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Development of a set of plasmid vectors for genetic manipulations of the pathogenic yeast *Candida parapsilosis*

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Abstract

A system for genetic transformation of the yeast *Candida parapsilosis*, recently developed in our laboratory, opened a venue for investigation of this pathogenic species at the molecular level. In this study we extend the range of available experimental tools by construction of a genomic DNA library suitable for screening and isolation of genes by functional complementation of yeast mutants and a set of replicative plasmid vectors for genetic manipulation of *C. parapsilosis* cells. The plasmids are based on auxotrophic (*CpGAL1*, *CpURA3*, *CpMET2*, *CpLYS4*) and dominant (*CalMH3*) selection markers. In addition, we constructed plasmid derivatives containing reporter genes *yEGFP3* and *KILAC4* coding for enhanced version of the green fluorescent protein and *Kluyveromyces lactis* β -galactosidase, respectively. The vectors facilitate propagation and expression of cloned genes in *C. parapsilosis* cells and allow intracellular localization of gene products and/or monitoring the activity of promoter sequences.

Keywords

yeast plasmids; genetic transformation; selection and reporter markers

1. Introduction

Candidiasis are important fungal diseases in humans and warm-blooded animals caused by yeasts from the genus *Candida*. Although *Candida albicans* predominates in these mycoses, recent studies revealed an increased incidence of non-*C. albicans* species (Cassone et al., 1995; Fridkin et al., 2006). Of them, the yeast *C. parapsilosis*, although considered as a harmless commensal organism in healthy individuals, represents important nosocomial pathogen attacking newborns and immunocompromised patients and causes severe cases of septic arthritis, peritonitis, vaginitis, endocarditis, endophthalmitis, nail and skin infections (Cassone et al., 1995; Huang et al., 2000; Alonso-Valle et al., 2003).

This species displays many interesting biological features. Its selective adherence to prosthetic materials and formation of biofilms on plastic surfaces (Branchini et al., 1994; Pfaller, 1995), resistance to drugs and inhibitors (Camougrand et al., 1986), secretion of extracellular proteases (Fusek et al., 1993; Pichova et al., 2001; Merkerova et al., 2006), phenotype switching (Lott et al., 1993; Laffey and Butler, 2005; Kim et al., 2006), colonization of human hands

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(Bonassoli et al., 2005) and proliferation in high concentration of glucose and lipids (Branchini et al., 1994) are presumed to be directly related to its virulence.

One of the most remarkable biological features of this species is the presence of a linear DNA genome terminating with specific telomeric structures in its mitochondria (Kovac et al., 1984; Nosek et al., 2004). This linear genome represents a unique model system for investigation of telomerase-independent mode of telomere maintenance (Nosek et al., 1995; Tomaska et al., 2000; Nosek et al., 2005). Moreover, the mitochondrial telomeres have a potential in the molecular diagnostics of this opportunist pathogen (Nosek et al., 2002b; Rycovska et al., 2004).

The ongoing sequencing project of the *C. parapsilosis* nuclear genome (<http://www.sanger.ac.uk/sequencing/Candida/parapsilosis>) provides an essential platform for further functional studies which are essential for understanding the biology of this pathogenic yeast species at the molecular level. However, the genetic analysis of *C. parapsilosis* is complicated due to diploid/aneuploid state of its genome (Doi et al., 1992; Fundyga et al., 2004) as well as the lack of sexual cycle (Logue et al., 2005). To overcome this problem, a set of auxotrophic host strains was isolated and the first system and optimized protocols for genetic transformation were developed in our laboratory (Nosek et al., 2002a; Zemanova et al., 2004). The system utilized the complementation of a galactokinase-deficient mutant of *C. parapsilosis* by the *CpGAL1* gene. Recently, a protocol based on the selection of transformed cells using an integrative plasmid with a dominant selection marker *CaIMH3* encoding resistance to mycophenolic acid (MPA) was described (Gacser et al., 2005).

To extend the range of available tools for genetic manipulation of *C. parapsilosis* cells we isolated two selection markers *CpMET2* and *CpLYS4* by functional complementation of corresponding mutations in auxotrophic strains. We used both genes, together with previously described markers *CpURA3*, *CpGAL1*, and *CaIMH3*, for construction of a set of plasmid vectors facilitating gene cloning and expression. In addition, we prepared vector derivatives allowing intracellular localization of the products of cloned genes and/or the monitoring of promoter activity.

