Yeast
Yeast 2010; **27**: 933–939.
Published online 2 July 2010 in Wiley Online Library
(wileyonlinelibrary.com) **DOI:** 10.1002/yea.1800

Research Article

Plasmids for *in vivo* construction of integrative *Candida albicans* vectors in *Saccharomyces cerevisiae*

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Received: 28 January 2010
Accepted: 13 May 2010

Abstract

A general system has been devised for the *in vivo* construction of *Candida albicans* integrative vectors in *Saccharomyces cerevisiae*. The system is especially useful for the integration of genes in *C. albicans* that cannot be propagated in *Escherichia coli*, possibly because of their toxic effects. The ligation of *S. cerevisiae* 2 μ sequences to a *C. albicans* integrative vector permits *in vivo* maintenance and gap repair cloning within *S. cerevisiae*. After the vector assembly, it can be purified from *S. cerevisiae* or amplified by PCR and then used for transformation of *C. albicans*. The *S. cerevisiae* 2 μ sequence is completely removed by linearization prior to *C. albicans* transformation, such that no unwanted DNA is transferred in the final construct. The system was successfully used to clone and reintegrate the *C. albicans JEN2* gene, which encodes a membrane protein that is apparently toxic to *E. coli*. Three popular *C. albicans* integrative vectors, CIp10, CIp20 and CIp30, are now available in versions that permit gap repair in *S. cerevisiae*. GenBank Accession Nos CIp10–2 μ (GU550119), CIp20–2 μ (GU550120) and CIp30–2 μ (GU550121). Copyright © 2010 John Wiley & Sons, Ltd.

Keywords: **yeast; *Candida albicans*; gap-repair; toxic genes; plasmid integration; *in vivo***

Introduction

Candida albicans is a major fungal pathogen especially for patients with compromised immune systems (Odds, 1988). Many of the molecular tools that have been developed for this organism are essentially based on those for the distantly related *Saccharomyces cerevisiae*. However, since *C. albicans* is an obligate diploid, gene disruptions must be carried out twice to inactivate both alleles for each gene (Berman and Sudbery, 2002; Noble and Johnson, 2007). Autonomously replicating plasmids exist for *C. albicans* (Cannon *et al*., 1992) but their use is limited, since they are less stable than their *S. cerevisiae* counterparts. The construction of expression plasmids for *C. albicans* is most often achieved by cloning the gene of interest into these plasmids using *Escherichia coli* before integrating them into the *C. albicans* genome by homologous recombination (Sonneborn *et al*., 2000; Tripathi *et al*., 2002).

In *C. albicans*, the position of marker genes (such as *URA3*) may substantially affect the expression level of the marker gene (Lay *et al*., 1998) and, more importantly, virulence in animal models of candidiasis (Sundström *et al*., 2002). Further research has shown that integration of the URA3 in the RPS1 locus leads to expression levels that can restore virulence levels comparable to that of URA3 at its wild-type locus (Brand *et al*., 2004). A series of vectors has been developed for integrating sequences at the *RPS1* locus (Dennison *et al*., 2005; Murad *et al*., 2000) to prevent *URA3* expression levels from influencing the virulence of these transformants. Since the effect of marker expression level on virulence is likely

Table 1. *S. cerevisiae, E. coli* and *C. albicans* strains used in this study

Strain	Genotype	Reference
S. cerevisiae		
CEN.PK 113-5D	*MAT***a** ura3	van Dijken *et al.*, 2000
E. coli		
XL1BLUE	*endA1 gyrA96(nal^R) thi-1 recA1 relA1 lac glnV44 F'[::Tn10 proAB$^+$ lacI^q Δ(lacZ)M15] hsdR17(r_K^- m_K^+)*	Bullock, 1987
C. albicans		
RM1000	*ura3::imm434/ura3::imm434, his1::hisG/his1::hisG*	Negredo *et al.*, 1997
CNV3	*ura3::imm434/ura3::imm434,his1::hisG/his1::G jen2::HIS1/jen2::URA3*	Vieira *et al.*, 2009
CNV3-1	CNV3 with *RPS1* – CIp20	Vieira *et al.*, 2009
CNF5	CNV3 with *RPS1* – CIp20–*JEN2*	This study

to be complex, the same vector integrated at the same genetic locus should be used for comparisons across genetic modifications if virulence is to be measured.

