Small subunit ribosomal RNA of *Blastomyces dermatitidis*: sequence and phylogenetic analysis

ANTONIA GEBER,1,* DESMOND E. HIGGINS,2 ANDREW P. WATERS,3 JOHN E. BENNETT1 and THOMAS F. MCCUTCCHAN1

1Laboratory of Clinical Investigation and Laboratory of Parasitic Diseases, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, MD 20892, USA
2European Molecular Biology Laboratory, Heidelberg, FRG
3Laboratorium voor Parasitologie, Universiteit Te Leiden, Postbus 9605, 2300 RC Leiden, The Netherlands

(Received 29 August 1991; accepted 23 October 1991)

We determined the small subunit (18S) ribosomal RNA sequence of the dimorphic fungus *Blastomyces dermatitidis*. The sequence was compared to that of fourteen other eukaryotic organisms, ten of which were higher fungi, and an evolutionary tree was constructed based on these sequences. *B. dermatitidis* aligned most closely with the Ascomycetes *Neurospora crassa* and *Podospora anserina*, in agreement with previous phylogenetic analysis based on morphological criteria. Phase-specific cDNA clones derived by reverse transcription of RNA isolated from the yeast and mycelial phases of *B. dermatitidis* were also sequenced. The 18S ribosome sequence was found to be the same in both phases. Heterogeneity was found at both the genomic and RNA level at position 1352.

Introduction

Studies of the evolution of fungi and their phylogenetic relationships have most often been based on biochemistry and morphology, including ultrastructure. Recent attempts to clarify fungal taxonomy have, however, used molecular data obtained from ribosomal nucleotide sequences (Foerster et al., 1990; Gunderson et al., 1987a). These molecules are well suited as taxonomic tools as they are universally present and, because of their function in protein synthesis, are highly conserved. The large size of the small subunit (SSU) rRNA (16-18S) makes it particularly well suited as an indicator of phylogeny (Olsen et al., 1986). In this paper, we report the sequence of the SSU rRNA of the dimorphic fungus *Blastomyces dermatitidis*, and construct an evolutionary tree based on this sequence and that of fourteen other eukaryotic organisms.

*B. dermatitidis* is the aetiological agent of blastomycosis, a multisystem illness whose pathogenesis involves inhalation of conidia and transformation to the yeast phase, followed by localized or disseminated disease. Dimorphism in *B. dermatitidis* is thermally regulated (Levine & Ordal, 1949). Differences in cell wall polysaccharide (Kanetsuna & Carbonell, 1971), protein composition (Roy & Landau, 1972), and rates of oxygen consumption (Nickerson & Edwards, 1949) have been described between the two phases. The amount of RNA has been found to differ between the yeast and the mycelial phase (Taylor, 1961), and two reports have suggested a difference in ribosomal size in dimorphic fungi (Gates & Brownstein, 1980; Bawdon et al., 1984). In *Plasmodium*, structurally distinct SSU rRNAs are known to exist at different stages of the parasite's life cycle (Gunderson et al. 1987b). We investigated the possibility that stage-specific SSU rRNA may exist in *B. dermatitidis* and be involved in the molecular events underlying the regulation of morphogenesis.

