The Possible Role of *SCO3388*, a *tmrB*-like Gene of *Streptomyces coelicolor*, in Germination and Stress Survival of Spores

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Abstract The *SCO3388* gene from *Streptomyces coelicolor* is homologous to *tmrB*, the tunicamycin resistance gene of *Bacillus subtilis*. The *SCO3388*-inactivation strain (SY-tbl-1) was generated by replacing *SCO3388* with thiostrepton resistance gene. Spores of *S. coelicolor* derivatives were prepared on mannitol-soy flour (MS) agar on which SY-tbl-1 displayed no significant defect in growth and development. When plated on R4 agar, spores of SY-tbl-1 displayed retardation in growth and sporulation, whereas its mycelium gave rise to normal growth. Thus, *SCO3388* is suggested to be involved in the dormant spore germination. Expression of *SCO3388* under the *ermE1* promoter restored but only partially the ability to sporulate in SY-tbl-1. Neither SY-tbl-1 nor SY-tbl-1/*ermE1p-SCO3388* showed a difference in tunicamycin resistance to the wild type whereas, interestingly, the introduction of *ermE1p-SCO3388* dramatically enhanced spore survival to heat and detergent treatments, suggesting that *SCO3388* might play a role in the maintenance of spore cell wall integrity.

Keywords *SCO3388* gene · spore germination · *Streptomyces coelicolor* · stress survival of spores · *tmrB*-like

Introduction

Tunicamycin is a mixture of nucleoside antibiotics produced from some *Streptomyces* strains (Takatsuki et al., 1971; Kenig and Reading, 1979). It targets peptidoglycan biosynthesis at the step of UDP-*N*-acetyl-D-hexosamine:polyprenyl-phosphate *N*-acetylhexosamine-1-phosphate translocase (Billot-Klein et al., 1997). Tunicamycin is also well known for its inhibitory activity on protein *N*-

glycosylation and is widely used as an endoplasmic reticulum stress-inducer in eukaryotic cell biology. As an antibiotic, tunicamycin was found to be most effective against *Bacillus subtilis* (Takatsuki et al., 1971). A tunicamycin-resistant mutant of *B. subtilis* harbored multiple copies of *tmrB*, which was thus assigned as tunicamycin resistance gene (Hashiguchi et al., 1986). It was later proposed that TmrB protein binds tunicamycin and is localized to cytoplasmic membrane (Noda et al., 1992; 1995). However, *B. subtilis* is sensitive to tunicamycin although it harbors a copy of *tmrB* on its chromosome. In addition, the tunicamycin biosynthetic gene cluster of *Streptomyces* spp. has no *tmrB* homologue (Wyszynski et al., 2010; Karki et al., 2011). Thus, TmrB may be involved in peptidoglycan biogenesis, which is a known target of tunicamycin. High levels of TmrB may confer tunicamycin resistance by potentiating cell wall (CW) metabolism and by enhancing CW integrity.

Homologues of *tmrB*, annotated as putative tunicamycin resistance genes or *tmrB*-like genes, can be found in genome sequences of many Gram-positive bacteria but this annotation is only given because TmrB-like proteins share no significant homology with any other proteins whose physiological functions are defined. As aforementioned, the role of TmrB in the physiology of *B. subtilis*, other than in tunicamycin resistance, is unknown. It is thus necessary to examine whether the *tmrB* homologues are related to tunicamycin resistance or have other roles in the cellular physiology of Gram-positive bacteria.

Streptomyces undergo morphological development, and each developmental stage adopts a distinctive cell type: substrate mycelium, aerial mycelium, and spore. This feature makes *Streptomyces* a model system in studying the regulatory network controlling bacterial cell differentiation. The *SCO3388* protein of *S. coelicolor* displays 38.9% identity to TmrB with 203-amino acid overlap and is annotated as a TmrB-like protein (Supplementary data Fig. 1). *SCO3388* is downstream of genes encoding a two-component system (*SCO3389* and *SCO3390*). Transcription of *SCO3388* is likely under the control of this two-component system. Among 29 analyzed genome sequences of

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Streptomyces spp., 9 have homologues of *SCO3388* with at least 64% identity in the deduced amino acid sequences. In this study, *SCO3388* was inactivated through gene-replacement and the phenotype of the resulting mutant was examined in terms of morphological differentiation, tunicamycin resistance, and stress survival of spores. Our study demonstrates that *SCO3388* is involved in germination and stress survival of spores but not in tunicamycin resistance.