The availability of vectors for efficient genetic manipulation of *C. parapsilosis* cells is particularly important as, similarly to other *Candida* species, this yeast employs a modified version of the genetic code which hampers the analysis of its genes in heterologous systems such as *S. cerevisiae* or *K. lactis*.

2. Material and Methods

2.1 Strains and media

The *C. parapsilosis* type strain CBS604 was kindly provided by Hiroshi Fukuhara (Institute Curie, Orsay, France). Auxotrophic strains *C. parapsilosis* SR23 gal1-7 (*ade*, *lys4*, *gal1*) and SR23 met-1 (*ade*, *lys4*, *met2*) were prepared in our laboratory (Nosek et al., 2002a). The *S. cerevisiae* L5366 (*MATa/MATa ura3/ura3*) strain was kindly provided by Cora A. Styles (Whitehead Institute for Biomedical Research, Cambridge, USA). *Escherichia coli* DH5α strain (Clontech) was used for cloning experiments. Yeast cells were cultivated at 28°C in YPD medium [1% yeast extract (Difco), 1% peptone (Difco), 2% glucose] or SD medium [0.67% Yeast nitrogen base w/o amino acids (Difco), 2% glucose] supplemented with appropriate amino acids or nitrogen bases. The carbon source in SGly and SGal/YPGal media was 3% glycerol and 2% galactose, respectively. Solid media were supplemented with 2% agar (Difco).

2.2 Preparation of the *C. parapsilosis* genomic DNA library

Genomic DNA of the strain CBS604 was isolated using a protocol described for *S. cerevisiae* (Phillippsen et al., 1991). Total DNA was partially digested with the restriction endonuclease *Sau3AI* and DNA fragments (2 – 15 kb) were cloned into the *Bam*HI site of the shuttle vector YEplac195 (Gietz and Sugino, 1988). The library contains approximately 86,000 independent *E. coli* clones and its analysis revealed 75% recombinant plasmids containing inserts with an average size of about 3 kb. As the size of *C. parapsilosis* diploid genome has been estimated to approximately 26 Mb (Doi et al., 1992) the library covers the genome with a probability above 99.9%. The library can be propagated in *E. coli* and *S. cerevisiae* cells. Moreover, we observed that plasmid vector YEplac195 replicates in *C. parapsilosis*. Therefore, the library can be employed for screening and isolation of genes by functional complementation of mutations in both yeast species.

2.3 Yeast transformation

The transformation of *S. cerevisiae* cells was performed essentially as described by Gietz and Schiestl (1995). *C. parapsilosis* cells were transformed using an optimized electroporation protocol developed in our laboratory (Zemanova et al., 2004). Transformants were selected in SD medium supplemented with appropriate amino acids and nitrogen bases at 28°C for 2-4 days. In the case of selection in media containing MPA, the electric pulse was followed by incubation of the cell suspension for additional 6 hours in YPGS (1% peptone, 1% yeast extract, 3% glycerol, and 1 M sorbitol) medium and transformants were selected on YPGal medium supplemented with 150 µg/ml MPA for 4-5 days at 28°C.

2.4 Isolation of CpMET2 and CpLYS4 genes by functional complementation

C. parapsilosis SR23-met1 cells were transformed with approximately 1 µg of the genomic library (see section 2.2) and selected on SD medium lacking either methionine or lysine at 28°C for 5 days. Plasmid DNAs isolated from transformants were amplified in *E. coli* and retransformed into *C. parapsilosis* SR23 met-1 to confirm the phenotype. Subsequent DNA sequence analysis of inserts from two isolated plasmids, pBP-M and pBP-L, revealed the presence of *CpMET2* and *CpLYS4* gene, respectively.

