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Separation of chromosomal DNA molecules from *C.albicans* by pulsed field gel electrophoresis

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ABSTRACT

Modifications have been made to standard pulse field gel electrophoresis (PFGE) systems to enable very large DNA molecules to be resolved. The single most important modification was to elevate the temperature of electrophoresis to 35°C. This enabled the largest Saccharomyces cerevisiae chromosome to be reproducibly resolved. More impressively, it enabled the DNA of Candida albicans to be clearly resolved into six bands, a feat which was very difficult at lower temperatures. Even so, optimal resolution could only be obtained by carefully adjusting field voltages and switching times. The DNA from the two largest C. albicans chromosomes, which was estimated to be at least 5-10Mbp in size, ran somewhat anomalously, giving fuzzy bands which did not migrate in the direction of the average electric field. That the highest molecular weight band was a distinct chromosome was demonstrated by specific hybridisation to the C. albicans ADE2 gene probe. With further fine tuning, the PFGE system described here should be capable of resolving DNA from the smallest human chromosomes.

INTRODUCTION

Genetic methods offer much promise as a tool for characterising the different strains of Candida albicans which infect humans (1). However, the genetics of C. albicans is in its infancy and it is not even certain how many chromosomes this yeast contains (2). Indeed it was established only recently that C. albicans is normally diploid (3,4). In contrast, much more is known about the yeast Saccharomyces cerevisiae with genetic analysis indicating that the genome consists of 17 linkage groups. That 16 of these groups actually represent distinct chromosomes was spectacularly confirmed by pulse field gel electrophoresis (PFGE) which separated S. cerevisiae DNA into 12 distinct bands (the identification of chromosome 17 has yet to be made by this technique) (5). We felt that extension of the technique to C. albicans would be of great value in determining the total number of chromosomes and, in addition, permitting the cloning of chromosome specific probes for use in genetic analyses.

The present paper describes the approach used to separate C. albicans DNA into chromosome size molecules. Unlike S. cerevisiae DNA which is very amenable to PFGE analysis, several problems were encountered with C. albicans, the chief of which was the very much larger size of some of the chromosomes. This necessitated the modification of the original PFGE system described by Cantor etal (6,7) and, later, Carle and Olson (8).

MATERIALS & METHODS

C. albicans diploid strain ATCC 10261 and the S. cerevisiae strain 2180 was used in our investigations. Cells were grown to stationary phase by incubating for 3 days at 30°C in YPD media (1 gm yeast extract, 2 gms bacto peptone, 2 gms dextrose in 100mls). Cells were then washed twice in 0.05M EDTA ph7.5, resuspended at $1x10^{10}$/ml in 0.05M EDTA, equilibrated to 42°C, mixed with 2.5% low gel agarose (Sea Plaque FMC) (0.125M EDTA ph7.5) containing Zymolyase 5000 (Seikagaku Kogyo Co.) at a 0.75mg/ml final concentration and the mixture then set in a mould giving a final dilution of approximately $2x10^9$cells per ml. Agarose was then cut to the required size, soaked for 12 hours in 0.45M EDTA, 7.5% B-mercaptoethanol, 0.01M Tris-HCl ph7.5 at 37°C. Agarose inserts were then placed in 0.45M EDTA 0.01M Tris-HCl (ph 9.5) 1% lithium dodecyl sulphate & 1 mg/ml protinase K (Boehringer)@ 50°C for 12 hours. They were stored in this solution at room temperature for up to 8 days.

The pulse field gel electrophoresis apparatus was similar to that of Schwartz and Cantor(6,7). A schematic diagram of our electrode layout is shown

in Figure 1. A 1.5% agarose slab gel (Biorad Ultra pure DNA grade) with dimensions of 9cm x 9cm was cast with 13mm long and 2mm wide wells. Inserts containing DNA were placed against the front face of the well and sealed in with agarose. Running buffer used was TBE/2 (1xTBE is 90mM Tris base, 90mM Boric acid 2.5mM EDTA ph8.2), maintained at a constant temperature by pumping it through a heat exchanger, either heating or cooling depending on the required conditions. In all experiments the voltages were maintained at 8.2 V/cm in the forward and 3.1 V/cm in the cross direction, with pulse time and temperature varied as detailed in the figure legends. After running for 18-22 hrs, gels were removed and stained in 2μgm/ml ethidium bromide for 12 hours then photographed with Polaroid type 665 film using an ultraviolet transilluminator (Ultraviolet Products Inc. TM36).

