Restriction fragment length polymorphisms in isolates of *Aspergillus fumigatus* probed with part of the intergenic spacer region from the ribosomal RNA gene complex of *Aspergillus nidulans*

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Differences in restriction fragment length polymorphisms (RFLPs) have been detected in isolates of *Aspergillus fumigatus*. Genomic DNA from 11 isolates was digested with *Eco*RI, separated by electrophoresis, Southern blotted and probed with DNA from the intergenic spacer or non-transcribed spacer region of the rRNA gene complex of *Aspergillus nidulans*. Three distinct RFLP patterns were detected which differed from the control patterns observed with *A. nidulans*, *Aspergillus flavus* and *Aspergillus niger* hybridized with the same probe. Furthermore, the differences in RFLP patterns in the *A. fumigatus* isolates were not detected when probed with DNA coding for the rRNA complex in *Saccharomyces cerevisiae*. These findings may be of use in the study of the epidemiology and pathogenesis of infections caused by *A. fumigatus*.

Introduction

Aspergillus fumigatus is an ubiquitous mould in the environment and is an important nosocomial pathogen, particularly in the immunocompromised host where it is often fatal (Meyer *et al.*, 1973). *A. fumigatus* infections are usually detected by standard cultural and/or histological methods. The organism is identified on the basis of its colonial morphology and characteristic microscopic features and is generally regarded as a single homogeneous species (Rippon, 1988).

The advent of molecular biology techniques has allowed a more precise examination of microbial taxonomy. Restriction endonucleases used to analyse restriction fragment length polymorphisms (RFLPs) of DNA from many species including the fungi *Candida albicans* (Magee *et al.*, 1987; Matthews & Burnie, 1989), *Histoplasma capsulatum* (Vincent *et al.*, 1986) and *Yarrowia lipolytica* (Fournier *et al.*, 1986) have clearly shown distinct differences between isolates that appear morphologically identical. Gene probes based on the rRNA gene complex have been particularly useful due to their sensitivity, which is a consequence of the presence of at least 100 copies of the complex (Bell *et al.*, 1977). In

addition there are conserved regions which result in heterologous hybridization allowing general gene probes to be developed for a variety of unrelated organisms. It has also been shown that there are variable regions, particularly in the intergenic spacer (IGS) regions, between the 25S and 18S genes, which contain promoters and enhancer sequences (Tautz *et al.*, 1987). Detection of differences between species and individual isolates may be possible using probes for these variable regions.

Recently we probed DNA from several *Aspergillus* species with a 6.35 kb DNA fragment coding for the rRNA complex in *Saccharomyces cerevisiae* and found each had a characteristic RFLP pattern (Bainbridge *et al.*, 1990). In this study we have analysed DNA from several *A. fumigatus* strains using a DNA probe coding for a 1.2 kb fragment from the IGS region of the rRNA complex from *Aspergillus nidulans*.

Methods

Strains and DNA extraction. We studied eight clinical isolates of *A. fumigatus* (H91, H95, H111, H119, H138, H139, H171 and H201) obtained from patients at the Hammersmith Hospital, one laboratory contaminant (H170) and two strains (NCPF 2109 and 2140) from the National Collection of Pathogenic Fungi, London, UK. An isolate of *A. nidulans* (H102, wild-type), a clinical isolate of *Aspergillus niger* (H114) and a clinical isolate of *Aspergillus flavus* (H92) were also used.

Abbreviations: IGS, intergenic spacer; RFLP, restriction fragment length polymorphism.

Fig. 1. Autoradiographs of *Aspergillus* isolates after digestion with *Eco*RI. Hybridization was done with the 6.35 kb *S. cerevisiae* probe (a) and the 1.2 kb *A. nidulans* probe (b). Lanes: 1, *A. fumigatus* H95; 2, *A. fumigatus* H119; 3, *A. nidulans*; 4, *A. niger*; 5, *A. flavus*.

Fungi were maintained on Sabouraud's media (Sab) slopes at room temperature or at 4 °C. For DNA extraction conidia were inoculated onto liquid GYEP media. [2% (w/v) glucose, 0.3% (w/v) yeast extract, 1% (w/v) peptone] and grown overnight at 37 °C. Small-scale extraction of high M_r DNA was obtained as described previously (Bainbridge *et al.*, 1990). Briefly, the mycelia were treated with a lysis solution to degrade the cell wall, followed by deproteinization with proteinase K (Sigma) and phenol/chloroform extraction. RNA was removed by RNAase digestion and the DNA precipitated with propan-2-ol. After washing and drying, the DNA pellet was dissolved in up to 50 μ l TE buffer and stored at 4 °C.

Analysis of DNA. All DNA samples were restricted with *Eco*RI (Gibco BRL) for 3 h at 37 °C. One gel (see Fig. 1) was prepared comprising two *A. fumigatus* samples (H95 and H119) and the DNA from the other three *Aspergillus* species. Another gel (see Fig. 2) was loaded with all of the *A. fumigatus* samples. DNA (2–3 μ g per well) was loaded onto the gels and electrophoresed in 0.7% agarose overnight. Samples were run with a 1 kb DNA ladder M_r marker (Gibco BRL). After staining with ethidium bromide (0.5 μ g ml⁻¹) the gels were photographed under UV light and then transferred to nylon membranes (Amersham) by Southern blotting. The DNA was bound by UV transillumination. Gene probes were prepared from the 6.35 kb *Hind*III fragment from a recombinant plasmid SCP7 containing the ribosomal RNA gene from *S. cerevisiae* (Bell *et al.*, 1977) and from a 1.2 kb *Pst*I fragment of the pAR1 *A. nidulans* rRNA complex (Borsuk *et al.*, 1982; Lockington *et al.*, 1982). The 1.2 kb fragment lies entirely within the IGS region. The DNA fragments were labelled with

