Review

Molecular Analysis of DNA Polymorphism of *Sporothrix schenckii*

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Abstract

Partial cDNA cloning of a putative membrane transporter protein gene expressed in *Sporothrix schenckii* and DNA polymorphism of the isolated gene are described here. DNA fragments were isolated from *S. schenckii*, and the deduced amino acid sequence from one of the fragments contained a region homologous to the conserved sequence of the membrane transporter protein family. 188-bp fragments encoding the homologous region were amplified from many strains of *S. schenckii*, and were subjected to polymerase chain reaction single strand conformation polymorphism (PCR-SSCP) analysis. The results demonstrated that the strains of *S. schenckii* were divided into three groups, and several base substitutions among these groups were observed. This finding agreed with the classification of *S. schenckii* strains based on the mitochondrial DNA diversity, because the three groups were clearly located on the branches of the phylogenetic tree constructed by digestion profiles of mitochondrial DNA with restriction enzymes. The correlation of the results of PCR-SSCP analysis with the mitochondrial DNA diversity might indicate linkage of the mutation of the membrane transporter protein gene with the evolution of *S. schenckii*, suggesting the protein plays an essential role in *S. schenckii*.

Key words: *Sporothrix schenckii*, membrane transporter protein, PCR-SSCP, classification

Introduction

Sporothrix schenckii is a causative agent of sporotrichosis, which affects the skin and subcutaneous tissue. Although genetic investigations of *S. schenckii* have not been well reported in comparison with other pathogenic fungi such as *Candida albicans* and *Cryptococcus neoformans*, phylogenetic analysis based on the mitochondrial DNA polymorphism of *S. schenckii* has already been presented^{1,2)}. Ishizaki *et al.* established the phylogenetic tree of *S. schenckii* based on the mitochondrial DNA digestion profiles of many strains from all over the world³⁾. In this paper, partial cDNA cloning of a *S. schenckii* component gene is reported⁴⁾, and DNA polymorphism of the isolated gene is demonstrated using polymerase chain reaction single strand conformation polymorphism (PCR-SSCP) analysis.

Materials and Methods

Fungal strains

S. schenckii was obtained from the American Type

Culture Collection (ATCC; No.14284, Rockville, MD, USA), and many other strains were generously provided by Dr. H. Ishizaki, Dr. M. Kawasaki, and Dr. T. Mochizuki (Kanazawa Medical School, Kanazawa, Japan). Several clinical isolates were also used. These strains were cultured and maintained in Sabouraud liquid medium.

DNA extraction

Cultured *S. schenckii* strains were pulverized in a mortar and pestle cooled with liquid nitrogen. DNA extraction was carried out according to the manufacturer's instructions (RapidPrep™ Genomic DNA Isolation Kits; Pharmacia, Uppsala, Sweden).

PCR

A pair of primers [5'-TACCGACATTTCCGC-GATAAAGTCGGCA-3', 5'-CGTCAACACATC-GTCAGTAGA-3'], which were originally designed to amplify a 157-bp DNA fragment from the *Mycobacterium leprae* *hsp* 70 gene⁵⁾, were used for nonspecific DNA amplification from *S. schenckii* DNA with lower annealing temperature. Amplification was performed in a PCR processor for 40 cycles, each consisting of denaturation for 1 min at 94°C, annealing for 1.5 min at 56°C, and extension for 1.5 min at 72°C. Another pair of primers [5'-GGCAATATATCCATCTGCAGTG-

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GGTGTG-3', 5'-GGCAAATTGCTCTTGCAGACCACGCTG-3'] was selected in the isolated DNA fragment to generate a 188-bp DNA fragment from many strains of *S. schenckii*. PCR condition was the same as above except for the annealing temperature of 58°C. One hundred nanograms of extracted DNA was utilized for each PCR as a template DNA.

DNA sequencing and database searches

PCR products purified by an ultrafiltration membrane (PM30; Amicon, Danvers, MA, USA) were subcloned according to the manufacturer's protocol (TA Cloning; Invitrogen, San Diego, CA, USA). DNA sequencing was carried out by the chain termination method using a non-radioactive sequencing kit (Plex Luminescent Kits; Millipore, Bedford, MA, USA). The European Molecular Biology Laboratory (EMBL; Heidelberg, Germany) and the Swissprot databases were utilized for database searches.

PCR-SSCP

PhastSystem (Pharmacia) semi-dry electrophoresis equipment was used for PCR-SSCP analysis. The mixture of the purified DNA and formaldehyde was denatured for 3 min at 95°C, and 1.5 μ l of the mixture was run on 12.5% homogenous polyacrylamide gels with native buffer strips (Pharmacia) at 15°C. Gels were stained with a Silver staining kit (Pharmacia).