The use of this system to overcome these positional effects of marker genes creates an implicit need to assemble genetic constructions in *E. coli.* However, this creates a problem if the *C. albicans* genetic modification cannot be propagated in this organism. Genes encoding eukaryotic membrane proteins are sometimes toxic to the extent that cloning is not possible in *E. coli*. Examples of these include *S. cerevisiae* hexose transporters *HXT15* and *HXT16* (Wieczorke *et al.*, 1999) and the *C. albicans JEN2* (Vieira *et al.*, 2009).

In *S. cerevisiae*, this can be overcome by direct cloning in yeast by *in vivo* gap repair. Orr-Weaver and Szostak (1983) were the first to report *in vivo* gap repair of two linear fragments into an episomal plasmid. This method has been widely used for direct high-throughput cloning of genes and libraries in yeast, where omitting *E. coli* in the cloning step simplifies the procedure and may increase the coverage of libraries. Therefore, we have created a set of three integrative *C. albicans* vectors, CIp10–2 μ, CIp20–2 μ and CIp30–2 μ, which permit *in vivo* assembly in *S. cerevisiae.* These vectors are based on the popular *C. albicans* integrative vectors CIp10 (Murad *et al.*, 2000), CIp20 and CIp30 (Dennison *et al.*, 2005). These vectors have one, two or three nutritional markers, respectively, for complementation of auxotrophic mutations (*URA3, HIS1, ARG4*), and they contain the *C. albicans RPS1* gene, which is used to direct integration of the plasmid to this genomic locus.

The CIp20–2 μ vector was used to clone the *C. albicans JEN2* gene in *S. cerevisiae* using gap repair, resulting in CIp20–2 μ–*JEN2*. Despite many attempts and the use of a range of alternative cloning strategies, we were unable to clone *JEN2* in *E. coli*. We tried unsuccessfully an *E. coli* strain that maintains a low copy number of the vector (CopyCutter™ EPI400™, EPICENTRE Biotechnologies, Madison, WI, USA), as well as constructing the vector by fusion PCR to avoid the *E. coli* cloning step. However, we were able to make CIp20–2 μ–*JEN2* in *S. cerevisiae* and the *JEN2* gene did not show any adverse effects in the yeast transformants. The cloned *JEN2* was then reintegrated in the *RPS1* genomic locus of a *C. albicans jen2* knockout mutant (Vieira *et al.*, 2009). The *JEN2* reintegration, which was confirmed by diagnostic PCR, suppressed the phenotypes of the *C. albicans jen2* knockout mutant. Growth on a medium containing succinic acid as sole carbon and energy source, as well as the ability to transport radiolabelled succinic acid by a mediated mechanism, was restored.

Materials and methods

Yeast strains, plasmids and growth conditions

Yeast strains and the plasmids used in this work are listed in Tables 1 and 2, respectively. Strains were maintained on solid YPD medium (Sherman, 1991). Growth of *C. albicans* strains was performed at 30 °C in synthetic complete (SC) medium

DOI: 10.1002/yea

Table 2. Plasmids used in this study

Plasmids	Source or reference
CIp20	Dennison *et al*., 2005
CIp20−2 μ−*JEN2*	This work
YEplac112	Gietz and Sugino, 1988

Table 3. Oligonucleotides used in this study

Name	Sequence
2 μ-CENARS_AarI_f	CCT TTG CAG GTG GTA TTT CAC ACC GCA TAT ATC G
2 μ-CENARS_AarI_r	CCT TTG CAG GTG TTT TGA AAA GCA AGC ATA AAA GAT C
cip20_3551_fwd	TTT TCA ATT TCA CGG CCA AT
2my_4936_fwd	GCA CAG AGA TAT ATA GCA AAG AGA TAC
cip20_5676_rev	CAA CAG ATC TAC CGG TTT AAA GAA
2my_4057_rev	GAA CCG GGG ATG CGA CGT
CaJEN2_rv_lo	AAA GGG AAC AAA AGC TGG GTA CCG GGC CCC CCC TCG AGG TCG ACC CGT CTC ATA TTT CTA ACC GAT TGT GCC AGT GGC TC
CaJEN2_fw_sh	CTT GTT TTT ACC GAC AGC CAT GTT GTA CTT GAG TTG GAT CTA CGC GTG AGC ACT AAC AAT TAG TTG TAC AGT TCA AA ACT
CaJEN2A1fwd	GGT GAT ACA TAT GGT AGA
CaJEN2A2rev	GTG ATC CAC ATT GGA TGG
DRPS10fwd	GTG GTT GGA GCT TTG ATG
DCaJEN2Rev	AGC CAT GAG AGC CAT CTC
HIS1_fw	TCA TCC TCC AGG TAC CGG ATC
RPS10_5ORF fwd	ACT TTG ATC AAC AGA TCT AC

