FEMS Microbiology Letters 115 (1994) 77-82

© 1994 Federation of European Microbiological Societies 0378-1097/94/$07.00
Published by Elsevier

FEMSLE 05749

Analysis of hybrids of *Candida albicans* formed by protoplast fusion

Carolyn Law *, Kevin Kavanagh [1] and Peter Whittaker

Department of Biology, Maynooth College, Maynooth, Co. Kildare, Ireland

(Received 16 September 1993; revision received and accepted 18 October 1993)

Abstract: A number of hybrids obtained by fusion of protoplasts of complementary auxotrophic strains of *Candida albicans* were analysed for growth rates, cell volume, DNA content, stability and adherence to human buccal epithelial cells. SDS-PAGE analysis of the cell wall proteins indicated that the hybrid cell walls contained many more of the proteins associated with one parent than the other. The adherence values of the hybrids were closest to the parent with which they shared most cell wall proteins and it is suggested that the hybrids contain the genome of this parent along with one or more of the chromosomes of the other.

Key words: Candida; Adherence; Protoplast fusion; Hybrid

Introduction

The yeast *Candida albicans* is a dimorphic, asexual fungal pathogen capable of initiating a range of superficial and systemic infections in humans. The incidence of *Candida* diseases has increased dramatically in recent years due to the advent of Acquired Immune Deficiency Syndrome (AIDS), the use of immunosuppressive therapy and the widespread use of broad-spectrum antibiotics [1]. A key stage in pathogenesis is the ability of cells of *C. albicans* to adhere to a range of host tissues [2]. Yeast wall mannoproteins [3], lipids [4] and chitin [5] have all been identified as putative adhesins.

C. albicans is classified among the fungi imperfecti because of the absence of a known sexual cycle for this diploid yeast, thus the organism is not amenable to conventional genetic analyses or manipulation. The application of protoplast fusion techniques to this yeast has allowed the creation of a parasexual cycle [6,7]. Fusion is generally achieved between protoplasts of complementary auxotrophic strains and selection imposed for the isolation of prototrophic hybrids.

The work presented here describes the characteristics of the hybrids formed from a single such fusion event between two doubly auxotrophic strains of *C. albicans*. The hybrids were analysed for growth rates, cell volume, DNA content, adherence values and electrophoretic profiles of their cell wall proteins. Adherence values were

* Corresponding author.

[1] *Present address:* The National Cell and Tissue Culture Centre, Dublin City University, Glasnevin, Dublin 9, Ireland.

SSDI 0378-1097(93)E0441-E

Downloaded from https://academic.oup.com/femsle/article-abstract/115/1/77/482980
by Maynooth University user
on 29 January 2018

considered along with the electrophoretic profiles of the cell wall proteins of parental and hybrid strains and we report here that the adherence properties and cell surface proteins of the hybrids have greater similarity to one parental strain than the other.

Materials and Methods

Strains

The following strains were used in this work: *Candida albicans* ATCC 44987 (his^-, arg^-) and *C. albicans* ATCC 44990 (ade^r, thr^-).

Media and culture conditions

Cells were routinely grown in 50 ml of YEPD (2% (w/v) glucose (BDH), 1% (w/v) bactopeptone (Oxoid), 1% (w/v) yeast extract (Oxoid)) at 30°C and 200 rpm in an orbital shaker.

Minimal medium (MM) contained 2% (w/v) glucose, 0.17% (w/v) yeast nitrogen base (without amino acids and ammonium sulphate (Difco)), and 0.5% ammonium sulphate. Where appropriate amino acid supplements were added at a final concentration of 20 mg l^{-1}.

All media were solidified by the use of 3% (w/v) Bactoagar (Oxoid).

Protoplast preparation and fusion

The method and conditions employed for protoplast isolation and fusion have been detailed elsewhere [8]. Briefly, exponential phase cells were harvested, washed and resuspended in protoplasting medium (1.45 mg ml^{-1} Novozym 234 (Novo Industri, Denmark) and 0.017 ml ml^{-1} Suc d'*Helix pomatia* (IBF Biotechnics, France) in osmotically stabilised MP Buffer at pH 5.5) for 45 min at 30°C. The resulting protoplasts were harvested and washed. Fusion was achieved by mixing equal numbers of protoplasts of the complementary strains in a fusogen mixture consisting of PEG and a 0.1 M solution of calcium acetate. After the appropriate incubation time the protoplasts were harvested, washed and plated out at a density of 1×10^6 complementary protoplast pairs per plate of selection medium (MM).

