Available online at www.sciencedirect.com
SCIENCE DIRECT
Biochemical and Biophysical Research Communications 343 (2006) 369–377
BBRC
www.elsevier.com/locate/ybbrc

ShyA, a membrane protein for proper septation of hyphae in *Streptomyces*

Shun-Fu Tseng [a], Tzu-Wen Huang [b], Carton W. Chen [b], Ming-Kai Chern [c], Ming F. Tam [c], Shu-Chun Teng [a,*]

[a] *Department of Microbiology, College of Medicine, National Taiwan University, Taipei 10063, Taiwan*
[b] *Institute of Genetics, National Yang-Ming University, Taipei 112, Taiwan*
[c] *Institute of Molecular Biology, Academia Sinica, Taipei 115, Taiwan*

Received 21 February 2006
Available online 10 March 2006

Abstract

The life cycle of *Streptomyces* involves the formation of filamentous substrate and aerial hyphae. Following cessation of growth of an aerial hypha, multiple septation occurs at the tip to produce a chain of unigenomic spores. A gene, *shyA*, which influences several aspects of this growth, was isolated and partially characterized in *Streptomyces coelicolor*. The gene product is a representative of a well-conserved family of small actinomycete proteins. The *shyA* mutant sporulates normally but displays hyper septum formation and altered spore-chain morphology. Biochemical separation experiments and immunofluorescence staining demonstrated that the *shyA* gene product locates at cell membranes. Moreover, yeast two-hybrid screen and GST-pull-down assay showed that ShyA can interact with itself. Altogether, ShyA belongs to a new family of membrane-associated proteins which plays a role in morphological differentiation in actinomycetes.

Keywords: *Streptomyces*; Actinomycetes; Hyphae; Spore; Membrane protein

The filamentous bacteria *Streptomyces* exhibit a complex life cycle [1–3]. Colonies germinate from spores and grow into the soil by forming a branching network of multinucleoid hyphae called the substrate mycelium. A hairy layer of specialized hyphae that project away from the surface of the colony into the air forms aerial mycelium. These aerial hyphae then undergo septation at regular intervals along the hyphae to form many unigenomic compartments that mature into pigmented spores, thereby completing the life cycle.

Critical aspects that distinguish the growth of substrate hyphae, aerial hyphae, and spore are the positioning, type, and number of septa. The combination of morphological mutants and gene cloning techniques is useful for the study of morphological differentiation, giving insight into structural and regulatory genes important for the developmental processes. Differentiation of a single filamentous aerial hyphal cell involves synchronous polymerization of the tubulin-like FtsZ protein on the inner surface of the cytoplasmic membrane, forming regularly spaced multiple ring-like structures, precursors of sporulation septa [4,5]. An *ftsZ* null mutant of *Streptomyces coelicolor* that produces no cross-walls at all is still viable but can only grow vegetatively without sporulation [6]. Multiple septation of an aerial hyphal cell is achieved by developmental control of *ftsZ* expression, involving upregulation of transcription of the gene specifically in the reproductive hyphae [7]. A C-terminal substitution in FtsZ leads to lack of proper septation among spores, but not in mycelia [8], suggesting mechanistic differences and developmental control of cytokinesis in the different hyphae. Aerial hyphae eventually convert into chains of spores by sporulation septation. Mutations in *bld* and *whi* genes cause mycelia failed to erect aerial hyphae and to form mature spores [2,9,10].

In this study, we describe a *Streptomyces* protein, ShyA, whose expression appears to be required for the morphological differentiation of *Streptomyces*. The *shyA* mutant

* Corresponding author. Fax: +886 2 23915293.
E-mail address: scteng@ha.mc.ntu.edu.tw (S.-C. Teng).

0006-291X/$ - see front matter © 2006 Elsevier Inc. All rights reserved.
doi:10.1016/j.bbrc.2006.02.178

sporulates normally but displays hyperseptation and altered spore-chain morphology. ShyA is an abundant membrane protein that interacts with itself in *Streptomyces*. The gene product, which is required for proper cell division and morphology, is a representative of a new family of conserved actinomycete-specific proteins.

Materials and methods

Bacterial strains and general methods. Streptomyces lividans TK64 and *S. coelicolor M130* have been described previously [11]. Standard methods were used for cell culturing, DNA cloning in *Escherichia coli* [12] and *Streptomyces* operations [11]. Southern blot hybridization was performed according to the standard procedure [12]. All primer sequences are available upon request.