Materials and Methods

Strains, culture conditions, and genetic procedure. *Streptomyces coelicolor* M145 was used in this study. *S. coelicolor* strains were grown at 28°C on R4 agar, which contains 10 g of glucose, 1 g of yeast extract, 0.1 g of casamino acids, 3 g of L-proline, 10 g of MgCl₂·6H₂O, 4 g of CaCl₂·2H₂O, 0.2 g of K₂SO₄, 5.6 g of TES (*N*-tris [hydroxymethyl] methyl-2-aminoethanesulfonic acid), 1 mL of trace element solution and 20 g of agar per liter (pH 7.2) or MS (mannitol-soy flour) agar, which contains 20 g of mannitol, 20 g of soya flour, and 20 g of agar per liter. For liquid culture, tryptic soy broth (TSB) medium was used. TSB contains 17 g of casein, 3 g of soybean, 2.5 g of D-dextrose, 5 g of NaCl, and 2.5 g of K₂HPO₄ per liter (pH 7.3). *S. coelicolor* and its derivatives were cultivated in TSB medium with 0.5% glycine and 5 mM MgCl₂·6H₂O for total DNA isolation. Total DNA was isolated by using bacterial genomic DNA isolation kit (Core-one™, CoreBio System, Korea). For spore collection, TSB culture was spread on MS agar plates and incubated at 28°C. After 5 days, spores were harvested with moistening sterile cotton soaked in sterile water. Five milliliter of sterile water was applied per one plate, wiping the surface of plates. Each stock was stored in 25% aqueous glycerol at -70°C. *Escherichia coli* DH5α strain was used for routine cloning and was grown at 37°C in Luria-Bertani medium. Ampicillin, apramycin or thiostrepton was added when necessary at the final concentration of 50 µg/mL except that thiostrepton was applied at 5 µg/mL in the liquid culture. Intergeneric conjugal transfer was conducted to introduce DNA into *S. coelicolor* by using *E. coli* ET12567/pUZ8002 as the donor strain (Paget et al., 1999). Germinated spore was used for recipient cell and conjugal transfer was performed on AS-1 agar as previously described (Bierman et al., 1992).

Inactivation and expression of *SCO3388* in *S. coelicolor*. The *SCO3388* inactivation plasmid pSY1101 was constructed by inserting thiostrepton resistance gene (*tsr*) between DNA fragments flanking *SCO3388* (nt 126,887 to 127,528), which were prepared by polymerase chain reaction (PCR) amplification from chromosomal DNA. The nucleotide numbers (nt) in this study are based on the sequence deposited in GenBank accession number AL939116. The oligonucleotide primers are listed in Table 1. The 2.0-kb upstream region (nt 127,478 to 129,540) was amplified by polymerase chain reaction (PCR) with the primer pair of d3388-1 and d3388-2. The 2.0-kb downstream region (nt 124,801 to 126,960) was amplified by PCR with the primer pair of d3388-3

Table 1 Primers used in this study

Pimer	Sequence*
d3388-1	5'- <u>ATTCTAGAG</u> GGTTACGTGCAGCGCCTCTA-3'
d3388-2	5'- <u>ATTAAAGCTT</u> GCGGGCAGCGACTGGATGGA-3'
d3388-3	5'- <u>ATTGAATTCC</u> GAACAACGAGGGCGCCCTG-3'
d3388-4	5'- <u>ATTACTAGT</u> TCTCGTTCTACAAGTCCCTC-3'.
3388f	5'- <u>ATTTCGTC</u> GGTAGGGTCTGCGCA-3'
3388r	5'- <u>ATTACTTCT</u> CGAGGGGATACTC-3'
3388ex-f	5'- <u>ATTAAAGCTT</u> ATGTCCAACGGCGAGATCGC-3'
3388ex-r	5'- <u>ATTCTAGAT</u> ACCTGGCCCGCAGCGCG-3'

*5' unmatched sequences and engineered restriction sites are underlined

and d3388-4. The upstream fragment was ligated into the *Xba*I/*Hind*III sites of pHJK-3-14C (Kim et al., 2008). To the resulting plasmid, the downstream fragment was ligated into the *Eco*RI/*Spe*I sites. The insert of the resulting plasmid was rescued as *Xba*I/*Spe*I fragment and ligated into the *Xba*I site of pOJ260 (Bierman et al., 1992). The resulting plasmid pSY1101 was introduced into *S. coelicolor* M145 by intergeneric conjugation. The double cross-over mutant of SY-tbl-1 (Δ *SCO3388::tsr*) was selected by thiostrepton-resistant and apramycin-sensitive phenotype. Inactivation of *SCO3388* in SY-tbl-1 was confirmed by PCR with the primer pairs of 3388f and 3388r (nt 126,819 to 127,555).