DNA transfer hybridisation & autoradiography

The gel was treated with 0.25M HCl for 20 minutes at room temperature then soaked in 0.5M NaOH, 0.15M NaCl neutralised and transferred to Hybond-N filters (Amersham) by standard methods (9).

Probes were prepared from both total C. albicans DNA and the plasmid containing the C. albicans ADE2 gene. The ADE2 C. albicans probe was obtained through Kurtz et al (10). This probe contains both the 2 micron DNA ori and the LEU2 gene from S. cerevisiae plus pBR322 sequences and the ADE2 gene from C. albicans . Total C. albicans DNA was prepared using spheroplast and lysis solutions under the same conditions as for the insert preparation, but the cells were not set in agarose. The DNA was purified from the lysates by phenol extractions and ethanol precipitation. ^{32}P-labelled probes were prepared by nick translation (9) of approximately 250 ngms of DNA in each case. Autoradiography was carried out using Kodak XAR-5 X-ray film with no intensifying screen for ~4 hours at room temperature.

RESULTS & DISCUSSION

From the gel shown in Figure 2 it is clear that C. albicans DNA electrophoreses as at least six distinct bands. The leading two of these bands have fluorescence levels compatible with that expected in doublets (That is, about twice the fluorescence of bands 3 and 4). This, however, could be an artifact as DNA content has been reported (8) to decrease in the bands of the larger chromosomes. We therefore take our

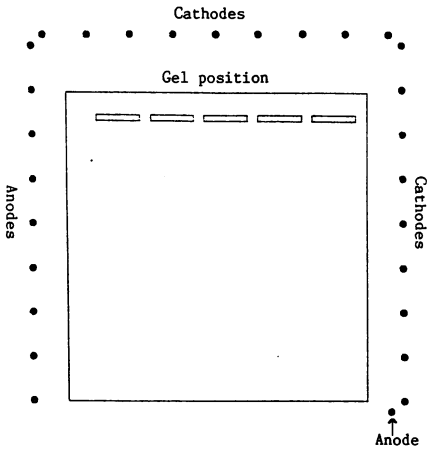

Figure 1

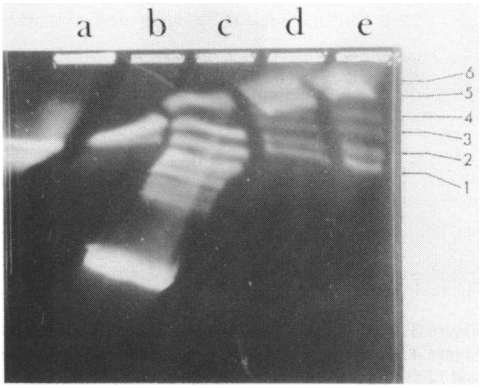

Figure 2

Figure 1 . A schematic illustration of the pulsed field gel electrophoresis electrode array and gel position.

Figure 2 . Separation of yeast DNA on a PFGE gel. Lanes marked b, d & e contain DNA from C. albicans strain 10261, lanes a & c DNA from S. cerevisiae strain 2180. Samples were electrophoresed for 21 hrs and then stained with ethidium bromide. The switching time was 125 sec. with a running temperature of 35°C. The six C. albicans bands are numbered on the right.

result to represent at least six chromosomes. The four fastest moving bands are relatively sharp while the two slowest bands are less distinct. These last two bands are of very high molecular weight DNA, somewhat larger than the largest S. cerevisiae band. Moreover, unless conditions were carefully optimised, these bands were not resolved at all, nor for that matter was the largest band in S. cerevisiae. Initially it was felt that the two high molecular weight bands, 5 and 6, could be an artifact, possibly consisting of an aggregate of DNA from the 4 lower molecular weight bands. In the absence of a battery of C. albicans single gene probes it was difficult to disprove this possibility. When the gel was blotted and then probed with ^{32}P labelled total C. albicans DNA, all six bands had some level of hybridisation, presumably due to highly repetitive DNA sequences (Fig.3). Apart from some variation in hybridisation from band to band, with band 3 being especially weak, this autoradiograph essentially confirms the ethidium bromide staining pattern. We did, however, find some cross hybridisation to S. cerevisiae band 12 DNA which is reported to represent chromosome 4 (5). This cross hybridisation could be to the ribosomal sequences reported to be on this S. cerevisiae chromosome (5).

