cycle progression. The *oriC* and *fbpB* genes were also identified as MtrAB target genes, indicating a major role for MtrAB in coordinating the fecundity and pathogenicity of *M. tuberculosis* (Rajagopalan et al. 2010). Furthermore, it was shown that *mtrA* gene transcription is activated by incubating *M. tuberculosis* with macrophages (Via et al. 1996) and that the *mtrA* expression level differed for virulent and avirulent strains grown in macrophages (Zahrt and Deretic 2000), suggesting that MtrAB function affects the infectivity of *M. tuberculosis*. More recently, the study has provided evidence for the role of MtrAB in the multidrug resistance of *Mycobacterium* species, thus providing new possibilities for drug design (Li et al. 2010). Meanwhile, MtrAB studies have also revealed the relationship between the two-component system and cell wall homeostasis in *Mycobacterium* species (Nguyen et al. 2010). In *C. glutamicum*, MtrA repressed the expression of *mepA* and *nlpC*, which encode putative cell wall peptidases (Brocker and Bott 2006), revealing that MtrAB is involved in regulating cell wall turnover and suggesting that MtrAB might contribute to multidrug resistance (Moker et al. 2004). Conversely, MtrA activated the expression of *betP* and *proP*, which encode transporters for the uptake of osmoprotectants (Brocker and Bott 2006; Hoskisson and Hutchings 2006; Moker et al. 2004), indicating that MtrAB is involved in osmoprotection (Moker et al. 2007). *Streptomyces* is the largest *Actinobacteria* genus and produces over two-thirds of clinically useful antibiotics. MtrA was found to regulate the expression of genes involved in antibiotic biosynthesis in *Streptomyces* (Som et al. 2017b). However, MtrAB research has so far been limited to *Corynebacteria*, *Mycobacteria*, and *Streptomycetes*; therefore, investigating the role of MtrAB in different bacteria would improve our understanding of the overall ability of *Actinobacteria* to respond to their environment (Hoskisson and Hutchings 2006).

Saccharopolyspora erythraea is an excellent strain for producing erythromycin A (Staunton and Wilkinson 1997), a widely used antibiotic against gram-positive bacteria. A detailed investigation of *S. erythraea* would therefore be highly valuable to the antibiotic industry. Previously, we found that the MtrA protein of *S. erythraea* E3 (a high erythromycin-producing strain) had two amino acids missing (H197 and V198) compared to *S. erythraea* NRRL2338 (a low erythromycin-producing strain), and hypothesized that this might be related to high erythromycin productivity (Li et al. 2013).

In this study, we investigated the effect of deleting the *mtrA* gene or the two amino acids in MtrA on erythromycin level in *S. erythraea*, finding that mutant $MtrA^{del}$ reduced the erythromycin yield by approximately 50%. MtrA repressed the expression of its target gene *mepA*, which encodes a membrane protein related to the metalloendopeptidases. The deletion of H197 and V198 resulted in the loss of MtrA DNA-binding activity for its target genes, its repressive activity, and affected various physiological functions consistent with *mtrA* deletion, including increased erythromycin biosynthesis, enhanced antibiotic resistance, deregulated osmoprotection, and improved transport of substances.

Materials and methods

Bacterial strains, plasmids, and growth conditions

The strains and plasmids used in this study are listed in Table S1. The collection number of *S. erythraea* NRRL23338 is DSM 40517.To prepare for seeding, *S. erythraea* was grown in tryptone soya broth at 30 °C and 200 rpm for 24 h. Then, 500 μL of the seed culture was inoculated into a 500-ml flask containing 50 ml of industrial fermentation medium (36 g/L soybean flour, 36 g/L corn starch, 2.4 g/L $(NH_4)_2SO_4$, 7.2 g/L $CaCO_3$, 5 g/L soybean oil, 20 g/L glucose, 6.7 g/L n-propanal, and distilled H_2O) and incubated at 30 °C and 200 rpm for 4 days in order to evaluate erythromycin production. *Bacillus subtilis* was grown in the test medium (5 g/L peptone, 3 g/L Beef Extract Powder, 3 g/L K_2HPO_4, and distilled H_2O) at 37 °C and 200 rpm for 14–16 h as the control organism for erythromycin production. A total of 500 μl of the seed culture was inoculated into a 500-ml flask containing 50 ml of tryptic soy broth (TSB) and incubated at 30 °C and 200 rpm for 48 h prior to RNA extraction. A total of 20 μL of the seed culture was inoculated onto MM agar plates (10 g/L glucose, 0.5 g/L K_2HPO_4, 1 g/L $(NH_4)_2SO_4$, 0.2 g/L $MgSO_4$, 0.01 g/L $FeSO_4$, 20 g/L agar, and distilled H_2O; pH 7.0) and incubated at 30 °C for 40 h before observing the cells using a scanning electron microscope (SEM). *Escherichia coli* strains were grown in Luria-Bertani (LB) medium at 37 °C. All media were sterilized by autoclaving at 121 °C for 20 min, except for MM agar which was autoclaved at 115 °C for 20 min.

Construction of the wild type mtrA complementation and deletion strain

The complementation experiments in *S. erythraea* E3 were performed as described previously (Liao et al. 2014). A 687 nt fragment of the mtrA gene (SACE_6447; protein ID: CAM05617.1) from *S. erythraea* NRRL2338 was amplified by PCR using the primer pairs mtrA-fw and mtrA-rev as shown in Table S2. The pIB-mtrA plasmid was created by cloning the PCR products into the NdeI/NotI sites of pIB139. The complementary plasmid was introduced into

S. erythraea E3 by polyethyleneglycol (PEG)-mediated transformation. The desired complementary strains (*S. erythraea* E3::mtrA strain) were confirmed using apramycin-resistance screening and PCR.