2.5 Construction of the plasmid vectors

Source plasmids pUC19-*CpGAL1B*-ARS7, pUC19-*CpGAL1B*, pUC19-*CpURA3*, and YEplac181-*CpGAL1* were described previously (Nosek et al., 2002a). The vector pPK1 was constructed as follows. First, the 503 bp *AccI*-*Clal* fragment containing the *CpARS7* sequence was excised from the pUC19-*CpGAL1B*-ARS7 plasmid and treated with DNA polymerase I (Klenow) to generate blunt ends. Next, the fragment was inserted into the plasmid pUC19-*CpGAL1B* which was linearized with the *Bsm*BI and treated with Klenow enzyme. The plasmid pPK2 was prepared analogously except that pUC19-*CpURA3* was used as the recipient vector. The plasmid pPK1 was used for construction of two derivatives, pBP1 and pBP2.

pBP1 was prepared by insertion of 2726 bp *Pml*I-*Bgl*II fragment of the clone pBG-M carrying the *CpMET2* gene into corresponding sites of pPK1. pBP2 was constructed from the 2974 bp *Xba*I-*Bam*HI fragment of the pBG-L clone containing the *CpLYS4* gene and pPK1 digested with *Pml*I and *Bgl*II endonucleases; both fragments were treated with Klenow enzyme prior the ligation reaction.

The vectors pPK2, pBP1 and pBP2 were subsequently used to design expression vectors containing the promoter of *CpGAL1* gene (pBP3, pBP3T, pBP5, pBP5T, pBP7) and/or the reporter markers, *KILAC4* (pPK3, pPK3-*P_{CpGAL1}*, pPK4, pPK4-*P_{CpGAL1}*) and *yEGFP3* (pBP4, pBP6, pBP8, pPK5).

The *CpGAL1* promoter (P_{CpGAL1}) was amplified from the plasmid YEplac181-*CpGAL1* using the primers 5'-CGGGATCCCCATGGTAATAATTTCAAC-3' and 5'-ATTTACACAGGAAACAGCTATGAC-3', digested with *Bam*HI and inserted into the unique *Bam*HI site of YE354 (Myers et al., 1986). Resulting plasmid YE354- P_{CpGAL1} contains *lacZ* driven by P_{CpGAL1} . Subsequently, the 576 bp *Bam*HI fragment of YE354- P_{CpGAL1} was inserted into corresponding site of pBP1, pBP2, and pPK2 and resulting constructs were named pBP3, pBP5, and pBP7, respectively. In addition, the 801 bp *Pvu*II-*Xba*I fragment from pPK1 containing presumed terminator sequence of the *CpGAL1* gene (T_{CpGAL1}) was treated with Klenow enzyme and cloned into *Pst*I linearized and blunt-ended plasmid molecules pBP3 and pBP5. These constructs were named pBP3T and pBP5T, respectively.

The reporter gene coding for *K. lactis* β -galactosidase was amplified from pKR1B-LAC4-1 (Sreekrishna and Dickson, 1985) using the primers 5'CCCCGGGTCTTGCCTTATTCCTGAGAATTTA3' and 5'ACTCTAGAATCGTTAATCAGCGGTGGTTA3', and digested with *Xba*I. The fragment was then inserted into the recipient vectors pBP3 and pBP5 which were linearized with *Nco*I, blunt-ended with Klenow enzyme, and digested with *Xba*I. The constructs were named pPK3- P_{CpGAL1} , and pPK4- P_{CpGAL1} . Subsequent deletion of the 580 bp *Sma*I fragment from both plasmids resulted in two vectors named pPK3 and pPK4, respectively.

The plasmids pBP4 and pBP6 were prepared as follows. The vector pUG35 (kindly provided by J. H. Hegemann, Düsseldorf, Germany) was linearized with *Kpn*I, treated with Klenow enzyme, and digested with *Sal*I. The 986 bp fragment containing the *yEGFP3* reading frame and the terminator of *S. cerevisiae* *CYC1* gene (T_{ScCYC1}) was then ligated into the recipient vector molecules pBP3 and pBP5 which were linearized with *Pst*I, treated with Klenow enzyme and digested with *Sal*I. Elimination of the 536 bp *Bam*HI fragment containing the P_{CpGAL1} sequence from pBP6 resulted in the construct named pBP6 Δ P.

To prepare the plasmid pBP8, the vector pPK2 was digested with *Xba*I and *Eco*RI, and the 1435 bp insert containing promoter of the *S. cerevisiae* *MET25* gene ($P_{ScMET25}$), *yEGFP3*, and the T_{ScCYC1} sequence was cleaved from pUG35 by *Kpn*I and *Sac*I. Termini of both DNA fragments were treated with Klenow enzyme and joined by T4 DNA ligase.