Results

Primary PCR generated several nonspecific DNA fragments from *S. schenckii* DNA (Fig. 1). Several recombinant clones were obtained by subcloning of the PCR products, and an uninterrupted 60-amino acid sequence was deduced from a 233-bp DNA fragment (Fig. 2). The fragment seemed to be a part of an expressed gene, because DNA amplification was observed in reverse transcription (RT)-PCR using the new sets of primers selected in the segment⁴⁾ (data not shown). GC content of the fragment was 60%, and the nucleotide sequence data of these fragments will appear in the DDJB, EMBL and GenBank nucleotide sequence databases with the accession number D86234. The database searches indicated the similarity to membrane transporter proteins, especially to the sugar transporter protein family (Fig. 3). The highest similarity was detected with a putative inorganic phosphate transporter in *Schizosaccharomyces pombe*, showing 40% identity in a 35-amino acid overlap. Other homologous proteins were also detected in *Haemophilus influenzae*, *Saccharomyces cerevisiae*, *Escherichia coli*, and so on⁶⁻⁹⁾.

A new set of primers amplified 188-bp DNA fragments from 31 strains of *S. schenckii* including

Fig. 1. DNA isolation from *S. schenckii* DNA⁴⁾. Lane M: DNA size markers (ϕ × 174 *Hae*III digestion). Lane 1: A 157-bp DNA fragment was amplified with *M. leprae* DNA. Lane 2: Several weak amplified products were observed with *S. schenckii* DNA using the same primers.

Fig. 2. Nucleotide and deduced amino acid sequence of the isolated DNA fragment⁴⁾.

Uninterrupted 60-amino acid sequence was deduced from a 233-bp isolated DNA fragment from *S. schenckii* DNA. Underlined sequences are PCR primers⁵⁾ used for primary amplification.

28 KMU strains (Table 1). Amplified fragments were applied to PCR-SSCP analysis with the result that all samples were grouped into three electrophoresis patterns (Fig. 4). Differences of DNA sequence among the three groups were demonstrated by sequencing these fragments, and three kinds of deduced amino acid sequences are shown (Fig. 5). The three groups of strains were clearly located on the branches of the phylogenetic tree previously established by Ishizaki³⁾ (Fig. 6). In additional analysis, among 58 strains which the 188-bp DNA fragment was able to amplify, only 3 were not located on the expected branches of the phylogenetic tree. An insufficient amount of DNA amplification was obtained from 11 other strains

S. sch. WVWRLLLGIGLIPCALTLYARLRIRET
S. pomb. GVWRLQMGLALVPALLVLIPRLTMKES
H. inf. 1 WAWRIPFLSSVILVAIGLYVRLKLTEA
H. inf. 2 WAWRIPFVSSILLVAVGLYVRLTLHES
S. cer. 1 QMWRILIGLGTVLGLACLYFRLTIPES
S. cer. 2 NGWRILVGLSLIPTVLQFSFFCFLPDT
E. coli. 1 WGWRIPFFTALPLGIIGLYLRHALEET
E. coli. 2 WGWRIPFALGAVLAVVALWLRRQLDET

Fig. 3. Amino acid sequences of the conserved regions in membrane transporter proteins. Identical amino acids are boxed.
S. sch. : *Sporothrix schenckii*, EMBL No. D86234; *S. pomb.* : *Schizosaccharomyces pombe*, putative inorganic phosphate transporter C23D3.12, EMBL No. Q09852; *H. inf.* 1 : *Haemophilus influenzae*, hypothetical metabolite transport protein HI0418, EMBL No. P44699; *H. inf.* 2 : *Haemophilus influenzae*, hypothetical metabolite transport protein HI0281, EMBL No. P44610; *S. cer.* 1 : *Saccharomyces cerevisiae*, inorganic phosphate transporter PH084, EMBL No. P25297; *S. cer.* 2 : *Saccharomyces cerevisiae*, myo-inositol transporter 2, EMBL No. P30606; *E. coli.* 1 : *Escherichia coli*, proline/betaine transporter, EMBL No. P30848; *E. coli.* 2 : *Escherichia coli*, alpha-ketoglutarate permease, EMBL No. P17448.