Growth rates

Strains were grown overnight in YEPD (37°C and 200 rpm), harvested, diluted and resuspended in the same medium at an initial concentration of 5×10^5 cells ml^{-1}. Growth was monitored by haemocytometer count over a period of 12 h with a final count at 24 h.

Determination of cell volume

The cell volumes of stationary phase parental and fusant strains were determined using the equation for the volume of a prolate ellipsoid:

$$V = 1.33 \Pi a b^2$$

where a is equal to half the length of the long axis and b is equal to half the length of the short axis. In all cases the dimensions of 50 cells were measured.

DNA estimation

DNA was extracted from mid-exponential phase cells with perchloric acid and quantified using the diphenylamine assay [9]. Salmon sperm DNA (Sigma) was used to prepare standard curves.

Adherence tests

The method used was that of Kimura and Pearsall [10] with some modifications. Yeast strains were grown overnight in YEPD to stationary phase, cells were harvested on a bench top centrifuge, washed once with Hank's Balanced Salt Solution (HBSS) and resuspended in 1 ml HBSS.

Buccal epithelial cells (BECs) were collected from five healthy human volunteers by gently scraping the mucosal surface of the cheeks with a sterile tongue depressor and agitating in 5 ml HBSS to remove attached cells. The cells were then pooled, harvested, washed once with HBSS and finally resuspended in 1 ml HBSS.

1 ml of the yeast cell suspension (10^7) was then added to the BEC suspension (10^5) and the mixture was incubated for 1 h at 37°C on an orbital shaker at 200 rpm. After incubation the cell suspension was filtered through a polycarbonate mesh (18 μm) and washed through with 10 ml of HBSS to remove unattached yeasts. The final 1

Downloaded from https://academic.oup.com/femsle/article-abstract/115/1/77/482980
by Maynooth University user
on 29 January 2018

ml of this suspension was mounted on a glass slide, air-dried, heat-fixed and stained with 1% Crystal violet for 1 min.

Adherence was determined microscopically by counting the number of yeasts adhering to at least 100 BECs. All assays were performed in duplicate.

Extraction of cell wall proteins

Cell wall proteins were extracted using techniques adapted from the methods of Hoberg et al. [11] and Catley [12].

Cells were pelleted and washed twice with HBSS. The pellet was resuspended in HBSS and a portion of glass beads (0.4 mm (40 mesh)) added to the suspension which was cooled on ice for 10–15 min. Phenylmethylsulphonylfluoride (PMSF) (Sigma) was added to the mixture to a final concentration of 1 mM and the cells broken up by agitating on a bench top whirly mix using 1 min pulses with cooling on ice in between pulses. Breakage was monitored by light microscopy and when $\geq 80\%$ breakage (estimated visually) was obtained supernatants were transferred to cooled centrifuge tubes by aspirating with a Pasteur pipette. The beads were then washed three times and the washes added to the original broken cell suspension.

Emulsions were pelleted (5000 rpm, 15 min, 4°C), washed four times with cold HBSS and then extracted at 100°C (10 min) with SDS-sample buffer (2.3% sodium lauryl sulphate (SDS), 5% β-mercaptoethanol, 10% glycerol, 62.5 mM Tris hydrochloride (pH 6.8)). The samples were centrifuged (6000 rpm) and supernatants transferred into 20 ml of cold ethanol and precipitated overnight at 4°C. The precipitates were washed twice with cold distilled water and then twice with cold acetone. Pellets were dried at room temperature, resuspended in SDS sample buffer and heated gently for 5 min (until maximal solubilisation had occurred). Samples were again pelleted and supernatants precipitated overnight with ethanol (−20°C). The precipitates were pelleted, washed and dried as before.

SDS polyacrylamide gel electrophoresis (SDS-PAGE)

Samples for SDS-PAGE were resuspended in SDS sample buffer and placed in a boiling water bath for 60 s. 1 drop of tracking dye (0.1% Bromophenol blue) was added to each sample which was then loaded onto a 15% polyacrylamide gel and subjected to electrophoresis at 120 V for 8–10 h. The proteins were fixed, stained with Coomassie brilliant blue overnight and the gels destained (10% acetic acid, 20% methanol) and rinsed with distilled water.

