CHAPTER 8

Pulsed field gel electrophoresis (PFGE) of yeasts

Teun Boekhout[1] and Elena Naumova[2]

[1]Centraalbureau voor Schimmelcultures
Uppsalalaan 8
3584 CT Utrecht
boekhout@cbs.knaw.nl

[2] State Institute for Genetics and Selection of Micro-organisms
I-Dorozhnyi 1
113545 Moscow

1. Introduction

The pioneering work of Carle and Olson (1) and Carle et al. (2) showed that the separation of chromosome sized DNA molecules of yeasts was possible using periodic inversion of electric fields. The resulting electrophoretic karyotypes have provided data of potential use in systematic studies of yeasts and have been widely applied in yeast systematics, genetics and epidemiology. In particular, the number and size of individual chromosomes, estimates of total genome size, chromosomal rearrangements and the assignment of genes to particular chromosomes have been among the characteristics studied. Taxonomically, these data have been used at or below the species level, e.g. to differentiate species, strains and/or populations. Electrophoretic karyotypes have been explored for many different yeast species, including *Saccharomyces cerevisiae* (3-5), *Candida albicans* (6-10), *Cryptococcus neoformans* (11-13), *Malassezia* species (14-16) and many others (17-20). These studies not only demonstrated that yeast species markedly differ in their chromosomal make-up, but also that chromosomal length polymorphism (CLP) occurs within most species (10, 11, 18). This renders PFGE a useful tool for the investigation of yeasts in epidemiology, nosocomial outbreaks, susceptibility and pathogenicity studies, property rights issues and in the tracking of industrial production strains.

A straightforward interpretation of electrophoretic karyotypes is sometimes complicated by the presence of chromosomal length polymorphism, aneuploidy and the occurrence of co-migrating bands. Ribosomal DNA-containing chromosomes may show considerable length variation, causing chromosomal heterogeneity and several species of yeasts in fact represent complexes of microspecies. For instance, in the *Saccharomyces cerevisiae* species complex, a correlation occurs between similarities of species based on DNA reassociation experiments and similarities of electrophoretic karyotypes (20). Interestingly, some species show rather variable karyotypes, e.g. *Candida albicans* (10) and *Cryptococcus neoformans* (11), whilst others have remarkably stable karyotypes, e.g. *Malassezia* species (14, 15). Also, chromosomal changes in some species have been found to be associated with phenotypic switching in *C. albicans* (10), and in *C. neoformans* after transfer through mice (12).

Several different technological platforms have been developed to separate yeast chromosomes, including field inversion gel electrophoresis (FIGE), transverse alternating field electrophoresis (TAFE), orthogonal field alternation gel electrophoresis (OFAGE) and rotating gel electrophoresis (e.g. ROTAPHOR). However, the preferred technique in the most recent studies is the contour clamped homogenous electric field (CHEF), which has proved to be technologically robust, combines good resolution over a wide range of sizes and yields straight-migrating lanes and good reproducibility. However, though chromosomal DNA of up to ca. 6 Mb can be easily separated, the separation of larger chromosomal DNA is still difficult, requiring long pulse- and run times.

Every species has a different chromosomal make up, therefore PFGE conditions have to be optimized for each species to be tested. Also, chromosomes may differ widely in size within a particular species, consequently different electrophoretic conditions may be required in order to separate the smaller and larger chromosomes of that species. Important parameters in

separating chromosomal DNA include the field strength, length of the pulse time, duration of the electrophoresis, concentration of the agarose and the temperature during electrophoresis. In general, the agarose concentration and field strength to be used is inversely proportional to the size of the chromosomal DNA to be resolved, whereas the pulse time is directly proportional to the chromosomal size.

For optimization ånd control purposes, chromosomal DNA of *Saccharomyces cerevisiae*, *Pichia canadensis* (= *Hansenula wingei*) and *Schizosaccharomyces pombe* are available commercially as size standards (Biorad, Richmond, U.S.A.).

Possible modifications of the standard PFGE protocol include the use of different and/or ramping pulse times in combination with different electrophoresis times (*see* Table 1), such modifications being particularly useful for separating chromosomes with a different range of size. A further modification is the possible use of restriction enzymes to reduce the size of large chromosomes (8, 9, 17, 21). PFGE gels have also been used to analyze the position of genes on individual chromosomes by hybridizing gene probes to blots of electrophoretic karyotypes (18).