without uracil or histidine (2% w/v glucose, 0.67% w/v yeast nitrogen base without amino acids and 2 g/l complete amino acid mixture without histidine or uracil; Kaiser, 1994). The same medium was also prepared with succinic acid (1% v/v, pH 5.0) as carbon source. Growth of *S. cerevisiae* was performed in synthetic defined (SD) medium with the same composition but leaving out the amino acid mixture. Solid media were prepared by adding agar (2% w/v).

Transport assays

Yeast cells were grown in SC medium to obtain glucose repressed cells. For conditions of glucose derepression, cells grown in SC medium were washed twice in ice-cold deionized water and inoculated into fresh SC medium with succinic acid instead of glucose. Cells were harvested during the exponential phase ($OD_{640\ nm} = 0.5$) by centrifugation, washed twice and resuspended in ice-cold deionized water to a final concentration of 25–35 mg dry weight/ml. Uptake rates of labelled succinic acid [(2,3-^{14}C) succinic acid (NEN Life Science); 5000 dpm/nM, pH 5.0] were estimated as described by Vieira *et al*. (2009). A computer-assisted non-linear regression analysis program (GraphPad Software, San Diego, CA, USA) was used in order to determine the best-fitting transport kinetics to the experimental data and to estimate the kinetic parameters. All the experiments were performed in biological triplicates and the data presented represent average values, with error bars corresponding to the standard deviation (SD).

Construction of the CIpX0−2 μ vectors

CIp10, CIp20 and CIp30 were digested with *Stu*I (AGG CCT), a restriction enzyme producing blunt ends that is normally used to linearize the vector before integration. The linearized vectors were ligated *in vitro* to a blunt PCR product of the *S. cerevisiae* 2 μ replication origin, containing one half *Stu*I site at each end. The PCR product was amplified from the vector YEplac112 (Gietz and Sugino, 1988) with the primers 2 μ-CENARS_AarI_f and 2 μ-CENARS_AarI_r (Table 3). Although many vectors carry the 2 μ sequence, the one in YEplac112 lacks a *Xba*I site due to directed mutagenesis, making this site available for manipulation elsewhere in the resulting vector. The ligation mixture was transformed directly to *S. cerevisiae* CEN.PK113-5D (ura3-52), using the high efficiency LiAc/ssDNA protocol (Gietz and Schiestl, 2007). Transformants were selected on solid SD medium on the rationale that the *C. albicans URA3* gene is able to complement the *S. cerevisiae ura3* mutation. Transformants were found to be almost exclusively the correct plasmids, which was as expected, since none of the DNA fragments alone should be able to both propagate in *S. cerevisiae* and complement the *ura3* mutation.