The deletion experiments in *S. erythraea* NRRL2338 were performed as described previously (Liu et al. 2018). KO6447Up, *tsr*, and KO6447Down were amplified using PCR with the primer pairs provided in Table S2. The sgRNAII sequence was designed as shown in Table S3. The 6447sgRNAII-(KO6447Up-tsr-KO6447Down) cassette was produced and ligated into the pKECas9 plasmid using the Hieff Clone Multi One Step Cloning Kit (Yeasen, Shanghai, China) to generate pKECas9-6447sgRNA-(KO6447Up-tsr-KO6447Down). *mtrA* deletion was confirmed by PCR and sequencing (Fig. S1).

Erythromycin determination by high-performance liquid chromatography

High-performance liquid chromatography (HPLC) was carried out as described previously (Liu et al. 2018). The seed cultures were inoculated into the industrial fermentation medium, incubated at 30 °C and 200 rpm for 7 days, and erythromycin production was evaluated by HPLC. Freeze-dried fermentation supernatant powder was dissolved in absolute ethanol, filtered using a hydrophilic PVDF membrane (0.22 mm pore size), and analyzed. The HPLC conditions were as follows: solvent B (55% acetonitrile) against solvent A (1 L water with 8.7 g K_2HPO_4; pH 8.2) at a flow rate of 1.0 mL/min and 40 °C. UV spectra were measured at 215 nm. A standard curve ($Y = aX + b$) was obtained by analyzing a series of erythromycin standard concentrations, allowing the erythromycin levels to be calculated. Y represented the peak area and X represented the erythromycin levels.

Overexpression, purification, and phosphorylation of MtrA

The primers for PCR were provided in Table S2. The *mtrA* (from *S. erythraea* NRRL23338 genomic DNA) and *mtrA*^{del} (from *S. erythraea* E3 genomic DNA) genes were amplified by PCR. The PCR products were cloned into pET-28a to generate recombinant pET28a-*mtrA*/pET28a-*mtrA*^{del} plasmids. The *cheA* gene (from *E. coli* K-12 MG1655 genomic DNA) was amplified by PCR and cloned into pET-42a to generate recombinant pET42a-*cheA* plasmids. The recombinant plasmids were then confirmed by DNA sequencing and introduced into *E. coli* BL21 (DE3). The *E. coli* cells were inoculated into 100 ml LB with 50 μg/mL kanamycin, grown at 37 °C and 220 rpm to an OD_{600} of 0.3, induced with isopropyl-beta-D-thiogalactopyranoside (IPTG) at a final concentration of 0.4 mM, and incubated at 20 °C for 12–16 h. For protein purification, cells were harvested by centrifugation, washed twice with PBS buffer (pH 8.0), and then disrupted using an ultrasonic cell crusher. Cell debris and membrane fractions were separated from the soluble fractions by centrifugation (10 min, 10,000 rpm, 4 °C). His6-proteins were purified using Ni-NTA Superflow columns (Qiagen). Purified proteins were eluted with 250 mM imidazole (50 mM NaH_2PO_4, 300 mM NaCl; pH 8.0), dialyzed in protein preservation Buffer D (50 mM Tris, 0.5 mM EDTA, 50 mM NaCl, 20% glycerol, 1 mM DTT; pH 8.0) at 4 °C overnight, and then stored at − 80 °C. The quality of the purified proteins was determined by SDS-PAGE and their concentrations were determined using the Bradford reagent.

MtrA phosphorylation was carried out as described previously (Deretic et al. 1992). CheA, a typical histidine protein kinase, was expressed, purified, and used to phosphorylate MtrA. CheA was autophosphorylated by incubating with ATP at room temperature for 20 min, and then mixed with 10 μM MtrA/MtrA^{del} for 1 h at 37 °C in a buffer containing 100 mM Tris HCl (pH 7.5), 50 mM KCl, and 5 mM $MgCl_2$, to allow the phosphoryl group to be transferred from CheA to MtrA/MtrA^{del}.

Electrophoretic mobility shift assays

The promoter regions (− 300 to + 50) of the predicted target genes were amplified using PCR with the primers listed in Table S2. The PCR products were labeled with a biotinylated universal primer (5′-biotinAGCCAGTGGCGATAAG-3′). The biotin-labeled PCR products were then analyzed by agarose gel electrophoresis and purified using a PCR purification kit (Shanghai Generay Biotech). The DNA probe concentrations were measured using a microplate reader (Biotek, USA). Electrophoretic mobility shift assays (EMSAs) were carried out as described previously (Yao et al. 2014) according to the protocol for the Chemiluminescent EMSA Kit (Beyotime Biotechnology, China). The binding reaction was carried out in 10 mM Tris HCl (pH 8.0), 25 mM $MgCl_2$, 50 mM NaCl, 1 mM DTT, 1 mM EDTA, 0.01% Nonidet P40, 50 μg/ml poly[d(I-C)], and 10% glycerol. A biotin-unlabeled specific probe (200-fold) and a non-specific competitor DNA (200-fold, sonicated salmon sperm DNA) were used as controls. After binding, the samples were loaded and separated on 6% non-denaturing PAGE gels in ice-cold 0.5X Tris-borate-EDTA at 100 V, and bands were detected by BeyoECL Plus.