Yeast two-hybrid system. The yeast strain used for the two-hybrid assay was PJ69-4A (*MATa trp1-901 leu2-3, 112 ura3-52 his3-200 gal4Δ gal80Δ LYS2*::*GAL1-HIS3 GAL2-ADE2 met2*::*GAL7-lacZ*) [13]. Medium was prepared as described [12]. Yeast transformations were performed using the LiAc method [12].

A genomic DNA library of *S. lividans TK64* was constructed on the yeast two-hybrid prey vectors pGAD-C1∼3. Chromosome DNA was partially digested by *Aci*I, *Msp*I, *Hin*PII, *Mae*II, and *Taq*I. The best partial digests were determined by gel running, and 500 ∼ 3,000-bp DNA fragments were purified by gel extraction and cloned into the *Cla*I digested pGAD-C1∼3. Ligations were transformed into ElectroMAX *E. coli* DH10B (Invitrogen, USA) by electroporation. The transformation reactions of each ligation were pooled, and a small aliquot was plated to count the number of primary transformants.

Yeast two-hybrid assay was performed as described by James et al. [13]. The bait pGBDU-*tpg* and pGBDU-*shyA* and prey pGAD-*shyA* were constructed as follows. The *tpg* and *shyA* were PCR amplified using *S. lividans TK64* genomic DNA as a template. The full-length *S. lividans tpg* and *shyA* PCR products were cloned into pGEMTeasy (Promega, USA) and sequenced. To construct pGBDU-*tpg*, the *Nde*I-digested Klenow-treated and *Bam*HI-digested full-length *tpg* from pGMTeasy-*tpg* was cloned into *Eco*RI-digested Klenow-treated and *Bam*HI-digested pGBDU-C3. To make pGBDU-*shyA* and pGAD-*shyA*, the *Nde*I-digested Klenow-treated and *Pst*I-digested full-length *shyA* from pGMTeasy-*shyA*(*Sl*) was cloned into *Sma*I/*Pst*I-digested pGBDU-C3 and pGAD-C3, respectively. In the pGBDU-*tpg* screening, we obtained 42 positive clones from 1.9×10^7 colonies. We sequenced seven of 42 and all seven clones contained different sizes of the *shyA* gene. Colony hybridization assay [12] demonstrated that 31 out of the rest 35 clones were all *shyA* gene. In the pGBDU-*shyA* screening, we obtained three positive clones from 5×10^6 colonies. Two of the three clones contained different sizes of the *shyA* gene and the other clone contained SCO3658, a probable aspartate aminotransferase.

Gene disruption and complementation. A *shyA* disruption plasmid was constructed by inserting the thiostrepton-resistance gene (*tsr*) at the *Aat*II site of *shyA*. The *shyA* coding sequence from the 857-bp upstream of the ATG start codon to the 853-bp downstream of the TGA stop codon was PCR amplified from *S. coelicolor M130* genomic DNA. The resulting 2.3-kb PCR fragment was cloned into pGEMTeasy, and the 2.3-kb *Eco*RI-digested fragment of *shyA* in pGEMTeasy-*shyA*(*Sc*2.3-kb) was further cloned into the *Eco*RI site of pBSKS(+). The 1.1-kb thiostrepton-resistance gene (*tsr*) from *Sma*I-digested pLUS1002 was cloned into the *Aat*II site present at the 263-bp downstream of the ATG start codon of *shyA*. The 1.26-kb kanamycin-resistance gene (*km*) gene from pUC4K [14] was then inserted into the *Bam*HI site of pBSKS-*shyA-tsr*. The resulting plasmid pST375 was then used for destroying the *shyA* gene. To mutate the chromosomal *shyA* gene, protoplasts of *M130* were transformed with pST375, which could not replicate in *Streptomyces* and contain the kanamycin-resistance gene. Transformants resistant to thiostrepton (Thio^r) were selected on R2YE [11] containing thiostrepton. Kanamycin-sensitive transformants were isolated by replica-plating to kanamycin-containing plates and confirmed by Southern blot analysis.

For complementation of the mutation by the *shyA* gene and *shyA* plus SCO5844, the fragments from the 857-bp upstream of the ATG start codon of *shyA* to the stop codon of *shyA* and of SCO5844 were PCR amplified from *M130* genomic DNA, respectively. The PCR products were cloned into pGEMTeasy. The *Eco*RI-digested 1.4-kb *shyA* and 2.6-kb*shyA* plus SCO5844 fragments from their pGEMTeasy plasmids containing the entire *shyA* gene and *shyA* plus SCO5844 were cloned into the *Eco*RI site of pSET152 [15], a *Streptomyces* integration plasmid, respectively.