The vector pPK6 was constructed in two steps. First, the 536 bp *Bam*HI DNA fragment was removed from pBP5T and the resulting plasmid was digested with *Kpn*I and *Sac*I. Next, the P_{CpGAL1} promoter and the *yEGFP3* coding sequence were amplified from the pBP6 template using the primers 5'CAGAGCTCGATCCATTAAGTTTATTTTTTTTCA3' and 5'CAGGTACCACCAGTAATTTGTACAATTCATCCA3', digested with *Kpn*I and *Sac*I, and inserted in corresponding sites of the vector.

In addition to vectors based on auxotrophic markers, the plasmid pPK5 carrying dominant selection marker *CalMH3* coding for resistance to MPA and *yEGFP3* gene was prepared. First, the 2710 bp *Eco*RI-*Bam*HI fragment from pSFI1 (kindly provided by J. Morschhäuser, Würzburg, Germany) was inserted into corresponding sites of pBP6. Subsequently, the 576 bp *Bam*^{HI} fragment containing P_{CpGAL1} (see above) was cloned into the *Bam*HI site.

2.6 Miscellaneous

Radioactive DNA labeling, Southern blot hybridization, DNA manipulation and cloning procedures were performed essentially as described by Sambrook et al. (1989). The *CpMET2* probe was amplified from the pBP1 template using the primers 5'GCAAAGGAGACGACGA3' and 5'AAACTACGCTCTACACGA3'. The *CpLYS4* probe was amplified from the pBP2 template using the primers 5'AGTGATGAGGATGCCAA3'

and 5'TTATCGGCATTACACAAGA 3'. The DNA sequences were determined using the BigDye Terminator Cycle Sequencing Kit (Applied Biosystems). The DNA sequences reported in this work were deposited in the EMBL/GenBank data library under following accession numbers: **X99635** (*CpURA3*), **Y14704** (*CpGAL1*), **DQ359727** (*CpMET2*), **DQ359726** (*CpLYS4*), and **AY074814** (*CpARS7*). The vectors and their sequences are available upon request from authors.

3. Results and Discussion

3.1 Isolation of auxotrophic selection markers *CpMET2* and *CpLYS4*

With the aim to isolate selection markers suitable for construction of plasmid vectors we complemented two auxotrophic mutations *met⁻* and *lys⁻* in the strain *C. parapsilosis* SR23-met1 using the genomic library prepared from the strain CBS604 (see section 2.2). This approach led to isolation of two plasmid clones pBG-M and pBG-L. Subsequent DNA sequence analysis of the 3270 bp insert of the pBG-M plasmid complementing *met⁻* mutation led to identification of an open reading frame (ORF) with a significant degree of sequence homology to *MET2* genes of diverse organisms. The sequence of deduced protein product with the length 461 amino acids displays 64.4 % and 55.5 % identity to L-homoserine-O-acetyltransferase of *C. albicans* and *S. cerevisiae*, respectively. The analysis of the 5608 bp insert of the pBG-L plasmid complementing *lys⁻* mutation revealed a homologue of the *S. cerevisiae* *LYS4* gene. The deduced protein consisting of 688 amino acids residues has 80.4 % and 70.6 % identity to homo-aconitase of *C. albicans* and *S. cerevisiae*, respectively. Next, the genes *CpMET2* and *CpLYS4*, together with *CpURA3* and *CpGAL1*, previously isolated in our laboratory (Nosek et al., 2002a), were used as auxotrophic markers in a set of cloning and expression vectors for *C. parapsilosis*.