RNA extraction and real-time RT-PCR

Cell pellets were collected by centrifugation at 4000×*g* and 4 °C for 20 min. Total RNA was extracted using the RNAprep

pure cell/bacterium kit (Tiangen Biotech Co., Ltd., Beijing, China). RNA quality was analyzed by 1% agarose gel electrophoresis and concentration was determined using a microplate reader (BioTek). Total RNA (1 μg) was reverse transcribed using a PrimeScript RT Reagent Kit with gDNA Eraser (Takara, Japan). Genomic DNA was extracted for 5 min at 42 °C by DNase digestion. Real-time RT-PCR was carried out using the primers listed in Table S2 with an SYBR Premix Ex Taq GC kit (Takara, Japan) and approximately 100 ng template cDNA. All procedures were performed according to the manufacturer's instructions. PCR was performed using the CFX96 real-time system (Bio-Rad, Hercules, CA, USA) with the following conditions: 95 °C for 10 min, then 40 cycles of 95 °C for 10 s, 60 °C for 10 s, and 72 °C for 30 s.

Antibiotic resistance assays

Seed cultures of *S. erythraea* NRRL2338, Δ*mtrA*, E3, and E3::*mtrA* (300 μL) were grown in a 250-ml flask containing 30 ml of tryptone soya broth at 30 °C and 200 rpm for 48 h, then their OD_{600} values (A) were measured. The OD_{600} values were measured again (B) after treatment with 30 μg/mL thiostrepton and 50 μg/mL vancomycin for 24 h. The survival percentage was calculated as follows:

$$\text{Survival percentage}\ (\%) = \frac{B}{A} \times 100.$$

Osmosis assays and $DiOC_2(3)$ fluorescent probe assays

Seed cultures of *S. erythraea* NRRL2338 and E3 (300 μL) were grown in a 250-ml flask containing 30 ml of tryptone soya broth at 30 °C and 200 rpm for 48 h. NaCl solution was then added to increase the osmotic pressure, resulting in a final concentration of 1 M NaCl. Cells were collected after 0 min, 90 min, and 180 min and the total RNA was extracted. The relative transcript levels of the target gene *mepA* (SACE_5687; Protein ID: CAM04873.1) were detected according to the "RNA extraction and real-time RT-PCR" method described previously.

Seed cultures of *S. erythraea* NRRL2338, Δ*mtrA*, E3, and E3::*mtrA* (300 μL) were grown in a 250-ml flask containing 30 ml of tryptone soya broth at 30 °C and 200 rpm for 48 h. A total of 1 mL of the cultures was transferred to sterile 50 mL tubes, then 10 μL $DiOC_2(3)$ fluorescent probe (3 mM) was added to the tubes and incubated at 37 °C and 200 rpm for 30 min. Cells were collected by centrifugation at 4000×*g*, washed twice with 500 μL PBS, and flow cytometry was carried out. Data were obtained using a BD FACSCalibur Flow Cytometer with a 488 nm argon ion gas laser and an FITC channel and were analyzed using FlowJo software.

Results

Wild type MtrA decreases erythromycin levels in high-producing S. erythraea with mutant MtrA

Previously, we revealed that the MtrA protein of high erythromycin-producing *S. erythraea* E3 had two amino acids missing (Li et al. 2013). In this study, we confirmed the deletion of 6 bp DNA (translated as H197 and V198) from MtrA ($MtrA^{del}$) in *S. erythraea* E3 by sequence alignment after gene sequencing (Fig. 1a, b). To investigate the effect of the $MtrA^{del}$ mutant on erythromycin biosynthesis, the wild type *mtrA* from *S. erythraea* NRRL2338 was transferred into *S. erythraea* E3 with the *mtrA* mutant, producing the *S. erythraea* E3::*mtrA* strain. As a control, the empty plasmid vector PIB139 was transferred into *S. erythraea* E3 with the *mtrA* mutant. The erythromycin levels produced by *S. erythraea* NRRL2338, E3, and E3::*mtrA* were measured by HPLC. The standard curve equation was as follows: $Y = 508281X - 120278$ (Y was the peak area, X was the erythromycin levels). The linear correlation coefficient R^2 was 0.9914. The mean erythromycin levels produced by *S. erythraea* NRRL2338, E3, E3::PIB139, and E3::*mtrA* were 7.00 mg/L, 296.50 mg/L, 282.95 mg/L, and 151.90 mg/L, respectively, when calculated using the standard curve (Fig. 1c). The erythromycin level of *S. erythraea* E3::*mtrA* was approximately 50% lower than that of *S. erythraea* E3, while the effect of the PIB139 plasmid on erythromycin level was shown to be negligible. The erythromycin level of *S. erythraea* E3::pSET152-*mtrA* was approximately 50% lower than that of *S. erythraea* E3 which was similar to that of *S. erythraea* E3::*mtrA* (Fig. S2). These results suggest that the wild type *mtrA* gene significantly decreased the erythromycin level of high-producing *S. erythraea* with the *mtrA* mutant.