DOI: 10.1002/yea

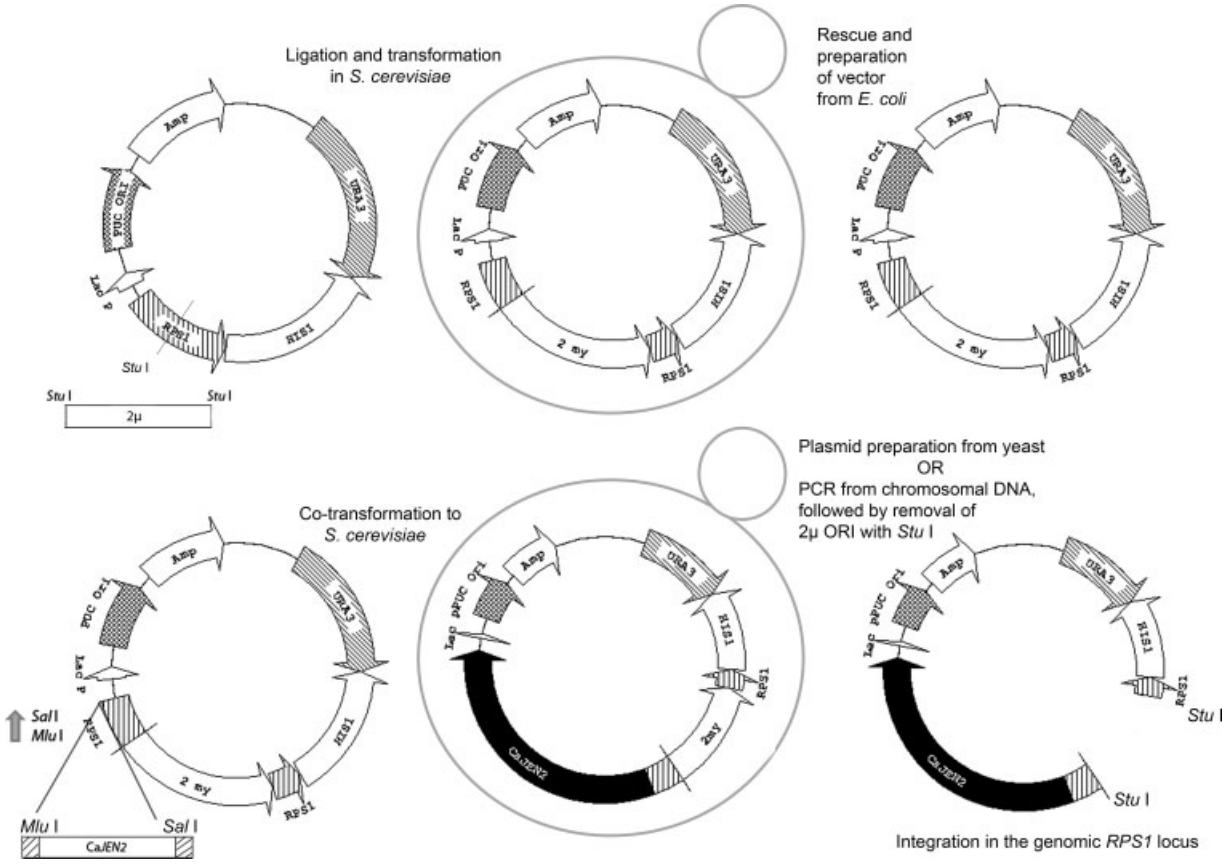

Figure 1. Schematic representation of the *in vivo* construction of CIp20−2 μ−JEN2 in *S. cerevisiae*. Only CIp20−2 μ is shown in this schematic overview but the methodology is similar for the CIp10−2 μ and CIp30−2 μ construction. CIp20 was initially digested with *Stu*I and ligated to the 2 μ sequence from YEplac112 *in vitro*. The CIp20−2 μ vector was isolated from yeast and amplified in *E. coli*. CIp20−2 μ was then digested with *Mlu*I and *Sal*I and co-transformed with a PCR-amplified *JEN2* fragment into *S. cerevisiae*. Transformants were selected for uracil prototrophy. The circular vector was purified and used as template for PCR amplification. Finally, *Stu*I digestion enabled the integration of the CIp20−2 μ plasmids at the *RPS1* genomic locus. The image was made with the vector drawing software Simvector from Premier Biosoft International (**http://www.premierbiosoft.com/plasmid_maps/index.html**)

The vectors were rescued from yeast by a combination of glass beads and *E. coli* plasmid mini preparation columns and transformed to *E. coli*. The vectors were purified from *E. coli* and analysed by digestion with *Stu*I to confirm the release of the 2 μ sequence. The location and orientation of the 2 μ sequence was confirmed by PCR, using the primer pairs cip20_3551_fwd/2my_4507_rev and cip20_4936_fwd/2my_5676_rev, producing PCR products of 764 bp and 525 bp across the vector insert junctions, respectively. The vectors were given the names CIp10−2 μ, CIp20−2 μ and CIp30−2 μ (Figure 1). The sequences are available from GenBank under AccessionNos GU550 119, GU550 120 and GU550 121, respectively.