3.2 Cloning vectors

We constructed a set of four cloning vectors pBP1, pBP2, pPK1, and pPK2, suitable for *C. parapsilosis* (Figure 1A). These plasmids are based on the *E. coli* vector pUC19 which possesses ColE1 origin of replication in bacteria and β -lactamase gene conferring the resistance to ampicillin. In addition, they contain auxotrophic selection markers *CpGAL1*, *CpURA3*, *CpMET2* or *CpLYS4*, and the autonomous replicating sequence *CpARS7* (Nosek et al., 2002a) ensuring their propagation and selection in appropriate auxotrophic mutants of *C. parapsilosis*. The *CpARS7* element permits the plasmid replication in multiple copies within *C. parapsilosis* cells and ensures the high efficiency of transformation. The vectors allow cloning of DNA fragments into several unique sites for restriction endonucleases originally present within the polylinker of pUC19 (e.g., *SacI*, *KpnI*, *SmaI*, *XmaI*, *BamHI*, *XbaI*, *SalI*, *PstI*, *SphI*). The mitotic stability of the plasmids in the yeast cells was determined after the growth of transformants for 10 generations in a non-selective medium (YPD). The results of three independent experiments show that 31.8% (± 4.4), 22.5% (± 1.6), and 36.6% (± 2.9) of cells retain the plasmid pBP1, pBP2, and pPK1, respectively. This indicates that the replicative plasmids can be easily eliminated from host cells by cultivation in non-selective media. In the case of pBP1 and pBP2, the number of plasmid copies per cell was estimated using Southern blot hybridization analysis (Figure 2) of total DNA prepared from three independent transformants grown overnight in liquid selective media and probed with corresponding marker (i.e., *CpMET2* or *CpLYS4*). Our results indicate that pBP1 and pBP2 are present in 17 ± 2 and 13 ± 2 copies per cell, respectively.

3.3 Vectors for gene expression

In our previous study (Nosek et al., 2002a) we isolated and characterized the P_{CpGAL1} sequence which was shown to control the expression of galactokinase by the presence/absence of appropriate carbon source (e.g., galactose, glucose, glycerol) in the cultivation media. Based

on these results, we constructed three plasmid vectors, pBP3, pBP5, and pBP7, containing the *CpMET2*, *CpLYS4*, and *CpURA3* marker, respectively, and the *P_{CpGAL1}* sequence (Figure 1B). Moreover, we prepared two vector derivatives, pBP3T and pBP5T, containing both the *P_{CpGAL1}* and the *T_{CpGAL1}* sequences (Figure 1B). These plasmids are suitable for the expression of homologous or heterologous genes in *C. parapsilosis* cells and permit an easy control of the gene expression by switching between repressed (glucose), non-repressed (glycerol) and induced conditions (galactose) by changing the carbon source in the cultivation medium. The vectors can be also used in genetic approaches such as screening for lethal genes by induction of gene expression (*e.g.* Espinet et al., 1995).

3.4 Vectors for intracellular localization of protein products

Based on the pBP3, pBP5, and pPK2 vectors, we constructed three derivatives pBP4, pBP6 and pBP8 (Figure 1C), respectively, containing the marker *yEGFP3* coding for yeast enhanced version of green fluorescent protein 3 that was shown to function in the closely related species *C. albicans* and *C. dubliniensis* (Cormack et al., 1997; Staib et al., 2000). In the plasmids pBP4 and pBP6, the *yEGFP3* expression is driven by *P_{CpGAL1}*, while in pBP8 its expression is under the control of *P_{ScMET25}*. To overcome the requirement for auxotrophic strains and synthetic media in experiments, the *IMH3* gene from *C. albicans* has been successfully employed as a dominant selection marker conferring the resistance to MPA in diverse *Candida* species (Staib et al., 2000; Du et al., 2004; Gacser et al., 2005). In this study we constructed the replicative plasmid pPK5 containing the *CaIMH3* gene (Figure 1C) which allows selection of transformants in complex media, such as YPD, supplemented with 150 µg/ml MPA, and the *P_{CpGAL1}* promoter driven *yEGFP3* marker for intracellular localization of C-terminally tagged fusion proteins. In addition, we constructed the vector pPK6 (Figure 1D). This plasmid contains *CpLYS4* as a selection marker and the *yEGFP3* coding sequence driven by *P_{CpGAL1}* promoter as a reporter. The unique *SmaI* site in pPK6 allows construction of fusion proteins N-terminally tagged with *yEGFP3*. To test these vectors we transformed them into appropriate *C. parapsilosis* strain. The transformants were then observed by fluorescence microscopy (Figure 3). In the case of plasmids pBP4, pBP6, pPK5, and pPK6, the transformants exhibit fluorescence within the cytoplasm. To illustrate targeting of *yEGFP3* into a specified cellular compartment, we amplified ORF of the *C. parapsilosis MTP1* gene coding for mitochondrial telomere-binding protein (mtTBP) (Nosek et al., 1999) from the genomic DNA of the strain CBS604 using the primers 5'GACTCTAGATTCTGTAGCTTCGGCTCTATCCTCA3' and 5'GACTCTAGAATGTTGCGAGCATTCCTAGATCA3'. The PCR product was then digested with *XbaI* and cloned in-frame with the *yEGFP3* sequence of the pBP6 vector. Resulting plasmid pBP6-mtTBP was subsequently transformed into *C. parapsilosis* cells. As expected from our previous study with a mtTBP-GFP construct in *S. cerevisiae* (Nosek et al., 1999), the mtTBP-*yEGFP3* fusion protein localizes into multiple foci corresponding to mitochondrial nucleoids. As the *ura3* mutant of *C. parapsilosis* is not yet available, the vector pBP8 was tested in *S. cerevisiae* cells with the same cytoplasmic fluorescence of the expressed marker as observed in the case of other vectors in *C. parapsilosis*.