For the expression construct, *SCO3388* was amplified by PCR with the primer pairs of 3388ex-f and 3388ex-r (nt 126,641 to 127,750). The PCR product was ligated into the *Xba*I/*Hind*III sites of the pLitmus28 to construct pSCOTBL2. The *Spe*I/*Xba*I fragment of pSCOTBL2 was then ligated into *Xba*I site of pSET152 (Bierman et al., 1992) harboring the *ermE* promoter (*ermE**p). Restriction digestion analysis was used to select the clone harbor *SCO3388* at the transcription direction of *ermE*1p to generate pSCO3388. A 270-bp *ermE* promoter region was amplified by PCR with engineered restriction sites of *Xba*I and *Hind*III at the transcriptional directions of *ermE*1p and *ermE*2p, respectively (Bibb et al., 1985). This divergent promoter fragment was cloned in *Xba*I and *Hind*III sites of pSET152.

Sodium dodecylsulfate (SDS) treatment and heat shock of spore. The concentrations of the spore suspensions were adjusted to be 5.0×10^8 /mL. The spore suspensions were diluted by 10^5 -fold with sterile water and exposed to 5% SDS for 5 h at ambient temperature. For the heat treatment, the diluted spores were incubated at 60°C for 10 min. The treated spores were spread on MS agar plates and were incubated at 28°C for 4 days to count colony forming unit (CFU). Survival rates were calculated by dividing the number of colonies on plates by the number of colonies generated from the spore sample without any treatment.

Scanning and transmission electron microscope (SEM and TEM) examination. The hyphase of *S. lividans* WT and SY-tbl-1 strain were examined under SEM. Blocks of the R4 agar cultures, which were incubated at 28°C for 7 days, were fixed with 1% osmium tetroxide for 12 h and then dehydrated by freeze-

drying. Each specimen was sputter coated with platinum-gold and examined under a Hitachi S4000 SEM.

TEM was used to observe spores of *S. lividans* derivatives. Spore pellets were fixed at 4°C for 4 h in 2% paraformaldehyde and 2% glutaraldehyde in 50 mM sodium cacodylate buffer (pH 7.2) and were then rinsed three times (at 4°C for 10 min) with the same buffer. The pellets were post-fixed at 4°C for 2 h with 1% osmium tetroxide OsO_4 in the same buffer and washed two times with distilled water. An en bloc staining was done at 4°C for 28 min with 0.5% uranyl acetate. Specimens were dehydrated through a graded ethanol solution, transferred to propylene oxide, embedded in Spur's resin and polymerized at 70°C for 24 h. Ultrathin sections were obtained with MT-X ultramicrotome (RMC, USA). To improve stain, the sections were stained with 2% uranyl acetate for 7 min and Reynolds' lead citrate for 7 min. The sections were examined using LIBRA 120 TEM (Carl Zeiss, Germany).

Results

Inactivation of *SCO3388* resulted in defective spore development. The *SCO3388*-inactivation strain (SY-tbl-1) was generated by replacing *SCO3388* with a thiostrepton resistance gene (*tsr*) (Fig. 1A). The *SCO3388*-inactivation construct pSY1101 was introduced into *S. coelicolor* by intergeneric conjugation; exconjugants were selected with thiostrepton resistance. pSY1101 is a derivative of pOJ260, which is incapable of replicating in *Streptomyces* (Bierman et al., 1992). The thiostrepton-resistant exconjugants were then tested for apramycin resistance and a double crossover mutant of SY-tbl-1 with the thiostrepton-resistant/apramycin-sensitive phenotype was selected. Exconjugants displaying the resistance to both thiostrepton and apramycin are single crossover mutants. PCR amplification was used to confirm the genotype of the mutants (Fig. 1B). PCR of *S. coelicolor* M145 wild-type strain (WT) resulted in a 0.74-kb band (lane 1), which was replaced with a 1.2-kb fragment in SY-tbl-1 (lane 2). Both the 0.74-kb and 1.2-kb fragments were found in the single crossover mutant (lane 3). This result substantiated that *SCO3388* was successfully knocked out in SY-tbl-1. Southern hybridization analysis further verified that *SCO3388* was eliminated in SY-tbl-1 (Supplementary data Fig. 2). Morphological comparison of mycelia, obtained from TSB liquid cultures of WT and SY-tbl-1, indicated that there was no significant difference when spread on R4 agar plates (Fig. 1C; -m). Spores of SY-tbl-1 were obtained on MS agar. When plated on R4 medium, these spores displayed a significant defect in growth (Fig. 1C; -s). SEM examination showed that SY-tbl-1 produced aerial hyphae thinner than WT (Supplementary data Fig. 3). It was found that SY-tbl-1 displayed a significantly lower level of growth than WT on R4 agar while its growth level was measured higher than WT on MS agar (Fig. 1D).