Results and Discussion

In this paper we present an analysis of the fusion products obtained from a single intra-

Table 1

Cellular dimensions and DNA content of parental and hybrid strains

Strain	Cell dimensions			DNA content (fg/cell)
	Length (μm) [a]	Width (μm)	Volume (μm^3)	
JJIA	6.25 ± 1.01	4.85 ± 1.01	80.7 ± 37.2	18 ± 0.9
ATCC 44987	6.12 ± 1.02	5.26 ± 1.22	88.4 ± 5.3	31 ± 1.7
ATCC 44990	7.49 ± 1.66	5.44 ± 1.43	122 ± 25	29 ± 1.0
CAH 1	9.04 ± 1.28	6.64 ± 0.74	208 ± 74	56 ± 2.4
CAH 2	9.06 ± 0.69	6.10 ± 0.51	176 ± 24	61.5 ± 1.5
CAH 3	8.90 ± 1.42	5.68 ± 0.96	150 ± 22	71 ± 2.2
CAH 4	8.60 ± 1.05	5.76 ± 1.32	149 ± 23	45 ± 1.1
CAH 5	8.86 ± 1.62	6.82 ± 1.69	215 ± 32	39.5 ± 1.8

Cells were grown at 30°C for 48 h in 50 ml YEPD in 125-ml conical flasks at 200 rpm. Values are the mean of 50 determinations for each strain. DNA was extracted and quantified as described in Materials and Methods. All values are the means of three separate determinations.

[a] Errors are the standard error of the mean.

Downloaded from https://academic.oup.com/femsle/article-abstract/115/1/77/482980
by Maynooth University user
on 29 January 2018

specific fusion event between two complementary auxotrophic strains of *Candida albicans*. Protoplasts of the strains were induced to fuse using a mixture of polyethylene glycol (PEG) 3350 (40% w/v) as fusogen and 0.1 M calcium acetate as a source of Ca^{2+} ions [8]. After incubation at 30°C for 7–10 days, hybrids were detected as colonies embedded in MM. Individual colonies were selected randomly for analysis and numbered arbitrarily from 1 to 5 (CAH1–CAH5). Growth of these strains and both parents was monitored over a period of 24 h and mean doubling times calculated. Both parental strains (ATCC 44987-86 ± 5 min and ATCC 44990-96 ± 20) had relatively similar doubling times while the hybrids had doubling times ranging from 50–68 min, in all cases shorter than those of the parents. These faster doubling times are probably due to the restoration of prototrophy in the hybrids as a result of protoplast fusion, while the slower rates of the auxotrophic parents may be due to blocks in the biosynthetic pathways for the particular metabolites through mutation and/or inefficient uptake of the pathway end product itself [13].

Cellular dimensions and DNA content of all the strains were also measured. *Saccharomyces cerevisiae* JJ1A, a known haploid strain, was included for reference purposes (Table 1). The hybrid strains all had larger cell volumes than either parent and there was considerable variation in the hybrid cell sizes themselves with the smallest hybrid, CAH4, being only slightly bigger than the larger parent while the largest hybrid, CAH5, was almost twice the volume of this parent. DNA measurements also showed similar variation, the hybrids all contained more DNA than either parent with no apparent correlation between cell volume and DNA content, showing aneuploid DNA levels ranging from 1.27 to 2.29 times the DNA content of parent ATCC 44987. The cell volumes of the hybrids were not directly proportional to their DNA contents; Sarachek et al. [6] proposed that this might be due to karyogamy in heterokaryotic cells generating diploid nuclei which either stabilise or undergo spontaneous chromosome loss to various states of aneuploidy. The data also point to CAH1 being the only chromosome loss to various states of aneu-

Table 2

Adherence capabilities of strains

Strain	Yeast cells/100 BECs	% Adherence [a]
ATCC 44987	365 ± 16	100
ATCC 44990	155 ± 11	42
CAH1	187 ± 7	51
CAH2	229 ± 12	63
CAH3	232 ± 10	64
CAH4	189 ± 13	52
CAH5	173 ± 9	47

Adherence was measured as described in Materials and Methods. Assays were carried out in duplicate and the results are the mean of three separate determinations.

[a] Percentage adherence relative to strain ATCC 44987.

ploidy. The data also point to CAH1 being the only probable diploid with approximately twice (2.34) the cell volume and twice (1.90) the DNA content of parent ATCC 44987. CHEF gel analysis showed no quantifiable differences in chromosome contents (unpublished observations).

Stability of the hybrids is indicated by the lack of detectable spontaneous segregation despite extensive screening. Even after either UV mutagenesis or growth in the presence of *m*-fluorophenylalanine there was less than 3% segregation in all colonies tested (results not shown). No segregants could be detected with CAH4 while the other hybrids produced segregants with an auxotrophic requirement for one of the four parental markers: CAH1 and CAH2 gave rise to segregants requiring adenine, and CAH3 and CAH5 produced segregants requiring histidine.