Different protocols have been developed for the study of chromosomal DNA of yeasts. One of the most widely used methods is the preparation of agarose plugs containing yeast cells with subsequent cell lysis within the plugs per se. A disadvantage of this procedure is that control of the protoplasting step is limited. An alternative is to protoplast the cells prior to the preparation of the plugs. It should also be kept in mind that the cell walls of ascomycetous and basidiomycetous yeasts differ widely in their biochemical composition. Consequently, different lysing enzymes are required for these two groups of yeasts. Protoplasts of ascomycetous yeasts (such as *Candida* and *Saccharomyces*) may be prepared with zymolyase, though this enzyme is not suitable for the preparation of protoplasts of basidiomycetous yeasts, such as *Cryptococcus* and *Malassezia* species. In the past, in our laboratory, good results have been obtained in protoplasting cells of these basidiomycetous yeasts using Novozym 234 (Novo Industri, Denmark). Unfortunately, this product is not commercially available anymore. However, alternative lysing enzymes from *Trichoderma harzianum* and *Rhizoctonia solani* (Sigma, St. Louis, U.S.A.) have now been tried and successfully used for protoplasting both asco- and basidiomycetous yeasts.

2. Methods for preparing agarose embedded protoplasts, electrophoresis, endonuclease digestion and hybridization with gene probes

2.1. Preparation of agar embedded protoplasts using the lysing enzymes of Trichoderma harzianum

1. Rotary-shaken (ca. 200 rpm) cultures are grown until late logarithmic phase in 50 ml 1% yeast extract-0.5% peptone-4% glucose (YPG) broth at 25°C. For basidiomycetous yeasts (e.g. Cryptococcus spp.), 0.05% yeast extract-0.5% peptone-0.7% malt extract (YPM) broth is preferred. Psychrophilic species require lower temperatures, e.g. 12° or 17°C.
2. Approximately 0.5 x 10^9 cells are harvested by centrifugation, and washed in 2 ml 0.05 M EDTA, pH 7.5.

3. The pellet is suspended in ca. 2 ml buffer made up of 0.05 M EDTA, 10 mM Tris-HCl, and 10 mM dithiothreitol (DTT), to achieve a final pH of 7.5.
4. After centrifugation, the pellet is washed with 2 ml CPE buffer (100 ml 40 mM citric acid, 120 mM Na2PO4 (pH 6.0) and 4 ml 0.5 M EDTA, (pH 7.5).
5. Centrifuge and suspend the pellet in 0.3 ml CPES buffer (CPE buffer containing 1.2 M sorbitol and 5 mM DTT).
6. Dissolve 2 mg lysing enzyme of Trichoderma harzianum (Sigma) in 0.5 ml 1% w/v low-melting agarose in CPE buffer at 38°C.
7. Mix equal volumes of the lysing enzyme/agarose solution and the cell suspension (N.B. when preparing protoplasts of hyphal species or large-sized cells, the volumes have to be adjusted!).
8. Pipet the cell/lysing enzyme/agarose mixture in a pre-cooled matrix and allow to gel on ice.
9. After solidification, the agarose/cell blocks are incubated for 1 h at 30°C in 2-5 ml CPE buffer.
10. The agarose blocks are rinsed in 2 - 5 ml NDS buffer (0.5 M EDTA, pH 7.5, 10 mM Tris-HCl, pH 7.5), 1% v/v sodium N-lauroylsarcosinate and subsequently placed for 16 h at 50°C in 2 ml NDS buffer to which 0.4 mg proteinase K and 1% v/v sodium N-lauroylsarcosinate have been added. Sodium N-lauroylsarcosinate and proteinase K should be added immediately prior to use.
11. After lysis, the agarose blocks are rinsed in NDS buffer containing 1% v/v sodium N-lauroylsarcosinate.
12. Store plugs in NDS buffer containing 1% v/v sodium N-lauroylsarcosinate and 0.2 mg ml-1 proteinase K.
13. The plugs may be stored for several months at 4°C.