The wild type *mtrA* of *S. erythraea* NRRL2338 was deleted to produce the *S. erythraea* NRRL2338 Δ*mtrA* strain. The level of erythromycin produced by *S. erythraea* NRRL2338 Δ*mtrA* was 11.25 mg/L (Fig. 1d), an increase of approximately 60% compared with *S. erythraea* NRRL2338 (7.00 mg/L), indicating that MtrA may be involved in erythromycin biosynthesis.

mepA is an MtrA target gene in S. erythraea

Several MtrA target genes, such as *dnaA*, *oriC*, and *fbpB* in *M. tuberculosis* (Fol et al. 2006; Rajagopalan et al. 2010) and *mepA*, *nlpC*, *betP*, and *proP* in *C. glutamicum* (Brocker and Bott 2006), have been reported previously. Furthermore, the 9-bp "GTCACAgcg-like" repeat has been identified as the MtrA-binding motif in *M. tuberculosis* (Rajagopalan et al. 2010). To identify the target genes of MtrA in *S. erythraea*, we used the "GTCACAgcg-like" MtrA-binding motif to screen the sequences upstream (+ 50 to − 300) of putative

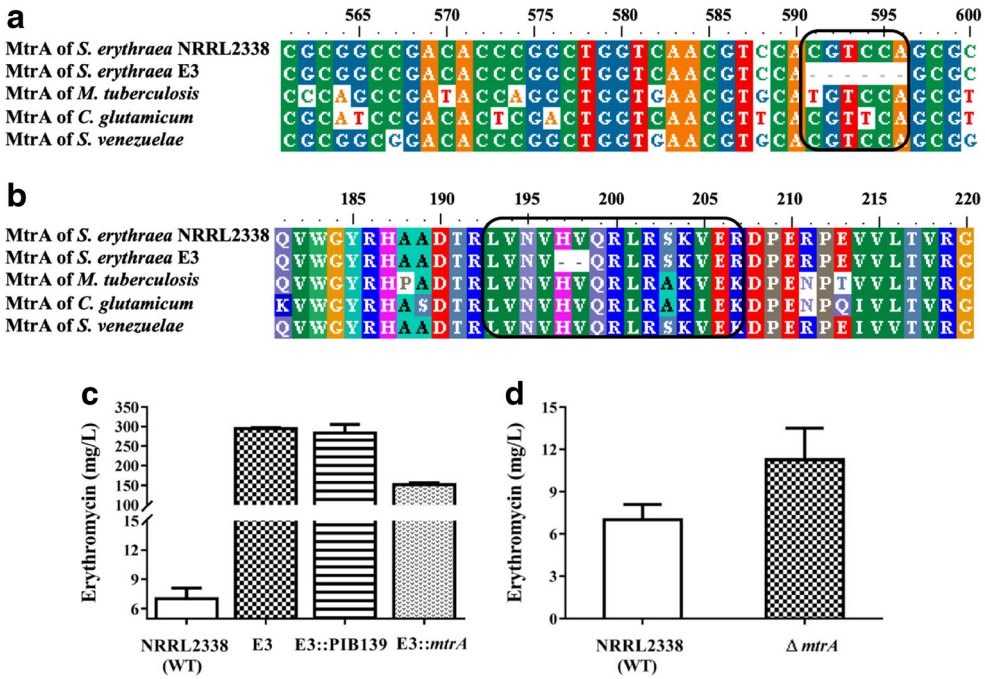

Fig. 1 Wild type MtrA decreases erythromycin levels in high-producing *Saccharopolyspora erythraea* with mutant MtrA. **a** Multiple DNA sequence alignment of *mtrA* genes from *S. erythraea* NRRL2338 and E3, *Mycobacterium tuberculosis*, *Corynebacterium glutamicum*, and *S. venezuelae*. The black rectangle indicates the 6-bp (591-596 sites) deletion in *mtrA*. **b** The amino acids H197 and V198 and the DNA-binding helix are indicated by the black rectangle. **c** The mean erythromycin yields of *S. erythraea* NRRL2338, E3, E3::PIB139, and E3::*mtrA* were 7.00 mg/L, 296.50 mg/L, 282.95 mg/L, and 151.90 mg/L, respectively. **d** Erythromycin yields of *S. erythraea* NRRL2338 and Δ*mtrA*. The mean erythromycin level of *S. erythraea* NRRL2338 Δ*mtrA* was 11.25 mg/L

MtrA target genes in *S. erythraea* NRRL23338 (Table 1). *mepA* is one such predicted target gene, encoding a membrane protein related to metalloendopeptidases whose target could be cell wall peptides. The MepA protein may play a role in cell wall metabolism, cell shape phenotype, antibiotic susceptibility, and osmoprotection (Papandreou et al. 2005; Stock et al. 2000). We hypothesized that MepA might improve erythromycin transport in high erythromycin-producing strains. Two "GTCACAgcg-like" motifs (GTCACGGTTCGGTCACGAGC) were located 76-bp upstream of the translational start site (− 77 to − 96) of the *mepA* gene (SACE_5687) (Fig. 2a). To validate this target gene, EMSA (Fig. 2b and Fig. S3) was performed with 1.8 μM MtrA, an unlabeled specific probe (200-fold), and a non-specific competitor DNA (200-fold, sonicated salmon sperm DNA) as controls (Fig. 2b). A range of purified MtrA protein concentrations (0.6 μM, 1.8 μM, and 3 μM) were investigated, with the results indicating that MtrA can bind directly to the upstream region containing the predicted motifs and suggesting that *mepA* might be an MtrA target gene in *S. erythraea*.