Table 1. Strains used for PCR-SSCP analysis

Types ^b	KMU No. ^a
Type 1	975, 2285
2	2286, 2924
3	2500, 2501
4	2052, 3112
5	3311, 3341
6	2515, 2525
7	3360, 2647
8	2741
9	2760, 2736
10	2763
11	2687
12	3314
13	2762
14	3486, 3488
15	3628, 3637
16	3653, 3659
17	3658

^a28 KMU strains were obtained from Kanazawa Medical University. ^bStrain typing is based on the mitochondrial DNA diversity¹⁻³).

(Table 2).

Discussion

The isolated DNA fragment from *S. schenckii* contained a segment encoding hydrophobic amino acid sequence which showed homology with the conserved transmembrane portion of membrane transporter proteins in many other microorganisms⁴). Although the primers were originally designed in the gene encoding heat shock protein 70 of *M. leprae*, most of the detected homologous proteins were not registered as heat shock proteins.

a: 2052, 3112, 3311, 3341, 2515 2525, 3360, 2647, 2741, 2760, 2736, 2763, 3314, 2762
b: 975, 2285, 2500, 2501, 2687, 3628, 3637, 3653, 3659
c: 2286, 2924, 3486, 3488, 3658

Fig. 4. Results of PCR-SSCP analysis
(1) Electrophoresis of 31 *S. schenckii* strains on a polyacrylamide gel. (2) Electrophoresed bands were grouped into three patterns. The three groups of strains are indicated by underlined italics (a), double lined (b) and bold letters (c).

Fig. 5. Nucleotide sequence (1) and amino acid sequence (2) of the three groups.

Fig. 6. A phylogeny of 18 types of *S. schenckii*. Correlation between the results of PCR-SSCP and phylogenetic tree of *S. schenckii*³⁾ is demonstrated. The three groups divided by PCR-SSCP are clearly located on the branches of the tree. The three groups of strains are indicated by underlined italics, double lined and bold letters.

The explanation of this phenomenon is unclear, however, it is speculated that the membrane transporter protein could have a protective role for microorganisms like the heat shock proteins. This hypothesis might be supported by recent investigations on the functional role of the membrane transporter protein in the mechanism of multi-drug resistance¹⁰⁾. Primary DNA amplification with low specificity was generated from the whole-cell DNA of *S. schenckii*. Although the membrane transporter protein gene should be contained in chromosomal DNA, the results of PCR-SSCP analysis agreed with the mitochondrial DNA diversity. PCR products could not be generated in 11 strains, and products from 3 strains were not grouped into the expected branches constructed by the mitochondrial DNA typing. However, coincidence of the results of PCR-SSCP analysis with the phylogenetic tree was observed in most all of the strains (Fig. 4,

Table 2. Strains used for PCR-SSCP analysis	
Types ^b	KMU No. ^a
Type 1	975, 2285, 2198
2	2286, 2924, (3114) ^c , 3487, 3694
3	2500, 2501, 3587, 3660, (3656) ^c
3'	3624 ^d , 3620, 3623, 3626, 3629
4	2052, 3112, 3509, 3631, 3657
5	3311, 3341, 3386, 3485
5'	3621, 3625, 3634, 3635, 3640
6	2515, 2525, (3515) ^c , 3593, (3705) ^c
7	3360, 2647, 2823, 2873, (3507) ^c
8	2741
9	2760, 2736, 2877, 339.
10	2763
11	2687
12	3314
13	2762
14	3486, 3488, (3580) ^c , (3590) ^c , (3600) ^c , (3501) ^c
15	3628, 3637, 3504 ^d , 3627
16	3653, 3659, 3652, (3654) ^c
17	3658 ^d , 3655, 3664
18	(3617) ^c

^aKMU strains were obtained from Kanazawa Medical University. ^bStrain typing is based on the mitochondrial DNA diversity. ^cPCR products were insufficient in 11 strains. ^d3 strains were not grouped into expected types.

Table 2). This finding indicated that the mutations of the membrane transporter protein gene occurred in relation to mitochondrial DNA mutation. Because mitochondrial DNA probably spontaneously mutates during the long period of *S. schenckii* evolution¹¹⁾, the possibility should be considered that the membrane transporter protein encoded by the isolated gene would also be an essential protein for the life of *S. schenckii*. Although the precise mechanism of this protein must be elucidated by further investigation, database searches also showed homology with the sugar transporter protein family, which must be involved in the basic metabolism of microorganisms (Fig. 3). The findings described here might be clues not only for structural and functional analysis but for the phylogeny of *S. schenckii*. Moreover, this paper has also demonstrated the usefulness and reliability of molecular biological methods because the results obtained from quite different methods clearly coincide.

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