[³²P]CTP using a random oligonucleotide kit ('Prime-a-Gene', Stratagene Scientific). The blots were prehybridized at 65 °C for 3 h, hybridized overnight and washed with 1 $\times$ SSC/0.1% SDS at 65 °C. (1 $\times$ SSC is 0.15 M-NaCl/0.015 M-trisodium citrate, pH 7.0). Autoradiographs were obtained after 3 d exposure. The first blot (see Fig. 1) was washed with 0.1 $\times$ SSC/0.1% SDS at 100 °C to remove the labelled probe and hybridized with the 1.2 kb *A. nidulans* spacer probe.

Results and Discussion

Genomic DNA from a range of *A. fumigatus* isolates and from *A. nidulans*, *A. niger* and *A. flavus* was extracted and analysed following digestion with *Eco*RI. As previously reported (Bainbridge *et al.*, 1990), the *Saccharomyces* probe gave distinctive RFLPs with all species and the *A. fumigatus* isolates could be distinguished readily from the other species. Differences in the degree of homology can be seen clearly as the 0.9 kb genomic band from *A. nidulans* hybridized strongly with the homologous 1.2 kb *Pst*I probe but poorly with the 6.35 kb heterologous probe from *S. cerevisiae* (Fig. 1a, b). Hybridization with the *Saccharomyces* probe revealed no differences between the two isolates of *A. fumigatus* (Fig. 1a).

Fig. 2. Autoradiograph of 11 *A. fumigatus* strains hybridized with the 1.2 kb *A. nidulans* probe. Lanes: 1, *A. fumigatus* strain H91; 2, H95; 3, H111; 4, H119; 5, H138; 6, H139; 7, H170; 8, H171; 9, H201; 10, NCPF 2109; 11, NCPF 2140.

In contrast, hybridization of the same blot with the 1.2 kb spacer probe showed clear differences in RFLPs of the *A. fumigatus* isolates (Fig. 1*b*). Three bands of 8.0, 4.9 and 3.2 kb were present in both tracks. The 3.2 kb fragment gave a strong signal with the 6.35 kb probe (Fig. 1*a*) but a weaker signal with the 1.2 kb probe (Fig. 1*b*). As the same blot was analysed, this was not due to differences in the amount of DNA. This observation therefore suggests that the 3.2 kb fragment contains only a short region of homology with the spacer probe, and that the 4.9 kb fragment must contain most of the spacer region. However, the 8.0 kb fragment (more clearly visible in Fig. 2) contains the complete rRNA complex of *A. fumigatus* (B. W. Bainbridge, unpublished data) and may represent incomplete digestion of the two *Eco*RI sites or the absence of one *Eco*RI site in some copies of

the complex. Since this 8.0 kb fragment will contain the complete IGS region, homology with the probe should be strong; the weak signal obtained suggests that there were fewer copies of the 8.0 kb type in contrast to the more frequent 4.9 kb plus 3.2 kb type. As differences in other faint bands of H95 and H119 were observed, the analysis was extended to more isolates of *A. fumigatus*.

Eleven *A. fumigatus* isolates were examined and three RFLP patterns were detected (Fig. 2). One pattern, designated A, which accounted for more than half of the isolates had the common bands 8.0, 4.9 and 3.2, but in addition bands at 4.2 and 3 kb. Pattern B, which was observed in H201 and NCPF 2140, lacked the 4.2 and 3 kb bands but had an extra band at 2.8 kb. Pattern C (H119 and H171) shared the same basic pattern as A but the 3 kb band was missing and a 2.6 kb band was

present. All the isolates had the 4.9 and 3.2 kb fragments and this appears to be the structure found in the majority of copies of the rRNA complex. The 8.0 kb fragment also occurs in all strains but must be present at a low copy number as discussed above. The other fragments of 4.2, 3.0, 2.8 and 2.6 kb could therefore represent copies of the complex with variable multiples of a repeat unit of approximately 200 bp.

Earlier workers have reported differences in RFLPs of fungal rRNA gene complexes by multiples of 200–400 bp (Buckner *et al.*, 1988) and by analogy with *Xenopus* (Busby & Reeder, 1983), this variation may occur in the IGS region. Fournier *et al.* (1986) have shown that copies of the complex in *Y. lipolytica* are not identical and that these differences, which segregate at meiosis, are probably generated by unequal crossing over and gene conversion. From our findings it appears that similar variation in the rRNA complex also occurs in *A. fumigatus*. This confirms the usefulness of the rRNA gene complex for the generation of probes to distinguish between fungal isolates of the same species (Vincent *et al.*, 1986; Magee *et al.*, 1987).

These results are important for two reasons. Firstly, they show that intra-species differences do exist between isolates of *A. fumigatus*, confirming observations made previously (Raper & Fennell, 1965). More recently, support for this has been obtained by studies using the 'killer biotyping' system of Fanti *et al.* (1989) and the immunoblot fingerprinting technique of Burnie *et al.* (1989). However, the RFLP typing reported here provides direct proof of variation at the level of genomic DNA. Secondly, these strain variations may prove useful as epidemiological markers for the investigation of hospital outbreaks and may also have implications for studies of the pathogenesis of aspergillosis.

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