The ability of all strains to adhere to human BECs was investigated as an indicator of the relative pathogenicities of the different strains (Table 2). Parent ATCC 44987 had the highest adherence values and it was therefore used as the reference strain. Parent ATCC 44990 had the lowest adherence values: at 42% exhibiting less than half the adherence capability of the reference strain. The hybrids all had adherence values intermediate between those of both parental strains ranging from 47 to 64% of the reference strain but generally closer in magnitude to that of parent ATCC 44990.

Downloaded from https://academic.oup.com/femsle/article-abstract/115/1/77/482980
by Maynooth University user
on 29 January 2018

On the basis of the adherence results and cytological analyses performed, three hybrid strains were selected for further study: CAH3 (the smallest of the hybrids with the highest DNA content and highest adherence values); CAH1 (the only probable diploid with adherence in the middle of the hybrid range), and CAH5 (the largest of the hybrids with the smallest DNA content and lowest adherence value). The cell wall proteins of these strains and the two parental strains were extracted and subjected to SDS-PAGE on a 15% polyacrylamide gel for 10 h at 120 V. The protein profiles so produced also point to the closer relationship between the hybrids and parent ATCC 44990 (Fig. 1). There were some bands in common to all the strains, of particular interest is the band above 30 kDa (in the region 31.5–34 kDa) which is characteristic of a mannoprotein with cross-reactivity between *S. cerevisiae* and *C. albicans* which is found in the cell walls of many ascomycetous yeasts and related anamorphic forms [14,15]. At molecular masses over 50 kDa the hybrids show more similarity to parent ATCC 44987 with four bands visible at approximately 55 kDa, 70 kDa, 73 kDa (doublet) and 75 kDa. At lower molecular masses ≤ 50 kDa there was more similarity to parent ATCC 44990 with nine bands visible ranging from 21 to 48 kDa. Overall, the protein profiles of the hybrids show greater similarity to that of parent ATCC 44990, suggesting that the hybrids are more closely related to this parental strain than the other. This phenomenon of greater similarity to one or other of the partners in a fusion has been reported before in both inter- and intraspecific and intergeneric crosses in yeast [16–20]. In the case of the intraspecific fusion reported here it appears that parent ATCC 44990 may be the dominant partner, and the resulting hybrids may be all aneuploids containing the entire chromosome complement of this parent along with one or more of the chromosomes from parent ATCC 44987.

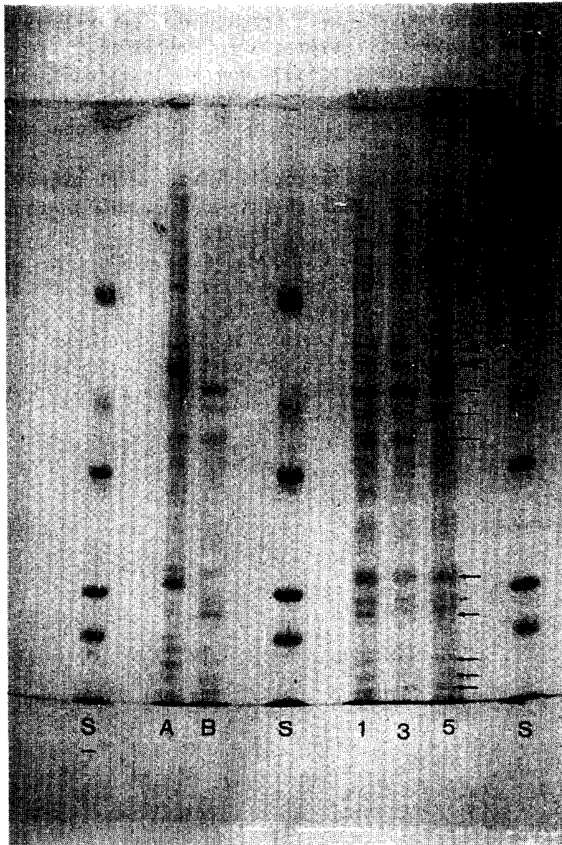

Fig. 1. SDS-PAGE analysis of cell wall proteins. SDS-PAGE (15% polyacrylamide) was carried out as described in Materials and Methods. Samples were subjected to electrophoresis for 8–10 h at 120 V. Samples loaded onto gel as follows: Lane A, ATCC 44987; lane B, ATCC 44990; lanes 1,3,5, hybrids CAH1, CAH3, CAH5, respectively. Lane S, Molecular mass standard markers (Sigma) as follows from top of gel (in kDa): bovine albumin, 66; egg alkumin, 45; glyceraldehyde-3-phosphate dehydrogenase, 36; carbonic anhydrase, 29; trypsinogen, 24; trypsin inhibitor, 20.1; and α-lactoalbumin, 14.2 (not visible).