Fortunately, the one available C. albicans single-copy probe, ADE2, did give a definitive result, specifically hybridising to band 6 (Fig.4). We therefore conclude that that band 6 is not an artifact and represents a C. albicans chromosome of high molecular weight. Furthermore, as the other bands contain lower molecular weight DNA it is unlikely that these represent artifactual aggregation of DNA. Thus we conclude there are at least 6 and possibly 8 distinct chromosomes in Candida albicans.

We are uncertain of just how large the DNA in these bands is because of the lack of suitable molecular

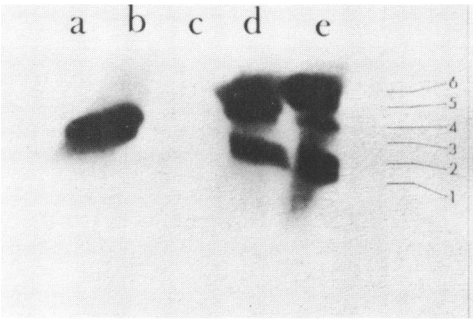

Figure 3

Figure 4

Figure 3. Hybridisation of total C. albicans DNA to the Southern blot of the gel in figure 2.
Figure 4. Hybridisation of the C.albicans ADE2 gene probe, (also containing S. cerevisiae 2 micron and LEU2 gene DNA) to the Southern blot of the gel in figure 2.

weight markers. However an approximate estimate can be made from the data of Riggsby et al (4) who measured the DNA content for a diploid strain of C. albicans as 37fg/cell. Assuming no DNA content difference between strains and an average molecular weight per base pair of 670 daltons gives a total of ~33x10^6 bp. The average molecular size for 6 and 8 chromosomes, respectively, is ~2.8x10^6 bp and ~2.1x10^6 bp. Not withstanding the inaccuracies of this calculation the largest chromosome probably approaches 10 Mbp in size.

As has already been mentioned PFGE conditions have to be critically adjusted to get the results shown in Fig 2. Alteration of the relative voltage conditions by 10%, the pulse time by 20%, or the temperature by 10% destroy the electrophoretic pattern. This exquisite sensitivity of the system is also reflected in the electrophoresis pattern in Figure 2 where DNA bands of different sizes electrophorese at very different rates as one moves across the gel. Thus on the left hand side the relative rates of migration for the smallest and largest bands are 1.1:1 increasing to about 2:1 in the centre and about 6:1 on the right. Another peculiarity of the system is that the bands do not all migrate along the lines of average electric force. This last effect is not so obvious when the gel orientation of Carle and Olson (8) is used with the average electric field parallel to the length of the gel as in conventional electrophoresis. Nevertheless we felt our field orientation (Fig.1) had advantages in the present application because it maximised the probability of achieving optimal electrophoresis conditions at some point across the gel.

The ADE2 probe also contains the LEU2 gene from chromosome 3 of S. Cerevisiae which is evident in lane c of Fig.4 as a partialy obscured band at the bottom of the pattern; the smear from this band back to the well is probably 2 micron DNA (5). The 2 micron DNA does not significantly stain with ethidium bromide and hence is not visible on Fig.2. Interestingly this DNA does not travel in the same direction as the chromosomes but in the direction of the net field. This implies that either the 2 micron DNA is slowly being released from the wells or that it has an unusual conformation. As the chromosome bands are not smeared it is probably the latter.