Gene complementation partially restored sporulation to SY-tbl-1. *SCO3388* was cloned in pSET152 under the control of

Fig. 1 Gene inactivation of *SCO3388* by double crossover-mediated gene replacement and the visual examination of SY-tbl-1. (A) Schematic representation of the homologous recombination that generates SY-tbl-1 and the restriction map of the gene-inactivation plasmid pSY1101 showing the predicted PCR product sizes with primer pair 3388f-3388r from WT and SY-tbl-1 strains. (B) Electrophoretic analysis of the PCR performed with the chromosomes of WT (1), dSCO3388 (2), and a single crossover mutant (3). Lane M indicates the DNA molecular weight marker with the size indication bars at the relevant fragments. (C) Visual examination of growth of WT and SY-tbl-1. Mycelia (-m), which were obtained from TSB liquid culture, or Spores (-s) were overlaid on R4 agar and incubated at 28°C for 4 days. (D) Growth of WT (filled circles) and SY-tbl-1 (open circles) on R4 (1) and MS (2) agar. The agar media were covered with autoclaved cellophane disks and then overlaid with 10^7 of spores. The cultures were incubated at 28°C and the mycelia were harvested with distilled water at the incubation time indicated. The mycelia were collected by centrifugation and dried at 60°C for 2 days before the dried cell weights were measured.

emrE1p. The resulting construct named pSCO3388 was introduced into SY-tbl-1. Morphology examination was done on either R4 or MS agar by applying approximately 500 spores on each plate. Introduction of pSCO3388 into SY-tbl-1 partially restored the ability to sporulate on R4 agar (Fig. 2A). The partial complementation with pSCO3388 may be due to the usage of an unnatural promoter (*emrE1p*) in *SCO3388* expression. Transformation of SY-tbl-1 with *SCO3388* without *emrE1p* failed to restore the sporulating

Fig. 2 Development of SY-tbl-1 and the gene complementation strain on R4 and MS agar. Time course (5 days, 7 days, and 9 days after culture initiation) examination of WT/pSET152 (1), WT/pSCO3388 (2), SY-tbl-1/pSET152 (3) and SY-tbl-1/pSCO3388 (4) on R4 agar (A) or MS agar (B). Approximately 500 spores were applied on each plate and incubated at 28°C.

Fig. 3 Stress survival test. Experiments were carried out three times; standard errors are provided as error bars.

phenotype (data not shown). In MS agar, SY-tbl-1 showed no defect in sporulation, displaying accelerated growth and a delay in spore pigmentation (Figs 1D and 2B).

Tunicamycin resistance was not affected by *SCO3388*. A preliminary test of tunicamycin resistance indicated that *S. coelicolor* had substantial resistance to tunicamycin and there was no inhibition zone in either WT or SY-tbl-1 when tunicamycin was tested at amounts up to 20 µg zone. Application of 200 µg tunicamycin gave rise to small inhibitions in both strains. To compare the tunicamycin resistance of WT/pSET152, SY-tbl-1/pSET152, and SY-tbl-1/pSCO3388, a paper disk (□, 1 cm) containing 1.2 mg of tunicamycin (Sigma, USA) was applied on a plate which was overlaid with approximately 3×10^7 spores. There was no significant difference in the inhibition zone between strains (Fig. S4), indicating that *SCO3388* is not a determining factor of tunicamycin resistance in *S. coelicolor*.