Methods

Cultures. *B. dermatitidis* (ATCC 26199) cultures were grown on brain heart infusion (BHI) media with 2% glucose. Yeast cells were grown on BHI agar at 37 °C for both DNA and RNA extraction. Hyphal cells were grown at 30 °C on BHI agar for DNA extraction and in BHI broth with constant agitation for RNA extraction. *E. coli* DH5αmax (Bethesda Research Laboratories) was the recipient for bacterial transformation.
Isolation of nucleic acids. Yeast and hyphal cells were pelleted and washed with 1 M-NaCl. Cells were broken by vortexing for 3–4 30 s intervals with 0.45 mm glass beads (PGC) for RNA extraction and 4 mm glass beads (PGC) for DNA extraction. The samples were kept on ice between vortexing. RNA was extracted by the method of Chomczynski (1987). Prior to DNA extraction, cells were lyophilized and stored at −20 °C. DNA was extracted in a sucrose buffer with 1 M-sarcosinate (ICN Biochemicals) and 1 M-sodium percholate (Fischer Scientific) by the method of Price (1978). Following two extractions with chloroform/isoamyl alcohol (24:1; J. T. Baker Chemical) the aqueous phase was replaced over a cesium chloride (optical grade; ICN Biochemicals) gradient and centrifuged at 45000 r.p.m. for 20–22 h. The DNA band was removed, extracted with butanol, and dialysed on ice between vortexing. RNA was extracted by the method of Washed with 1 M-NaCl. Cells were broken by vortexing for 3–4 30 s intervals with 0.45 mm glass beads (PGC) for RNA extraction and stored at −20 °C. DNA was extracted in a sucrose buffer with 1 M-sarcosinate (ICN Biochemicals) and 1 M-sodium percholate (Fischer Scientific) by the method of Price (1978). Following two extractions with chloroform/isoamyl alcohol (24:1; J. T. Baker Chemical) the aqueous phase was replaced over a cesium chloride (optical grade; ICN Biochemicals) gradient and centrifuged at 45000 r.p.m. for 20–22 h. The DNA band was removed, extracted with butanol, and dialysed against 10 mM-Tris/1 mM-EDTA (TE) for 2 h. DNA was precipitated in ethanol, washed with 70% ethanol, and resuspended in TE.

Restriction enzyme digestion and Southern blot analysis. Genomic DNA was digested with EcoRI, XhoI, HindIII, and Sphi restriction endonucleases (BRL) according to the manufacturer’s instructions. Fragments were separated by electrophoresis through a 0.8% agarose gel. DNA was transferred to nylon membranes (DuPont) by the method of Southern (1975). Nylon blots were hybridized to oligonucleotides complementary to conserved areas of 18S ribosome which had been end-labelled with [32P]ATP (Amersham) using T4 polynucleotide kinase (BRL).

Cloning and sequencing. Phase-specific cDNA clones of the SSU rRNA were obtained in the following manner. First strand cDNA was synthesized using the MMLV reverse transcriptase enzyme (BRL) and primers complementary to a conserved region of the 3′ end of the SSU rRNA as described by Sambrook et al. (1989). The sample was heated to 95 °C for 5 min and amplified by the polymerase chain reaction (PCR) using primers containing EcoRI sites complementary to conserved areas at the 5′ and 3′ ends of the SSU rRNA. The PCR product was digested with EcoRI (BRL), electrophoresed in low melting agarose gel, excised from the gel, and ligated into pUC19. Ligated material was transformed into E. coli DH5α max.

Genomic clones were obtained from EcoRI fragments, size selected based on Southern analysis, and ligated into pUC19. Ligation products were transformed into E. coli DH5α max. Transformants were screened with radiolabelled oligonucleotide complementary to conserved areas of the SSU rRNA. Plasmid isolation was carried out by the method of Holmes (1987). Prior to DNA extraction, cells were lyophilized and centrifuged at 45000 r.p.m. for 30 h. DNA was precipitated from genomic clones is shown in Fig. 1. The sequence is 1800 bases long. No variability was found between yeast- and mycelial-phase cDNA clones. Unlike Plasmodium, B. dermatitidis does not appear to have phase-specific, structurally distinct small subunit ribosomes. Previous reports have suggested a ribosomal size difference between the yeast and mycelial phases in B. dermatitidis (Bawdon et al., 1984) and in the dimorphic fungus Histoplasma capsulatum (Gates & Brownstein, 1980; Bawdon et al., 1984), and it is possible that differences in other components of the ribosome account for these observations. A number of point mutations which were not phase-specific were detected in the cDNA clones (data not shown), but only one, the base substitution at position 1352, could be demonstrated to be within the genome and not the result of reverse transcription or PCR amplification error. Heterogeneity has previously been described in the 18S ribosomal subunit of several fungal species (Selker et al., 1985), but not in the 18S rRNA. The functional significance of this polymorphism is not known.

