Abstract In solid-state cultures (SC), Aspergillus oryzae shows characteristics such as high-level production and secretion of enzymes and hyphal differentiation with asexual development which are absent in liquid (submerged) culture (LC). It was predicted that many of the genes involved in the characteristics of A. oryzae in SC are differentially expressed between SC and LC. We generated two subtracted cDNA libraries with bi-directional cDNA subtractive hybridizations to isolate and identify such genes. Among them, we identified genes upregulated in or specific to SC, such as the AOS (A. oryzae SC-specific gene) series, and those downregulated or not expressed in SC, such as the AOL (A. oryzae LC-specific) series. Sequencing analyses revealed that the AOS series and the AOL series contain genes encoding extra- and intracellular enzymes and transport proteins. However, half were functionally unclassified by nucleotide sequences. Also, by expression profile, the AOS series comprised two groups. These gene products’ molecular functions and physiological roles in SC await further investigation.

Keywords Aspergillus oryzae · Solid-state culture · cDNA subtraction · Differential gene expression

Introduction

In Japan, the filamentous fungus Aspergillus oryzae is used widely to produce traditional fermented foods such as sake, shoyu (soy sauce), miso, and so on. It is also employed in commercial enzyme production. In many of these industrial utilisations, A. oryzae is cultured on cereals such as rice, barley, wheat bran, and soybean. This cultivation style is one of solid-state culture (SC), the so-called koji culture. During SC, A. oryzae produces large amounts of various enzymes and secretes them into the medium. Mycelia also show hyphal differentiation and asexual development. Conidia resulting from asexual development simplify inoculation for the cultivation and passage of strains, as culture starter. These A. oryzae characteristics are not expressed in liquid (submerged) culture (LC). That is the reason why SC is mainly employed in the industrial utilization of A. oryzae. In addition to the viewpoint of industrial utilization, the behavior of A. oryzae in SC is also interesting as a growth model for filamentous fungi in nature, because SC is similar for many natural filamentous fungi.

The SC-specific characters and their expression mechanism in A. oryzae are therefore interesting and important subjects. However, most studies of Aspergillus SC approached the subject from the viewpoint of culture engineering (Gervais and Bensoussan 1994; Sato and Sudo 1999). Molecular biological studies on the SC system are very few and their applications practically limited to LC systems.

The high glucoamylase productivity of A. oryzae in SC is a rare example of a well investigated molecular mechanism. Study revealed that SC-specific expression of the glucoamylase-encoding gene glaB of A. oryzae was responsible for much higher glucoamylase activity in SC than in LC (Hata et al. 1997, 1998); and glaB was the first gene found to express specifically in SC.

It is expected that glaB is not the only SC-specific gene in A. oryzae, since mycelial characteristics differ greatly between SC and LC. Further SC-specific genes may exist. Also, some LC-specific genes, genes repressed in SC, could be involved in generating different characteristics. Although functional and physiological categories of products of these genes may be distributed over
a wide range, they have not been specified at present. cDNA subtraction is a very effective method to identify transcripts of such genes with differential expression profiles (Sagerstrom et al. 1997).

In this report, we describe the identification of genes specifically transcribed or transcriptionally repressed in SC, compared with LC, by cDNA subtraction from *A. oryzae*. The genes are then divided into a few groups by their expression profiles.

### Materials and methods

**Strain, medium, and cultivation**

A wild-type strain, *A. oryzae* RIB40, was employed for cultivation and mRNA isolation. As a solid medium, autoclaved wheat bran was used. *A. oryzae* RIB40 conidial suspension (4 ml, at 1.25 × 10⁶ conidia/ml) was inoculated onto 5 g of solid medium (giving 1.0 × 10⁶ conidia/g wheat bran), and incubated at 30 °C. A 3% (w/v) wheat bran suspension was autoclaved for extraction at 121 °C for 15 min, cooled to room temperature, and then centrifuged at 2,500 × g for 10 min. The supernatant was used as a medium for LC. *A. oryzae* RIB40 conidial suspension (200 ml, at 5 × 10⁶ conidia/ml) was inoculated into 100 ml of the liquid medium in a 500-ml baffled flask (giving 1 × 10⁷ conidia/ml) and then cultured at 30 °C with rotary shaking (120 rpm).