Expression of *SCO3388* enhanced stress survival. It was previously reported that inactivation of cell wall hydrolases resulted in heat sensitivity, defects in spore formation, delay in spore germination and vegetative growth suggesting a key role of these enzymes in cell wall metabolism throughout *S. coelicolor* life cycle (Haider et al., 2009). To assess the possibility that *SCO3388* was also involved in spore cell wall metabolism, stress survival was tested with spores of the recombinant strains. Following heat shock and 5% SDS treatment, SY-tbl-1/pSCO3388 displayed a dramatic increase in spore survival (Fig. 3), suggesting that the expression of *SCO3388* enhanced spore cell wall integrity. WT/pSCO3388 also displayed a dramatic increase in the heat shock-resistance up to the level observed in SY-tbl-1/pSCO3388 (Fig. 3). Spore survival tests with 2% and 10% SDS showed patterns similar to those seen with 5% SDS (data not shown). These observations suggest that *SCO3388* is playing a role in spore cell wall metabolism and its integrity. This assumption led us to examine spore cell wall structure of SY-tbl-1 with TEM (Fig. 4). The cell wall of SY-tbl-1/pSET152 appeared darker (denser) and thinner than that of the WT/pSET152 and SY-tbl-1/pSCO3388 but the physiological significance of this apparent modification of the cell wall structure is still unknown.

Discussion

Multiple alignment analysis of several TmrB homologues indicates that there are some strictly conserved residues, the most conserved of which are the N-terminal regions (Supplementary data Fig. 1). The N-terminal region contains the characteristic Walker A ATP-binding motif (GXXGXGKT) (Walker et al., 1982), which is not found in *SCO3388*. The possibility that *SCO3388* is a variant TmrB-like protein that lost its ATP-binding activity cannot be excluded, but it is unclear whether TmrB-like

Fig. 4 Transmission electron microscopy. Microscopic inspection of WT/pSET152 (A), SY-tbl-1/pSET152 (B), and SY-tbl-1/pSCO3388 (C) at 60,000 $\times$ magnification. Cell wall (CW) and periplasmic space (PS) are indicated by arrows.

proteins, including TmrB of *B. subtilis*, employ ATP-binding to perform their biological roles.

The Δ SCO3388::tsr mutant (SY-tbl-1) displayed defects in growth and sporulation on R4 agar (Figs. 1 and 2), which was partially complemented when *ermE1p-SCO3388* (pSCO3388) was introduced into this mutant (Fig. 2A). Significantly, this result is the first demonstration that a TmrB-like protein has a role in bacterial development. Morphological differentiation of SY-tbl-1 was normal in MS agar (Fig 2B), suggesting that SY-tbl-1 is a conditional mutant.

Germination of dormant spores demands CW remodeling, involving CW hydrolysis (Haiser et al., 2009). Development of spores also requires CW assembly in addition to component synthesis, as shown in *mre* mutants (Mazza et al., 2006). The CW of *rpfa* (CW hydrolase gene) mutant is thinner and denser than WT (Haiser et al., 2009), while the *mre* mutants display severely deformed spores (Mazza et al., 2006). Spores of SY-tbl-1 possessed a dense (dark) CW with a limited PS layer compared to WT, resembling the *rpfa* mutant (Fig. 4). This observation may indicate that the loss of *SCO3388* does not affect CW assembly itself but rather CW remodeling in the spore germination stage. The true nature of the layer underlying the CW layer was unclear, but this layer was assumed to be PS in the present study, according to the electron microscopic dissection study of *B. subtilis* CW (Matias and Beveridge, 2008).

A mutation in either CW hydrolase or *mre* gene (spore CW assembly) makes the cognate mutant sensitive to stress conditions, such as heat shock (Mazza et al., 2006; Haiser et al., 2009), but SY-tbl-1 displayed no decrease in stress survival (Fig. 3). Stress survival of SY-tbl-1 was moderately higher than WT (Fig. 3), whereas its spore was defective in morphological development (Figs. 1 and 2). Introduction of *ermE1p-SCO3388* dramatically enhanced stress survival of *S. coelicolor* (Fig. 3), while the expression strain (SY-tbl-1/pSCO3388) displayed no significant difference to WT in the spore CW appearance under TEM (Fig. 4). Thus, stress survival was not related to the appearance of the

spore CW structure under TEM. We therefore propose that SCO3388 is a novel factor in spore CW remodeling and morphological differentiation in *S. coelicolor* and that TmrB-like proteins have a role in CW metabolism in Gram-positive bacteria.

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