An evolutionary tree was derived from 1625 nucleotide bases which could be unambiguously aligned with the SSU rRNA sequences of fourteen other eukaryotic organisms (Fig. 2). This tree includes a broader range of fungi than prior publications containing phylogenetic analyses of SSU rRNA. B. dermatitidis is closely aligned with the Ascomycetes N. crassa and P. anserina. Ascomycetes are distinguished from other fungi primarily on the basis of their sac-like meiosporangia or asci, which usually contain four or eight haploid ascospores at maturity (Kendrick, 1985). Within the phylum Ascomycota, organisms have been further classified based on their type of ascus and the manner with which they discharge their ascospores (Kendrick, 1985). B. dermatitidis has protunicate asci which disintegrate prior to the release of ascospores. N. crassa and P. anserina both contain unitunicate-inoperculate asci which are single-walled and contain lids which open at maturity, allowing the ascospores to be ejected. The presence of B. dermatitidis on a separate branch of the dendrogram from N. crassa and P. anserina is thus supported by previous classification schemes based on morphological criteria.

Results and Discussion

The nucleotide sequence of the SSU rDNA as derived from genomic clones is shown in Fig. 1. The sequence is 1800 bases long. No variability was found between yeast- and mycelial-phase cDNA clones. Unlike Plasmodium, B. dermatitidis does not appear to have phase-specific, structurally distinct small subunit ribosomes. Previous reports have suggested a ribosomal size difference between the yeast and mycelial phases in B. dermatitidis (Bawdon et al., 1984) and in the dimorphic fungus Histoplasma capsulatum (Gates & Brownstein, 1980; Bawdon et al., 1984), and it is possible that differences in other components of the ribosome account for these observations. A number of point mutations which were not phase-specific were detected in the cDNA clones (data not shown), but only one, the base substitution at position 1352, could be demonstrated to be within the genome and not the result of reverse transcription or PCR amplification error. Heterogeneity has previously been described in the 18S ribosomal subunit of several fungal species (Selker et al., 1985), but not in the 18S rRNA. The functional significance of this polymorphism is not known.

An evolutionary tree was derived from 1625 nucleotide bases which could be unambiguously aligned with the SSU rRNA sequences of fourteen other eukaryotic organisms (Fig. 2). This tree includes a broader range of fungi than prior publications containing phylogenetic analyses of SSU rRNA. B. dermatitidis is closely aligned with the Ascomycetes N. crassa and P. anserina. Ascomycetes are distinguished from other fungi primarily on the basis of their sac-like meiosporangia or asci, which usually contain four or eight haploid ascospores at maturity (Kendrick, 1985). Within the phylum Ascomycota, organisms have been further classified based on their type of ascus and the manner with which they discharge their ascospores (Kendrick, 1985). B. dermatitidis has protunicate asci which disintegrate prior to the release of ascospores. N. crassa and P. anserina both contain unitunicate-inoperculate asci which are single-walled and contain lids which open at maturity, allowing the ascospores to be ejected. The presence of B. dermatitidis on a separate branch of the dendrogram from N. crassa and P. anserina is thus supported by previous classification schemes based on morphological criteria.
Small ribosomal subunit RNA sequence of *B. dermatitidis* 397

Fig. 1. The nucleotide sequence of the SSU rRNA gene of *Blastomyces dermatitidis*.

Fig. 2. Evolutionary tree based on the SSU rRNA sequences of fifteen eukaryotic organisms. Evolutionary distance between organisms is indicated by the horizontal branch length, which reflects the number of nucleotide substitutions per site along that branch from node to endpoint. Where the branches are not drawn to scale, numbers reflecting the branch length are given above the branch. Percentage of bootstrap samplings, derived from 1000 samples, supporting the interior branches are given below these branches.