2.1.2. Preparation of cell protoplasts before embedding in agarose (alternative to method 2.1.1 above)

1. Follow Protocol 2.1 (above) up to and including step 2.1.5.
2. Add 2 mg lysing enzyme in 0.5 ml CPE to the suspended cells in 0.3 ml CPES, and incubate for 2-3 h at 30°C (to allow for degradation of the cell wall).
3. Check protoplast formation microscopically and in case of sufficient cell wall degradation, centrifuge for 3 min at 13,000 rpm.
4. Suspend the protoplasts in 0.3 ml CPES, after which an equal volume 1% low-melting agarose in CPE buffer is added.
5. Pipet the protoplast/agarose mixture in a pre-cooled matrix and allow to gel on ice.
6. Rinse the agarose blocks in 2 ml NDS, 1% v/v lauroylsarcosinate, and incubate for 16 h at 50°C in NDS to which 1% v/v lauroylsarcosinate and 0.02% proteinase K are added.
7. Proceed according to protocol 2.1.11 and onwards (see above).

2.2. Preparation of agar embedded protoplasts of ascomycetous yeasts using Zymolyase

1. Grow 5-10 ml yeasts in a rich medium, such as Yeast extract Peptone Glucose (YPG) broth, to stationary phase (OD600 = 10-14).
2. Transfer 1.0 ml to a 2 ml microcentrifuge tube.
3. Centrifuge 10 sec at 14.000 rpm (or 3 min at 2500 rpm).
4. Suspend pellet in 1 ml 50 mM EDTA, 10 mM Tris, pH 7.5
5. Wash twice with 1 ml 50 mM EDTA, 10 mM Tris, pH 7.5
6. Centrifuge 10 sec at 14.000 rpm.
7. Suspend pellet in 0.2 ml 50 mM EDTA, 10 mM Tris, pH 7.5 containing 4 _g ml-1 Zymolyase (Kirin Brewing Co., Japan).
8. Incubate the cells briefly 5-10 min at 42°C.
9. Add 0.8 ml 1% low melting agarose in H2O or 125 mM EDTA, pH 7.5 at 42C.
10. Mix gently by pipetting in a large-bore pipette tip.
11. Pipette the yeast cell-agarose mix to the wells of the pre-cooled matrix and allow to set on ice (ca. 15 - 60 min).
12. Transfer the plugs to a glass tube and add 1-4 ml LET buffer (0.5 M EDTA, 10 mM Tris, pH 7.5) to totally cover the plugs.
13. Incubate for 8-10 h or overnight at 37°C.
14. Remove LET buffer with a Pasteur pipette and add 1-4 ml NDS buffer (0.5 M EDTA, 10 mM Tris, pH 7.5 with 1% N-lauroylsarcosinate, pH 9.5 and 2 mg ml-1 proteinase K (add Proteinase K immediately prior to use).
15. Discard NDS buffer and dialyze the agar plugs 4 times (1 hour each wash) by soaking in 2 ml 50 mM EDTA, 10 mM Tris pH 7.5. Remove each wash with a Pasteur pipette.
16. Store plugs at 4°C in 50 mM EDTA, 10 mM Tris, pH 7.5. Agar plugs may be stored for several months.

2.3.1.Preparation of agar embedded protoplasts using the lysing enzyme of *Rhizoctonia solani* (22)

This protocol has been successfully used in our laboratory to produce plugs for the electrophoretic karyotyping of a wide range of both asco- and basidiomycetous yeasts.

1. Grow 2 x 10^9 cells overnight at 25°C in 20 ml YEPD broth (2% glucose, 1% yeast extract, 1% peptone).
2. Harvest and wash twice at 4°C with water and 50 mM EDTA pH 7.5.
3. Resuspend cells in 1ml sodium phosphate glycerol (SPG) buffer (10 mM NaH2PO4 in 50% glycerol) containing 5 mg ml-1 lytic enzymes of Rhizoctonia solani (Sigma, U.S.A.).
4. Incubate 30 min at 40°C.
5. Add 0.9 ml of a 1.8% low melting point agarose solution in 125 mM EDTA.
6. Pipette the mix in a pre-cooled matrix.
7. After solidification place the plugs in small vials and cover with ca. 2 ml LET buffer (450 mM EDTA, 10 mM Tris-HCl, pH 7.5).
8. Incubate for 30 min at 37°C (N.B. some basidiomyceteous yeast may need longer times!).