To further investigate the effect of MtrA on erythromycin production, we determined the transcriptional levels of SACE_0721, SACE_0723, and SACE_0724 of the ery cluster involved in erythromycin biosynthesis in *S. erythraea* NRRL2338 (WT), NRRL2338 Δ*mtrA*, E3, and E3::*mtrA* (Fig. S4). The results indicated that MtrA has a significant effect on erythromycin biosynthesis. To identify direct MtrA targets that might be involved in erythromycin biosynthesis, ChIP sequencing was performed using MtrA-3 x Flag. SACE_0438, SACE_3737 (*ama2*), SACE_4140, SACE_5429 (*coaE*), SACE_6295 (*rho1*), and SACE_6447 (*mtrA*) were identified as MtrA target genes, but not

Table 1 Target genes predicted using the search tool MEME

Predicted target genes	Function
SACE_0047 (*ftsW2*)	Putative cell division protein
SACE_2125	Putative membrane protein
SACE_2312 (*tnp9B*)	Transposase, IS4 family
SACE_2544 (*proP*)	Proline/betaine transporter
SACE_3525	Cytochrome P450 CYP125
SACE_3737	Putative alpha-mannosidase
SACE_5044	Transport system ATP-binding protein
SACE_5687 (*mepA*)	Membrane proteins related to metalloendopeptidases
SACE_6295	Putative transcription termination factor Rho
SACE_6447 (*mtrA*)	Response regulator MtrA

The motif matches shown have a position *p* value of less than 0.0001

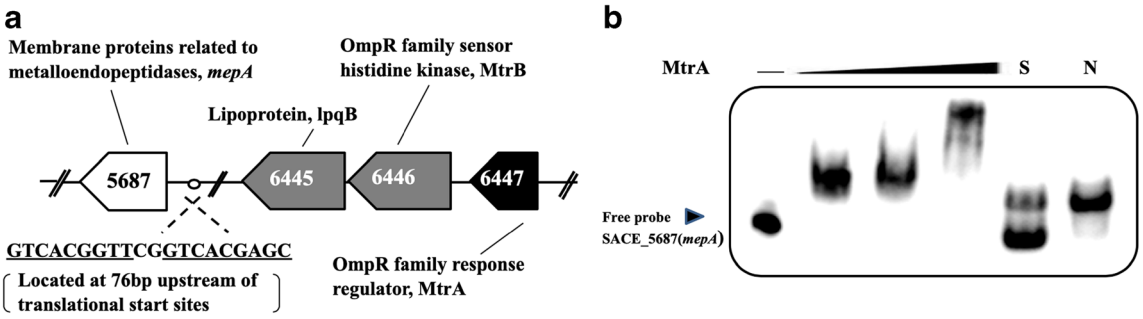

Fig. 2 MtrA directly regulates the *mepA* gene in *Saccharopolyspora erythraea*. **a** Genetic structure of the *mepA* gene in *S. erythraea* and its predicted MtrA-binding site. **b** Electrophoretic mobility shift assays of a range of pure *S. erythraea* MtrA protein concentrations (0.6 μM, 1.8 μM, and 3 μM) with biotin-labeled PCR products. The concentration of PCR products was 2 nM. S represents the unlabeled specific probe (200-fold) and N represents the non-specific competitor DNA (200-fold; sonicated salmon sperm DNA) used as controls, and the corresponding MtrA concentration was 1.8 μM

SACE_5687 (*mepA*) (Fig. S5 and Table S4). No proteins directly regulating erythromycin biosynthesis were found.

Deletion of two amino acids in MtrA (MtrA^{del}) results in loss of MtrA DNA-binding activity

MtrA, a response regulator of the OmpR family, contains a conserved DNA recognition helix (HTH domain) (Martinez-Hackert and Stock 1997). The deletion of two amino acids (H197 and V198) in the DNA recognition helix of the C-terminal domain altered the α-helical structure of the DNA recognition helix as predicted (Fig. 3a, b).

To confirm the effect of the deletion of these two amino acids in MtrA, EMSAs were performed on purified *S. erythraea* MtrA and MtrA^{del} proteins with biotin-labeled *mepA* (Fig. 3c). The concentration of the PCR products was 2 nM. A range of MtrA^{del} protein concentrations (0.6 μM, 1.8 μM, 3 μM, 4.2 μM, and 5 μM) were used. As shown in Fig. 3c, the MtrA^{del} protein could not bind the region upstream of *mepA*, suggesting that the deletion of the two amino acids in MtrA (MtrA^{del}) resulted in the loss of MtrA DNA-binding activity by altering the structure of the DNA recognition helix.

Introducing mtrA into S. erythraea with mtrA^{del} represses mepA transcription

To determine the regulatory effects of MtrA on *mepA* in *S. erythraea*, the transcriptional levels of *mepA* in *S. erythraea* NRRL2338, Δ*mtrA*, E3, E3::PIB139, and E3::*mtrA* were measured using real-time RT-PCR with the internal 16S rRNA gene as a control. Total RNA was extracted from the samples collected at 48 h. In *S. erythraea* Δ*mtrA*, E3, and E3::*mtrA* the transcription of *mepA* increased by approximately 3.5-fold, 2-fold, and 1.5-fold, respectively, compared with *S. erythraea* NRRL2338. Furthermore, deleting *mtrA* in the *S. erythraea* NRRL2338 strain promoted the transcription of *mepA* (Fig. 4 and Fig. S6), suggesting that MtrA is a transcriptional repressor of *mepA*.