3.5 Vectors for monitoring the activity of gene promoters

β -galactosidase represents another reporter marker which activity can be detected by several methods: quantitative liquid assays using permeabilized cells, colorimetric assays of colonies replicated to paper filters, and *in situ* coloration of colonies growing on medium containing the indicator X-Gal (Guthrie and Fink, 2002). However, β -galactosidase encoded by the *E. coli lacZ* gene routinely used in many species (El Barkani et al., 2000; Juretzek et al., 2001), cannot be employed in the case of *C. parapsilosis* as it contains 51 'CTG' codons that are translated as serine instead of leucine (Ohama et al., 1993). In contrast, the genes coding for β -galactosidase from *Streptococcus thermophilus* and *K. lactis* (*KILAC4*) with only one and two 'CTG' codons, respectively, were successfully tested as reporters in *C. albicans* and *C.*

tropicalis (Leuker et al., 1992; Uhl and Johnson, 2001). Therefore, we constructed two plasmids (pPK3 and pPK4) containing the *KILAC4* gene (Figure 1E) which can be utilized for investigation of various aspects of gene regulation in a range of cellular processes of *C. parapsilosis*. Both vectors lack the translation initiation codon 'ATG' of *KILAC4* which has been replaced by the site for *SmaI* endonuclease permitting cloning of blunt-ended PCR-amplified promoters terminating with 'ATG' codon. To test pPK3 and pPK4 we cloned the *P_{CpGAL1}* sequence into both vectors. Resulting constructs named pPK3-*P_{CpGAL1}* and pPK4-*P_{CpGAL1}* were transformed into *C. parapsilosis*. Subsequently, the transformants were assayed for β -galactosidase activity. The colonies grown in media (SGal) where the expression of the *KILAC4* marker is induced by galactose were blue. In contrast, the transformants cultivated in the repressed (SD) and non-repressed (SGly) conditions remained white, although light blue coloration developed in both cases after prolonged time of incubation. Next, we performed quantitative β -galactosidase assays. Our results clearly indicate that in both cases galactose induces, while glucose effectively represses, *P_{CpGAL1}* driven expression of the *KILAC4* marker (Figure 4). We observed about 5-fold difference in β -galactosidase activities between cells transformed with pPK3-*P_{CpGAL1}* and pPK4-*P_{CpGAL1}* grown on galactose (Figure 4). Although this might result from a difference in plasmid copy number, the estimated number of copies of the parental vectors (pBP1 and pBP2) did not differ significantly (17 ± 2 vs. 13 ± 2).

3.6 Conclusion

In this report, we constructed a collection of replicative shuttle vectors suitable for molecular genetic studies of *C. parapsilosis* and functional analysis of its genes (Table 1). Our vectors allow expression of the cloned genes, intracellular localization of protein products or monitoring of a promoter activity. These plasmids are based on auxotrophic or dominant selection markers facilitating their propagation either in the auxotrophic host strains or wild type strains of *C. parapsilosis* such as clinical isolates. These vectors open new experimental opportunities and may significantly contribute to investigations of diverse biological phenomena related to *C. parapsilosis*.