9. Incubate the plugs in 1.5 ml NDS buffer (450 mM EDTA, 500 mM Tris, 1% lauryl sarcosine) containing 1 mg proteinase K for 4-16h, (N.B. usually 4 hours is sufficient for ascomycetous yeasts, but up to 16 h may be needed for basidiomyceteous yeasts).
10. Wash plugs twice with 50 mM EDTA pH 7.5.
11. Plugs may be stored at 4°C for several weeks.

2.4. Preparation of gels and electrophoresis

1. The percentage of agarose gel required is dependent on the size of the chromosomal DNAs to be separated, prepare 0.5 - 1.5% agarose gels (e.g. chromosomal grade Biorad) in 0.25-0.5X TBE.
2. Flood the inserts with 0.5XTBE buffer and position the agarose/cell blocks in the gel.
3. Use 0.25 - 0.5X TBE as electrophoretic buffer and maintain the running buffer temperature between 12° and 15°C.
4. After completion of electrophoresis, stain the gels in 0.5 µg ml-1 ethidium bromide for 30-120 min, destained with demineralized water, and visualize the DNA with a UV transilluminator.
5. Electrophoretic parameters for several different yeast species are presented in Table 1.

N.B. Sometimes the DNA banding may be obscured by a smear. In this case an RNase treatment is recommended (4) as follows: incubate the gel for 2 h at 37°C with gentle shaking in a sealed bag containing 30 ml 0.5 x TBE and 1.5 ml RNase solution (500 µg ml^{-1} pancreatic RNase (100 units ml^{-1} T1 Rnase) in 10 mM Tris-HCl, pH 7.5, 15 mM NaCl, heated for 10 min at 100°C and slowly cooled to room temperature). Rinse twice with electrophoresis buffer, each wash lasting 60 min.

2.5. Restriction endonuclease digestion of agarose embedded chromosomal DNA of *Candida albicans, C. glabrata, C, parapsilosis* and *C. rugosa* (8, 9, 17, 21)

1. Agarose embedded chromosomal DNA is incubated with the required restriction enzyme (e.g. as *Not*I, *Sfi*I, *Sma*I or *BssH*II) (8, 9, 17, 21), according to the manufacturer instructions.
2. Restriction DNA fragments may be separated using the CHEF DRII (Biorad, Richmond, U.S.A.), or by TAFE (Beckman Geneline I, Palo Alto, U.S.A.),
3. NotI digests are run for 24 h in 1% agarose in TBE buffer (pH 8.5) at 6 V cm-1 with an initial switch time of 5 sec and a final switch time of 90 sec. (8).
4. *Sfi*I digests are run for 17 h in 1% agarose and 0.5 TBE buffer at 185V with pulse times ramping from 1 to 38 sec (17).
5. *BssH*II digests are run for 24 h in 1% agarose and 0.5 TBE at 13°C and 200V with pulse times ramping from 5 to 35 sec (9).
6. *Sfi*I and *Sma*I digests are run for 12 h in 0.8% agarose and 0.5 TBE at 14°C and 150V with a pulse time of 120 sec, 3 h at 150 V for a pulse time of 300 sec, and 16 h at 115V using a pulse time of 300 sec (9).

Table 1. Electrophoretic conditions for separating chromosomal DNA in a variety of yeasts as analyzed by CHEF (unless otherwise indicated)

Species	Run time (h)	Pulse time (sec)	Agarose %	Reference
Candida albicans	30	120-300 (70 V)	1.0	(7)
	66	420-900 (70 V)		
Candida glabrata, C. lusitaniae, C. parapsilosis, C. kefyr	22	180 (150 V)	?	(19)
	26	360 (150 V)		
Candida tropicalis	22	120 (150 V)	?	(19)
	6	360 (150 V)		
	24	360 (113 V)		
Candida rugosa	20	130 (140V)	1.0	(17)
	14	300 (150V)		
	22	360 (110V)		
Cryptococcus neoformans	30	100-300 (100 V)	1.0	(11)
	40	400-600 (100 V)		
Malassezia species	5	100 (3 V cm^{-1})	1.0	(16)
	10	40-200 (3 V cm^{-1})	1.0	
	50	300 (3 V cm^{-1})	1.0	
Malassezia furfur, M. sympodialis	36	300 (80 V)	1.0	(14, 15)
	36	300-600 (80 V)		
Malassezia pachydermatis	48	50-300 (120 V)	1.0	(14)
Saccharomyces cerevisiae (TAFE protocols)	18	60	1.0	(5)
	6	35		
Saccharomyces cerevisiae	15	60 (200 V)	1.0	(20)
	9	90 (200 V)		
Yarrowia lipolytica (TAFE protocols)	162	4200	?	(18)