**RNA preparations**

Total RNAs from SC were prepared, based on a modified method of Cathala et al. (1983). About 4–6 g of whole SC of *A. oryzae* RIB40 (mycelia with wheat bran) was immediately frozen with media in liquid N₂, ground to a fine powder, and then suspended in 6–10 vol. of GuSCN buffer (5 M guanidine isothiocyanate, 50 mM Tris-HCl (pH 7.5), 10 mM EDTA, 10% 2-mercaptoethanol) pre-warmed at 50 °C. The suspension was then mixed with 5 vol. of 4 M LiCl and allowed to precipitate overnight at 4 °C. After the removal of mycelia and wheat bran debris by low-speed centrifugation, it was centrifuged at 11,000 × g for 90 min at 4 °C. The pellet was collected, washed with 3 M LiCl, and centrifuged again. Then, the pellet was suspended in 5–10 ml of a buffer containing 10 mM Tris-HCl (pH 7.5), 1 mM EDTA, and 0.1% SDS. The suspension was extracted 3–4 times with phenol/chloroform/isoamyl alcohol (25:24:1) and once with chloroform/isoamyl alcohol (24:1). These aqueous layers were combined and total RNA was precipitated with ethanol or isopropanol. *A. oryzae* RIB40 mycelia harvested from LC were immediately frozen in liquid N₂ and ground to a fine powder. Then, total RNAs were isolated with Isogen (Nippon Gene), based on the acid guanidium thiocyanate-phenoil-chloroform extraction method (Chomczynski and Sacchi 1987; Chomczynski 1993), according to the manufacturer’s instructions. Poly(A)⁺ RNAs were isolated from total RNAs using Oligotex dT30 super (Takara Shuzo), according to the manufacturer’s instructions.

**cDNA subtractions**

Double-stranded cDNAs were synthesized from mRNAs, followed by restriction digestion with Rsal. Fragmented cDNAs were bi-directionally subtracted with the PCR-select cDNA subtraction kit (Clontech), based on the suppression subtractive hybridizations (SSH) method (Diatchenko et al. 1999), according to the manufacturer’s instructions. The PCR products generated from subtracted cDNAs were directly ligated into the pCR2.1 TOPO vector (Invitrogen), transformed into *Escherichia coli* TOP10 (Invitrogen), and plated onto Luria-Bertani/ampicillin agar. Then, the resultant colonies carrying a plasmid containing a cDNA insert were collected for screening.

**Reverse Northern analysis**

Approximately 0.1 µg of each cloned plasmid DNA with an insertion of a subtracted cDNA fragment was diluted to 200 µl with transfer solution (0.4 M NaOH) and then blotted onto duplicated positively charged Nylon membranes, Hybond-N⁺ (Amersham Pharmacia). Sets of DNA-blotted membranes were hybridized with digoxigenin (DIG)-labeled PCR products of two species of subtracted cDNAs, respectively, instead of the reverse transcripts normally used. This modification gives clearer results which are independent of the original population of each transcript, although false positive clones may be screened to some extent. Reverse Northern analysis was done according to a protocol supplied with the PCR-select differential screening kit (Clontech). Hybridizations and subsequent washing were carried out as described by Church and Gilbert (1984) with some modifications. Immunodetections of hybridized DIG-labeled probes were performed using the DIG luminescent detection system (Roche); DIG-labeled random-primed DNA probes were prepared using DIG-High Prime (Roche), according to the manufacturer’s instructions.

**Northern analysis**

Denatured total RNAs (approximately 10 µg/lane) were electrophoresed on formaldehyde-agarose gel and transferred in 20 × SSC onto Hybond N⁺ membranes (Amersham Pharmacia) with the VacuGene XL (Amersham-Pharmacia) vacuum blotting system. Each blotted membrane was hybridized with the Dig-labeled cDNA probe. Hybridizations were carried out in hybridization solution [5 × SSC, 50% deionized formamide, 7% SDS, 50 mM sodium phosphate (pH 7.0), 0.1% lauryl sarcosine, 2% blocking reagent (Roche), 0.005% yeast RNA (Roche)] at 50 °C for 16 h. Then, membranes were washed once at room temperature for 10 min in 2 × SSC containing 0.1% SDS and then twice at 55 °C for 20 min in 0.1 × SSC containing 0.1% SDS. Immunodetection of hybridized DIG-labeled probes was performed using the DIG luminescent detection system (Roche). DIG-labeled cDNA probes were prepared using the PCR DIG probe synthesis kit (Roche), with cloned plasmid DNAs as templates, according to the manufacturer’s instructions. Synthesized oligo-DNA NP1 and NP2, supplied with the PCR-select cDNA subtraction kit (Clontech) were used as a primer set for the PCR-labeling reaction.

**Nucleotide sequencing**

Nucleotide sequences of isolated DNA clones were determined by the dideoxynucleotide chain termination method, using a BigDye terminator cycle sequencing kit (Perkin Elmer) and an ABI Prism 310 automated DNA sequencer (Perkin Elmer).

**Sequencing analysis of DNA**

DNA sequences were analyzed using sequencing analysis software: DNASIS (Hitachi Software Engineering) and GENETYX (Software Development). Homology searches against databases were performed using BLAST algorithms (Altschul et al. 1997) on the National Center for Biotechnology Information web server (http://www.ncbi.nlm.nih.gov/BLAST). Comparison of DNA sequences with the *A. oryzae* expressed sequence tag (EST) database in our laboratory was also done with BLAST algorithms.

**General techniques for nucleic acid manipulation**

Nucleic acid manipulations not described above were performed using standard methods as described by Sambrook et al. (1989).