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Abbreviations

CpARS7	<i>C. parapsilosis</i> autonomously replicating sequence 7
MPA	mycophenolic acid
mtTBP	mitochondrial telomere-binding protein
ORF	open reading frame
P_{CpGALI}	promoter of the <i>CpGALI</i> gene
P_{ScMET25}	

promoter of the *ScMET25* gene

PCR

polymerase chain reaction

T_{CpGAL1}

terminator of the *CpGAL1* gene

T_{ScCYC1}

terminator of the *ScCYC1* gene

yEGFP3

yeast enhanced green fluorescent protein 3

Figure 1.

A set of cloning (A) and expression vectors (B, C, D, E) developed for *C. parapsilosis*. (A) The cloning vectors pBP1, pBP2, pPK1, and pPK2 contain *CpMET2*, *CpLYS4*, *CpGAL1* and *CpURA3*, respectively, as auxotrophic selection markers. All four vectors possess multiple cloning sites (MCS) localized within *E. coli lacZ'* gene. (B) The expression vectors pBP3, pBP5, and pBP7 contain *CpMET2*, *CpLYS4*, and *CpURA3* selection marker, respectively. The expression of cloned genes is driven by *P_{CpGAL1}*. In addition, plasmids pBP3T and pBP5T derived from pBP3 and pBP5, respectively, contain the *T_{CpGAL1}* sequence. (C) The plasmids pBP4, pBP6, pBP8, and pPK5 designed for intracellular localization of protein products are based on *CpMET2*, *CpLYS4*, *CpURA3*, and *CalMH3* selection marker, respectively. The

expression of proteins C-terminally tagged with yEGFP3 is driven either by P_{CpGAL1} (pBP4, pBP6, pPK5) or $P_{ScMET25}$ (pBP8) promoters. (D) The vector pPK6 contains *CpLYS4* as a selection marker and is suitable for intracellular localization of proteins N-terminally tagged with yEGFP3. The gene expression of fusion proteins is driven by the P_{CpGAL1} promoter. (E) The vectors pPK3 and pPK4 suitable for monitoring the activity of gene promoters contain *CpMET2* and *CpLYS4* selection markers, respectively. Promoter sequences can be inserted into unique *SmaI* site.

Figure 2.

Southern blot hybridization analysis of transformants harboring pBP1 (A) and pBP2 (B) plasmids. Cells of *C. parapsilosis* SR23-met1 were transformed with the plasmids and selected in appropriate medium. In both cases, total DNA was prepared from three individual transformants, digested with the restriction endonuclease *Eco*RI, electrophoretically separated in 0.7 % agarose gel, blotted onto a Nylon membrane and hybridized with radioactively labeled probes derived from the genes *CpMET2* (A) and *CpLYS4* (B). Note that the faint upper bands (9.5 and 10.1 kb) correspond to the gene copies in presumably diploid nuclear genome.

Figure 3.

Fluorescence microscopy of yeast cells transformed with plasmids expressing yEGFP3 alone or in the fusion with mtTBP. A, B, C, D, and E are *C. parapsilosis* cells transformed with pBP4, pPK5, pBP6, pPK6 and pBP6-mtTBP, respectively. F and H show fluorescence of *C. parapsilosis* cells transformed with pBP6ΔP lacking P_{CpGAL1} promoter grown in the presence of glucose (F) and galactose (G). H shows *S. cerevisiae* expressing yEGFP3 from pBP8. The bar represents 5 μm.

Figure 4.

C. parapsilosis expressing *K. lactis* β -galactosidase under the control of the P_{CpGAL1} promoter. *C. parapsilosis* SR23-met1 cells were transformed with pPK3-P_{CpGAL1} (A) and pPK4-P_{CpGAL1} (B). Transformants were grown in liquid selection media containing 2 % glucose (glu), 2 % galactose (gal), and 3 % glycerol (gly) as the sole carbon source, and the activity of β -galactosidase was measured essentially as described by Rose and Botstein (1983). The results are based on three independent replicates of the experiment. In a control experiment, lysates from cells transformed with the plasmid pPK3 lacking the P_{CpGAL1} promoter exhibited 6.7, 15.4, and 10.0 Miller units of β -galactosidase in media containing 2 % glucose, 2 % galactose, and 3 % glycerol, respectively. In the case of pPK4, the activities were 5.3, 8.1, and 6.3 Miller units.