Overexpression of recombinant ShyA protein in E. coli. The 588-bp chromosomal *shyA* gene was PCR amplified from *S. coelicolor* genomic DNA and cloned into pGEMTeasy to form pGEMT-*shyA*(*Sc*0.6 kb). The*Nde*I–*Eco*RI fragment containing the full-length of *shyA* gene from pGEMT-*shyA*(*Sc*0.6-kb) was further cloned into the *Nde*I/*Eco*RI-digested pET28(c) (Novagen, Germany), which was used for the production of the fusion protein containing a histidine tag at the N-terminus. Recombinant ShyA in *E. coli* was overexpressed and purified by Ni^{2+}-column chromatography as described by the manufacturer (Qiagen, Germany). Expression of the fusion protein was confirmed by Western blotting using an anti-His-tag antibody (Amersham/Pharmercia, USA), and the fusion protein was further used for the production of rabbit anti-ShyA polyclonal antibodies. To generate GST-ShyA fusion protein, the 0.6-kb *Bam*HI-digested, Klenow-filled, and *Eco*RI-digested*shyA* gene from pGEMT-*shyA*(*Sc*0.6-kb) was further cloned into pGEX-4T, which was used for production of GST fusion protein as described by the manufacturer (Amersham/Pharmacia). The molecular mass of the His6-tagged recombinant ShyA protein was analyzed by LC/MS [16]. Thus, the Ni^{2+}-column purified protein was resolved on a Vydac (C_{18}) reverse-phase column and electrospray mass spectrum was obtained on a VG Quattro-Bio-Q mass spectrometer (Micromass, Altrincham, Cheshire, UK). Proteins were estimated by Western blotting.

Microscopic methods and immunofluorescence assay. Living vegative hyphae were mounted for microscopy and observed by spotting about 20 μl of liquid culture in YEME [11] directly on microscope slides coated with 1% agarose in phosphate-buffered saline (PBS) and covered with coverslips. For staining of the DNAs, vegetative mycelium was fixed in the final concentration of 3.7% of formaldehyde at room temperature for 45 min, washed twice in PBS, digested in 2.5 mg/ml of lysozyme in GTE buffer (50 mM glucose, 20 mM Tris–HCl, pH 7.5, and 10 mM EDTA) at room temperature for 10 min, and attached to poly(L-lysine) pretreated slide at room temperature for 10 min. The slides were washed with GTE buffer, methanol, and acetone, sequentially, and allowed to air-dry. The dried slides were stained in 2 μg/ml DAPI before observation. Length of hyphae was measured from a tip to the other tip in each septum in the computer imaging. The statistical significance was determined by a pooled variance *t* test. For visualization of substrate and arial mycelium from solid plates, strains were streaked for single colonies on YMD plates [11] grown at 30 °C for 3 days. Single colonies were soaked with 10% glycerol and examined by microscopy. For visualization of arial spore chain from solid plates, cultures for microscope were set up by inoculating spore suspensions in the acute angle of a sterile coverslip inserted at a 45° angle in the YMD agar. After the desired incubation time for spore to form, the coverslip was removed and cells on its surface were fixed by methanol. The fixed cells were incubated in poly(L-lysine), allowed to air-dry, and stained with DAPI. For immunofluorescence, spores were cultured in YEME [11] for 2 days until the OD_{600} reached 0.5. Immunofluorescence was performed as described [5].

All microscopy was carried out using a Leica DMLB microscope equipped with appropriate filter sets, a Princeton Instruments microMAX cooled CCD camera, and MetaMorph version 6.1 software (Universal Imaging Corp., USA). Digital images for the figures were assembled using Adobe Photoshop software.

Protoplast preparation and protein fractionation. Protoplast preparation was performed as described in Kieser et al. [11]. Cell fractionation from protoplasts was performed as described by Meijer et al. [17]. The protoplasts were pelleted and lysed in 1 ml of hypotonic TM buffer (20 mM Tris–HCl, pH 8.0, 5 mM $MgCl_2$) containing 2 mM of phenylmethylsulfonyl fluoride (PMSF). After 20 min of incubation at room