Effects of MtrA on antibiotic resistance

In order to understand the effects of MtrA on antibiotic resistance, we tested the susceptibility of *S. erythraea* NRRL2338, Δ*mtrA*, E3, E3::PIB139, and E3::*mtrA* to thiostrepton and vancomycin (Fig. 5 and Fig. S7). Vancomycin inhibits transpeptidation by inhibiting transpeptidases and binding to the terminal D-Ala-D-Ala residues of non-cross-linked peptides in the peptidoglycan network and in lipid II (Hubbard and Walsh 2003). Thiostrepton prevents protein synthesis on procaryotic ribosomes by inhibiting the GTPase activation of elongation factor. Thus, both antibiotics are related to the cell wall. When treated with thiostrepton (30 μg/mL), the survival percentage of *S. erythraea* NRRL2338, Δ*mtrA*, and E3 was approximately 63%, 26%, and 39% at 24 h, respectively. When treated with vancomycin (50 μg/mL), the survival percentage of *S. erythraea* NRRL2338, Δ*mtrA*, and E3 was approximately 83%, 47%, and 68% at 24 h, respectively. *S. erythraea* Δ*mtrA* and E3 were both more susceptible to thiostrepton and vancomycin than *S. erythraea* NRRL2338. *S. erythraea* E3::*mtrA* was slightly more resistant to thiostrepton and vancomycin than *S. erythraea* E3 (Fig. 5 and Fig. S7), and its antibiotic resistance was more similar to that of *S. erythraea* NRRL2338. The effects of the plasmid were shown to be negligible. These results demonstrate that MtrA has a clear effect on antibiotic resistance in *S. erythraea* NRRL2338.

Effects of MtrA on cell osmoprotection

To investigate the effects of MtrA on osmoprotection, *S. erythraea* NRRL2338 was subjected to high osmotic pressure. As shown in Fig. 6a, the transcriptional level of *mepA* increased by approximately 2-fold at 90 min compared to 0 min and was then recovered at 180 min, suggesting that *mepA* transcription responds to osmotic changes in *S. erythraea* NRRL2338. However, the transcriptional level of *mepA* in *S. erythraea* E3 showed no obvious changes due to

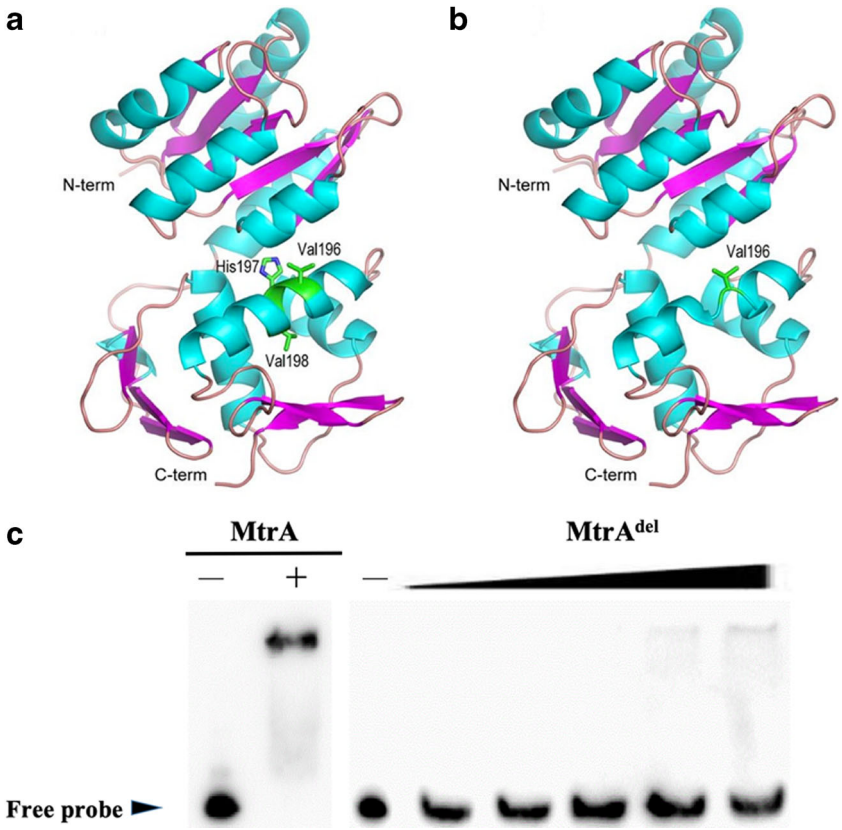

Fig. 3 Deletion of two amino acids in MtrA (MtrA^{del}) results in loss of MtrA DNA-binding activity. **a** The structure of MtrA from *Saccharopolyspora erythraea* NRRL2338 was predicted based on the crystal structure of Rv3246c in *Mycobacterium tuberculosis* (PDB: 2gwr). **b** The deletion of His197 and Val198 disrupts the α-helix of the DNA-binding region. **c** Electrophoretic mobility shift assays of pure *S. erythraea* MtrA and MtrA^{del} proteins with biotin-labeled *mepA*. The concentration of PCR products was 2 nM, the concentration of MtrA was 3 μM, and a series of MtrA^{del} concentrations were used (0.6 μM, 1.8 μM, 3 μM, 4.2 μM, and 5 μM)

high osmotic pressure (Fig. 6b), indicating that *S. erythraea* E3 had lost the MtrA-mediated regulation of *mepA* in response to osmotic stress. These results were consistent with the loss of *mepA* DNA-binding activity in MtrA^{del}.