2.6.Southern hybridisation of electrophoretic karyotypes of *Saccharomyces* yeasts with gene probes

1. Chromosomal DNA separated by PFGE can be quickly transferred onto nylon or nitrocellulose membranes using the Model 785 vacuum blotter (Biorad, Richmond, U.S.A.).
2. Gel and membrane are assembled with a window gasket which is cut according to the gel size.
3. Pre-treatment of the gel is carried out under vacuum and includes depurification (0.25 M HCl) for 15 min, denaturation (1.5 M NaCl, 0.5 M NaOH) for 30 min and neutralisation (1.5 M NaCl, 1M Tris pH 7.5) for 30 min. All solutions should cover the surface of the gel.
4. After pre-treatment, 20x SSC transfer solution (3 M NaCl, 0.3 M Na-citrate, pH 7.0) is poured to approximately twice the thickness of the gel and incubated for 60-90 min (depending on chromosome sizes) at room temperature.
5. Remove the transfer solution and turn off the vacuum.
6. Allow the membrane to air dry and proceed according to the instructions recommended by the manufacturer.
7. Probes for Southern hybridizations are labelled with digoxigenin-11-dUTP using the "Boehringer Mannhein Non-radioactive DNA Labelling Kit" (Boehringer Mannheim, Mannheim, Germany).
8. To a microfuge tube on ice, add 10 ng - 3 μg linearized purified DNA (this should be denaturated for 10 min at 95° C and then quickly chilled on ice for 10 min), 2 μl hexanucleotide mixture, 2 μl dNTP labelling mixture, 1 μl Klenow enzyme, and sterile bidistilled H2O to make a total volume of 20 μl.
9. Incubate the mixture overnight at 37° C. To stop the reaction, add 2 μl of 0.2 M EDTA solution (pH 8.0) and precipitate the DNA with 2.5 μl of 4 M LiCl and 75 μl of cold ethanol (-20 C).
10. Place the mixture at -20° C overnight and centrifuge at 12,000 g for 10 min. Wash the pellet with 50 μl cold 70% ethanol, dry in air and dissolve in 50 μl of TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0) for 30 min at 37° C.
11. Hybridize the blot in 5 x SSC containing 0.1% N-lauroylsarcosine, Na-salt, 0.02% SDS (sodium dodecyl sulphate, also called sodium laryl sulphate) and 1% blocking reagent at 65-68° C overnight.
12. Wash twice in 2 x SSC containing 0.1% SDS at room temperature for 5 min and subsequently with 0.1 x SSC containing 0.1% SDS at 65-68° C for 15 min.
13. For the colorimetric detection all incubations, except for the colour reaction, require shaking.
14. Wash the blot briefly in buffer 1 (0.1 M maleic acid, 0.15 M NaCl), then incubate for 30 min in ca.100 ml of buffer 2 (1% blocking reagent in buffer 1).
15. Dilute the anti-digoxigenin antibody conjugate to 150 mU ml-1 (1:5000) in buffer and cover the blot and incubate for 30 min at room temperature.
16. Remove unbound antibody conjugate by washing twice with 100 ml of buffer 1 for 15 min at room temperature.

17. Soak the membrane for 5 min in 20 ml of buffer 3 (100 mM Tris-HCl, 100 mM NaCl, 50 mM MgCl2).
18. Pour off buffer 3 and add ca. 10 ml freshly prepared colour solution (10 ml buffer 3 and 200 µl NBT/BCIP solution (nitroblue tetrazolium salt/5-bromo-4-chloro-3-indolyl phosphate toluidinium salt)). Incubate the blot in the dark for 4-16 h until the desired spots or bands are detected.
19. Stop the reaction by washing the membrane with double distilled water.
20. The wet blot may be photocopied or photographed to document the results. A dried filter may be (indefinitely) stored in the dark and the colour restored by wetting the membrane with double distilled water.

3. References

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