Acknowledgement

This work was supported in part by Eolas, the Irish Science and Technology Agency.

References

1 Odds, F.C. (1988) Candida and Candidosis. 2nd edn. Balliere Tindall, London.

Downloaded from https://academic.oup.com/femsle/article-abstract/115/1/77/482980
by Maynooth University user
on 29 January 2018

2 Kennedy, M.J. (1988) Adhesion and association mechanisms of *Candida albicans*. Curr. Topics Med. Microbiol. 3, 73–169.
3 McCourtie, J. and Douglas, L.J. (1984) Relationship between cell surface composition adherence and virulence of *Candida albicans*. Infect. Immun. 45, 6–12.
4 Ghannoum, M.A., Burns, G.R., AbuElteen, K. and Radwan, S.S. (1986) Experimental evidence for the role of lipids in adherence of *Candida* spp. to human buccal epithelial cells. Infect. Immun. 54, 189–193.
5 Segal, E., Lehrer, N. and Ofek, I. (1982) Adherence of *Candida albicans* to human vaginal epithelial cells: inhibition by amino sugars. Exp. Cell Biol. 50, 13–17.
6 Sarachek, A., Rhoads, D.D. and Schwarzhoff, R.H. (1981) Hybridization of *Candida albicans* through fusion of protoplasts. Arch. Microbiol. 129, 1–8.
7 Poulter, R., Jeffrey, K., Hubbard, M.J., Shepherd, M.G. and Sullivan, P.A. (1981) Parasexual genetic analysis of *Candida albicans* by sphaeroplast fusion. J. Bacteriol. 146, 833–840.
8 Kavanagh, K., Walsh, M. and Whittaker, P.A. (1991) Enhanced intra-specific protoplast fusion in yeast. FEMS Microbiol. Lett. 81, 283–286.
9 Stewart, P.R. (1975) Methods Cell Biol. 12, 111–147.
10 Kimura, L.H. and Pearsall, N.N. (1978) Adherence of *Candida albicans* to human buccal epithelial cells. Infect. Immun. 21, 64–68.
11 Hoberg, K.A., Cihlar, R.L. and Calderone, R.A. (1986) Characterisation of Cerulenin-resistant mutants of *Candida albicans*. Infect. Immun. 51, 102–109.
12 Cately, B.J. (1988) Isolation and analysis of cell walls. In: Yeast. A Practical Approach. (I. Campbell and J.H. Duffus, Eds.), pp. 163–183.
13 Kirsch, D.R. and Whitney, R.R. (1991) Pathogenicity of *Candida albicans* auxotrophic mutants in experimental infections. Infect. Immun. 59, 3297–3300.
14 Cassone, A. (1989) Cell wall of *Candida albicans*: Its functions and impact on the host. In: Current Topics in Medical Microbiology (M.R. McGinnis and M. Borgers, Eds.), Vol. 3, pp. 248–314.
15 Herrero, E., Sanz, P. and Sentandreu, R. (1987) Cell wall proteins liberated by zymolyase from several ascomycetous and imperfect yeasts. J. Gen. Microbiol. 133, 2895–2903.
16 Groves, D.P. and Oliver, S.G. (1984) Formation of intergeneric hybrids of yeast by protoplast fusion of *Yarrowia* and *Kluyveromyces* species. Curr. Genet. 8, 49–55.
17 Kobori, H., Takata, Y. and Osumi, M. (1991) Interspecific protoplast fusion between *Candida tropicalis* and *Candida boidinii*: Characterisation of the fusants. J. Ferment. Bioeng. 72, 439–444.
18 Kucsera, J. and Ferenczy, L. (1986) Interspecific hybridisation between *Candida albicans* and *Candida tropicalis*. FEMS Microbiol. Lett. 36, 315–318.
19 Suzuki, T., Rogers, A.L. and Magee, P.T. (1986) Inter- and intra-species crosses between *Candida albicans* and *Candida guillermondi*. Yeast 2, 53–58.
20 Whittaker, P.A. and Leach, S.M. (1978) Interspecific hybrid production between the yeasts *Kluyveromyces lactis* and *Kluyveromyces fragilis* by protoplast fusion. FEMS Microbiol. Lett. 4, 31–34.

Downloaded from https://academic.oup.com/femsle/article-abstract/115/1/77/482980
by Maynooth University user
on 29 January 2018