To a certain extent, osmosis represents the transport of solvent molecules. To determine whether MtrA was involved in diffusion and transport, $DiOC_2(3)$ fluorescent probe assays were performed. $DiOC_2(3)$ is a lipophilic fluorescent dye used to label cell membranes as its fluorescence intensity increases when bound to the cell membrane,. The fluorescence intensity of *S. erythraea* Δ*mtrA* and E3 was stronger than that of *S. erythraea* NRRL2338 (Fig. 6c, d), indicating that more $DiOC_2(3)$ fluorescent probes were bound to the cell membrane. The fluorescence intensity of *S. erythraea* E3::*mtrA* was weaker than of *S. erythraea* E3, and similar to the intensity of *S. erythraea* NRRL2338. The effects of the plasmid were found to be negligible (Fig. S8). MtrA may therefore affect diffusion and transport, and thereby improve the transport of erythromycin in high erythromycin-producing strains.

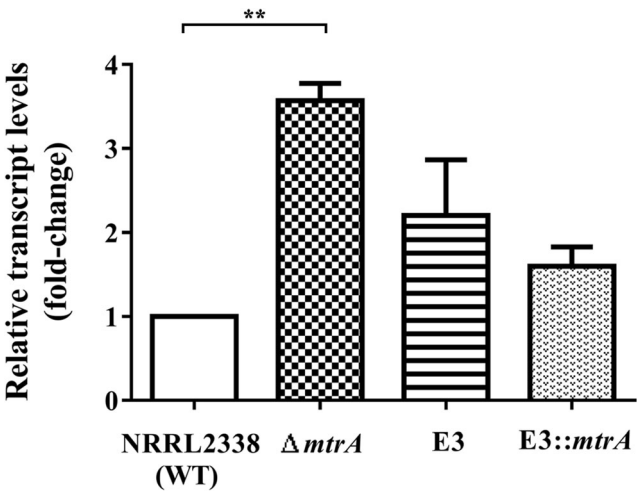

Fig. 4 *mepA* transcript levels in *Saccharopolyspora erythraea* strains *mepA* transcript levels in *S. erythraea* Δ*mtrA*, E3, and E3::*mtrA* were increased by approximately 3.5-fold, 2-fold, and 1.5-fold compared with *S. erythraea* NRRL2338, respectively. The fold change for *mepA* expression levels in *S. erythraea* NRRL2338 was set to 1.0. Error bars indicate the SD of three independent experiments

Discussion

MtrAB is one of the major two-component signal transduction pathways through which *Actinobacteria* sense and respond to their environment in order to survive. MtrAB is involved in cell cycle progression, cell morphology, antibiotic resistance, and osmoprotection (Fol et al. 2006; Li et al. 2010; Moker et al. 2007; Nguyen et al. 2010). Previously, we found that MtrA had a two amino acid (H197 and V198) deletion in erythromycin-overproducing *S. erythraea* E3 compared with low-producing *S. erythraea* NRRL2338, indicating that MtrA might be associated with erythromycin production (Li et al.

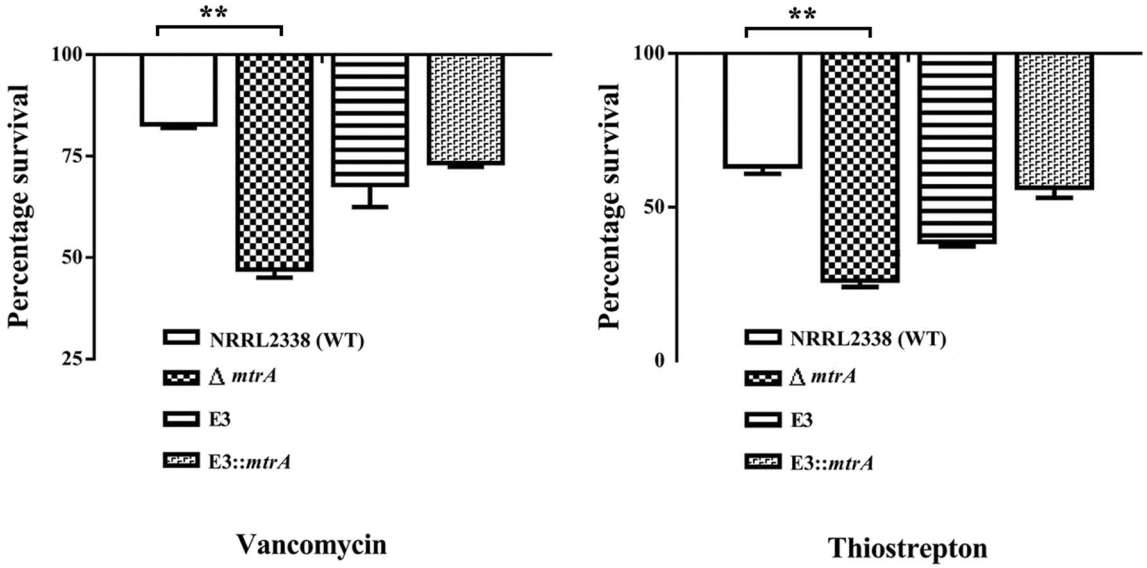

Fig. 5 Effects of MtrA on antibiotic resistance percentage survival of *Saccharopolyspora erythraea* NRRL2338, Δ*mtrA*, E3, and E3::*mtrA* after treatment with 30 μg/mL thiostrepton and 50 μg/mL vancomycin for 24 h. Error bars indicate the SD of three independent experiments