By running gels at 35°C it was also possible to resolve S. cerevisiae DNA detail in the very high

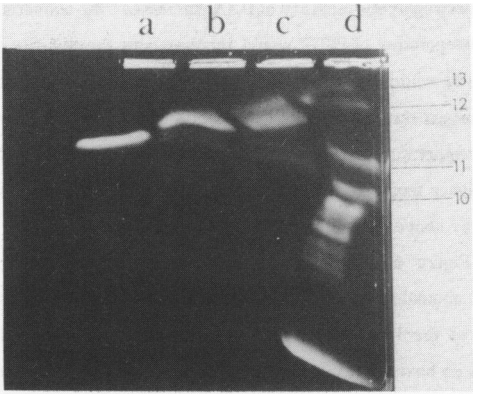

Figure 5. Resolution of the largest S. cerevisiae chromosome on a PFGE gel. Lane d shows the 3 largest S. cerevisiae chromosome bands (nos. 10-12) resolved by Carle & Olson (5) plus another large band of high M.W. (labeled 13). Lanes (a-c) are C. albicans DNA. The gel was run for 21 hrs at 35°C with a switching time of 115 sec.

molecular weight region that proved a problem in Carle and Olsen's (5,8) work. The highest molecular weight band in their work was designated no.12 and it was not always clearly resolved, often remaining in the region of the well. Under our PGFE conditions the DNA band no.12 is clearly resolved and another, slower moving band which we designate no.13, is also visible (Figure 5). We did not have the necessary probes to test whether or not this band corresponded to chromsome 17 but nevertheless, the present results clearly show that modifying the PFGE method does permit larger DNA molecules to be analysed. The theoretical basis of these improvements is not clear.

A crucial aspect of the experiments described here is the temperature of the gel, so it is worthwhile discussing this parameter in some detail. If one accepts the theory of PFGE put forward by Schwartz etal (6), the fact that the viscoelastic relaxation time of DNA decreases with temperature (11) is just as

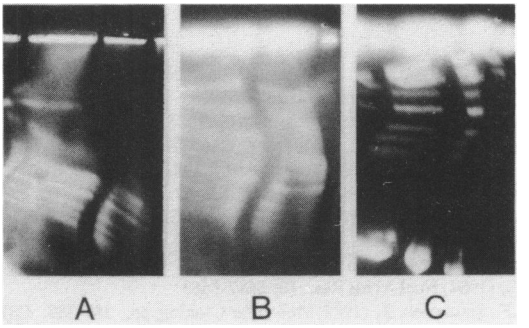

Figure 6. Effect of temperature on PFGE of S. cerevisiae DNA Gel A was run at 40°C, B at 30°C and C at 11°C. All were run for 21 hrs at a switching time of 80 sec.

important as the fact that electrophoretic mobility of DNA increases with temperature (12). Thus one would expect that increasing the temperature of PFGE would increase both the mobility of the DNA and the size of the DNA molecules which would enter the gel. These expectations were borne out in our preliminary experiments in which the largest chromosomes of C. albicans and S. cerevisiae only entered the gel and were clearly resolved at temperatures above 25°C. Although the mobility of these larger chromosomes increased even further at higher temperatures, that of the smaller chromsomes did not, so that the overall resolution began to fall away above 35°C (Figure 6).

Close examination of Figure 6 also shows that the high molecular weight bands, which at low temperature migrate in an anomalous orientation with respect to the net electric field, rotate into an orientation similar to that of the lower molecular weight bands at high temperatures. Nevertheless, these high molecular weight bands do have some odd properties, namely they are much fuzzier than low molecular weight bands and under some conditions (Figure 2) they actually travel in an orientation so different from the lower molecular weight bands that they partially overlap with them so that, for example, in Figure 2 lane d there is an overlap between bands 4 & 5 . Whether these phenomena reflect a different DNA conformation or simply the anomalous electrophoresis of very high molecular weight DNA under sub-optimal PFGE is not clear.

In summary, the data presented here show that it is possible to resolve C.albicans DNA into at least six chromosomal bands. This result has two immediate applications. First, by isolating DNA from the individual C. albicans DNA bands it will be possible to prepare chromosome specific DNA probes. Second, different strains of C. albicans can be analysed by PFGE using these probes to determine translocations, aneuploidy etc in much the same way as been done for S. cerevisiae (8). Finally, the modified PFGE technique described here suggests that by carefully controlling the electrophoresis parameters it may be possible to separate DNA molecules of several tens of megabases such as in the smallest human chromosomes.

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