Members of the genera *Candida* and *Torulopsis* lack a sexual cycle and have been grouped with other imperfect fungi in the phylum Deuteromycota. Based on physiological criteria, *Candida* and *Torulopsis* appear closely related to the Ascomycetes and are felt to have evolved from an Ascomycete ancestor following the loss of a sexual state. SS rRNA (Chen et al., 1984) and 18S rRNA (Hendriks et al., 1989; Barns et al., 1991) sequence data support the close affiliation of these yeasts with the Ascomycetes. These same studies demonstrate a close evolutionary relationship between *K. marxianus var. lactis* and *S. cerevisiae*. 18S rRNA sequence analysis has shown *T. glabrata* to be more closely related to *S. cerevisiae* than to *C. albicans* (Barns et al., 1991), which
favour maintaining *Torulopsis* in a genus distinct from *Candida*. Our dendrogram confirms these observations, and supports the close evolutionary relationship between *S. cerevisiae* and *T. delbrueckii* demonstrated in the recent analysis by Hendriks *et al.* (1991). Based on ribosomal sequence data, the genera *Candida*, *Torulopsis*, *Torulaspora*, *Kluyveromyces* and *Saccharomyces* can appropriately be placed in the class Saccharomycetes, order Endomycetales.

*S. pombe* is an ascosporogenous yeast which, based on morphological criteria, has been grouped with *S. cerevisiae* within the family Saccharomycetaceae. *S. pombe*’s position between *B. dermatitidis* and *B. emersonii* in our dendrogram, which is well-supported by bootstrap analysis, conflicts with this taxonomic assignment. Sequence analysis of SSU RNA, however, places *S. pombe* in a distant relationship with *S. cerevisiae*, aligning it more closely with the Zygomycetes and Chytridiomycetes (Huysman et al., 1983). *S. pombe* differs from *S. cerevisiae* in that it multiplies by binary fission, as opposed to budding. At a molecular level, the two yeasts differ in (1) the regulation of gene transcription, (2) autonomously replicating sequences, (3) ras gene structure and protein function, and (4) the mitotic cell cycle (reviewed by Russell & Nurse, 1986). Our dendrogram supports the hypothesis that the phylogenetic relationship between *S. pombe* and *S. cerevisiae* is more distant than has been previously thought.

The divergence of the Zygomycete *M. racemosus* prior to the Chytridiomycete *B. emersonii* was unexpected. When the fungi were given their own kingdom by Whittaker (1959), he placed the Chytridiomycetes in this kingdom and suggested that the Zygomycetes had evolved from a Chytridiomycete by loss of cilia (Whittaker, 1969). He later removed the Chytridiomycetes from the Fungal kingdom and placed them in the Protocista kingdom (Whittaker & Margulis, 1978). Cavalier-Smith (1981), who advocates a seven-kingdom classification of eukaryotic organisms that does not recognize the Protocista kingdom, places the Chytridiomycetes in the kingdom Fungi, phylum Archemyota. The Zygomycetes and other non-ciliated higher fungi are proposed to have evolved from a ciliogenous ancestor (Cavalier-Smith, 1987). While a close phylogenetic relationship between the Chytridiomycetes and higher fungi is supported by recent SSU rRNA sequence analysis (Foerster *et al.*, 1990), the ancestral relationships of the Zygomycetes and the Chytridiomycetes remain unknown.

The two Oomycetes, *Lagenidium giganteum* and *Phytophthora megasperma*, form a monophyletic group in agreement with previous SSU rRNA analysis (Foerster *et al.*, 1990). There has been much speculation regarding the phylogenetic relationship of the Oomycetes and fungi. The Oomycetes have been variously grouped with the higher fungi (Whittaker, 1959), the Protocista (Margulis, 1988), and with the chromophyte algae in the plant kingdom Chromista (Cavalier-Smith, 1981). Recent SSU rRNA sequence analysis supports this latter classification.

As other analyses of SSU RNA sequences of fungi are completed, dendrograms such as that reported here with *B. dermatitidis* should become increasingly helpful in fungal systematics.

The authors thank K. J. Kwon-Chung and M. Geber for their review of the manuscript, O. Gigiotti for his technical assistance, and C. Bennett for his help in the preparation of this manuscript.

**References**


Small ribosomal subunit RNA sequence of B. dermatitidis