Table 1
Vectors for genetic manipulation of *C. parapsilosis*

Cloning vectors						
Plasmid	Plasmid size (bp)	Selection marker	Autonomously replicating sequence (ARS)	Reference		
pPK1	6177	<i>CpGAL1</i>	<i>C. parapsilosis</i> ARS7	This study		
pPK2	5027	<i>CpURA3</i>	<i>C. parapsilosis</i> ARS7	This study		
pBP1	6652	<i>CpMET2</i>	<i>C. parapsilosis</i> ARS7	This study		
pBP2	6900	<i>CpLYS4</i>	<i>C. parapsilosis</i> ARS7	This study		
YEplac181- <i>CpGAL1</i>	8717	<i>CpGAL1</i>	<i>S. cerevisiae</i> 2μm	Nosek et al., 2002a		
pUC19- <i>CpGAL1B</i>	5670	<i>CpGAL1</i>	-	Nosek et al., 2002a		
pUC19- <i>CpGAL1E</i>	5275	<i>CpGAL1</i>	-	Nosek et al., 2002a		
pRM100- <i>CpGAL1</i>	~ 11300	<i>CpGAL1</i>	<i>C. albicans</i> ARS	Nosek et al., 2002a		
pUC19- <i>CpGAL1B</i> - ARS2	5871	<i>CpGAL1</i>	<i>C. parapsilosis</i> ARS2	Nosek et al., 2002a		
pUC19- <i>CpGAL1B</i> - ARS3	6016	<i>CpGAL1</i>	<i>C. parapsilosis</i> ARS3	Nosek et al., 2002a		
pUC19- <i>CpGAL1B</i> - ARS4	5837	<i>CpGAL1</i>	<i>C. parapsilosis</i> ARS4	Nosek et al., 2002a		
pUC19- <i>CpGAL1B</i> - ARS7	6276	<i>CpGAL1</i>	<i>C. parapsilosis</i> ARS7	Nosek et al., 2002a		
pMPA	5665	<i>CaIMH3</i>	-	Gacser et al., 2005		
Expression vectors*						
Plasmid	Plasmid size (bp)	Selection marker	Expression cassette			Reference
			Promoter	Reporter gene	Terminator	
pBP3	7228	<i>CpMET2</i>	P _{<i>CpGAL1</i>}	-	-	This study
pBP3T	8013	<i>CpMET2</i>	P _{<i>CpGAL1</i>}	-	T _{<i>CpGAL1</i>}	This study
pBP4	8208	<i>CpMET2</i>	P _{<i>CpGAL1</i>}	<i>yEGFP3</i>	T _{<i>ScCYC1</i>}	This study
pBP5	7476	<i>CpLYS4</i>	P _{<i>CpGAL1</i>}	-	-	This study
pBP5T	8261	<i>CpLYS4</i>	P _{<i>CpGAL1</i>}	-	T _{<i>CpGAL1</i>}	This study
pBP6	8456	<i>CpLYS4</i>	P _{<i>CpGAL1</i>}	<i>yEGFP3</i>	T _{<i>ScCYC1</i>}	This study
pBP7	5603	<i>CpURA3</i>	P _{<i>CpGAL1</i>}	-	-	This study
pBP8	6435	<i>CpURA3</i>	P _{<i>ScMET25</i>}	<i>yEGFP3</i>	T _{<i>ScCYC1</i>}	This study
pPK3	10157	<i>CpMET2</i>	-	<i>KILAC4</i>	T _{<i>KILAC4</i>}	This study
pPK4	10405	<i>CpLYS4</i>	-	<i>KILAC4</i>	T _{<i>KILAC4</i>}	This study
pPK5	7343	<i>CaIMH3</i>	P _{<i>CpGAL1</i>}	<i>yEGFP3</i>	T _{<i>ScCYC1</i>}	This study
pPK6	9006	<i>CaLYS4</i>	P _{<i>CpGAL1</i>}	<i>yEGFP3</i>	T _{<i>CpGAL1</i>}	This study

* these plasmids contain *C. parapsilosis* ARS7 element