2013). In this study, we investigated the effects of *mtrA* deletion and the deletion of the two MtrA amino acids on erythromycin production, antibiotic susceptibility, osmoprotection, and transport of substances. The MtrA^{del} mutant and Δ*mtrA* affected various physiological functions, enhancing antibiotic resistance, deregulating osmoprotection, and improving the transport of substances by reversing the inhibition of *mepA* expression by MtrA. The *mepA* gene is predicted to encode a protein with an extracytoplasmic metallopeptidase domain belonging to the M23B family (Rawlings et al. 2002), which could target cell wall peptides (Moker et al. 2004). An obvious relationship between MtrA and the expression of the *ery* cluster has been observed in *S. erythraea*. However, the molecular mechanisms underlying the effect of MtrA on erythromycin production remains unknown. MtrA has been reported to directly regulate the production of secondary metabolites in

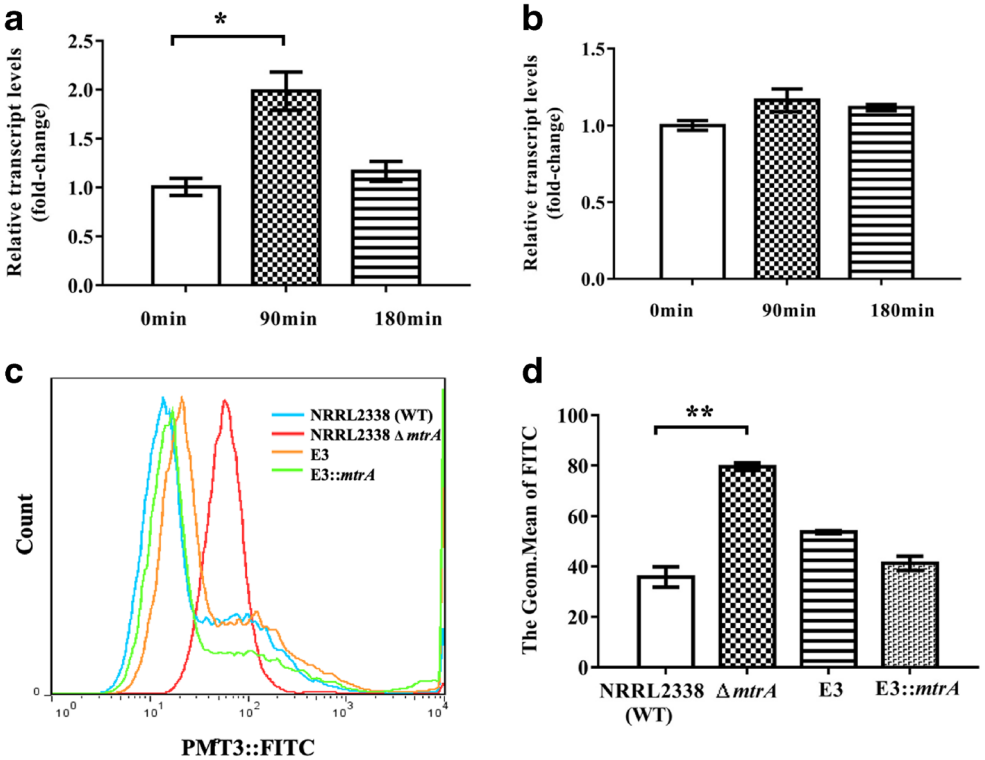

Fig. 6 Effects of MtrA on cell osmoprotection. **a** *mepA* transcription levels in *Saccharopolyspora erythraea* NRRL2338 at 0 min, 90 min, and 180 min after increased osmotic pressure, normalized using the internal 16S rRNA gene. The fold change in *mepA* expression level at 0 min was set to 1.0. Error bars indicate the SD of three independent experiments. **b** *mepA* transcription levels in *S. erythraea* E3 were measured at 0 min, 90 min, and 180 min after increased osmotic pressure, normalized using the internal 16S rRNA gene. **c** Representative measurements of the $DiOC_2(3)$ fluorescent probe absorbed by *S. erythraea* NRRL2338, Δ*mtrA*, E3, and E3::*mtrA* cells obtained using BD FACSCalibur. **d** The FITC geometric means were produced using FlowJo software. Error bars indicate the SD of three independent experiments

Streptomyces species (Som et al. 2017b), and can bind to sites spanning more than 70% of the biosynthetic gene clusters in *S. coelicolor* and *S. venezuelae* to activate antibiotic production. For example, in *S. venezuelae* MtrA can bind to the intergenic region between the *cmlF* and *cmlN* genes, activating the expression of *cmlN* which encodes an efflux permease to transport chloramphenicol (Fernandez-Martinez et al. 2014). These observations demonstrate that MtrA plays an important role in the biosynthesis of secondary metabolites in *Streptomyces* (Som et al. 2017a). However, there is no evidence that MtrA directly regulates the expression of the erythromycin biosynthesis gene cluster in *S. erythraea*, and exactly how $MtrA^{del}$ increases erythromycin production remains to be elucidated.

In summary, our results demonstrated that MtrA is an important pleiotropic regulator of secondary metabolism, antibiotic susceptibility, and osmoprotection in *S. erythraea*. Inactivation of MtrA affected various physiological functions, increasing erythromycin biosynthesis, enhancing antibiotic resistance, deregulating osmoprotection, and improving transport of substances. Future studies linking the molecular effects of MtrA to these phenotypes will improve our understanding of the MtrA-MtrB two-component regulatory system in *Actinobacteria*.

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Compliance with ethical standards

This article does not contain any studies with human participants or animals performed by any of the authors.

Conflict of interest The authors declare that they have no conflict of interest.

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