Construction of the CIp20−2 μ−JEN2 vector

The CIp20−2 μ vector was digested with *Mlu*I and *Sal*I in the presence of shrimp alkaline phosphatase to prevent religation of the vector. The enzymes were heat-inactivated according to the manufacturer's instructions. A 4295 bp fragment containing the *C. albicans JEN2* locus was PCR-amplified from *C. albicans* genomic DNA, using the primers CaJEN2_rv_lo and CaJEN2_fw_sh (Table 3). The primers add 41 and 45 bp of homology to the CIp20−2 μ vector, up- and downstream of the *Mlu*I and *Sal*I sites. The linear vector and the PCR product were co-transformed into *S. cerevisiae* CEN.PK113-5D *(ura3-52)* and transformants were

DOI: 10.1002/yea

selected for uracil prototrophy on SD medium. The presence of the *C. albicans JEN2* gene sequence in the transformants was confirmed by colony PCR, using the primers CaJEN2A1fwd and CaJEN2A2rev (Table 3), which amplify a 764 bp PCR product internally in the *JEN2* locus. The correct location of the *JEN2* relative to the CIp20−2 μ vector was confirmed by PCR with the DRPS10fwd and DCaJEN2Rev primers (Table 3), resulting in a 387 bp PCR product across the junction between the *RPS1* gene of the vector and the *JEN2* fragment.

Isolation of the CIp20−2 μ−CaJEN2 vector from *S. cerevisiae*

Several protocols have been described for the isolation of plasmids from *S. cerevisiae*. Most protocols rely on transformation of *E. coli* of a crude yeast DNA preparation. Since it was not possible to propagate the *JEN2* gene, a method was needed for direct isolation of plasmid DNA from yeast. *S. cerevisiae* has a strong cell wall and expresses more nuclease activities than the normally used laboratory strains of *E. coli*, so direct plasmid purification poses a technical problem. Direct isolation of plasmid DNA and separation of circular and linear DNA has been described using CsCl density gradient centrifugation, alkaline lysis of spheroplasts and partitioning of linear DNA using acid phenol. In our hands it was difficult to prepare DNA of sufficient quantity and quality using these methods. We therefore chose to use a combination of plasmid DNA preparation from *S. cerevisiae* and amplification of the vector using long PCR. We prepared a small quantity of plasmid DNA from the *S. cerevisiae* strain, using the same protocol combining glass beads and plasmid mini-preparation columns described earlier. We used 4% v/v of the isolated DNA as template for PCR, using the Long PCR Enzyme Mix (FERMENTAS) according to the manufacturer's recommendations and the primers 2my_4936_fwd and 2my_4057_rev. Four 50 μl PCR reaction were pooled, purified using the SureClean DNA cleanup kit (BIOLINE) and resuspended in 50 μl TE buffer. The PCR product was then digested with *Stu*I by adding 7.4 μl buffer 2.5 μl *Stu*I to 17.1 μl concentrated PCR product and used to transform *C. albicans* (Walther and Wendland, 2003). Three independent transformants were obtained and the correct localization of the CIp20−JEN2 vector was confirmed by PCR, using primers HIS1_fw and RPS10_5ORF fwd (Table 3). The first primer is plasmid-specific, whereas the second is located in the genomic *RPS1* locus, producing a PCR product of 672 bp from the correct integrated plasmid (Figure 2).

Results and discussion

We have constructed a series of *C. albicans* integrative vectors (CIp10−2 μ, CIp20−2 μ and CIp30−2 μ) that permit plasmid construction *in vivo* by gap repair in *S. cerevisiae*. The vectors were constructed by combining a series of widely used *C. albicans* vectors (CIp10, −20 and −30) and a PCR product of the yeast 2 μ sequence. This sequence was inserted within the part of the vector that normally directs the chromosomal integration to a specific locus by ends-in homologous recombination. The enzyme used to linearize the vector before transformation in *C. albicans* was retained on each side of the 2 μ sequence, so that it is lost by linearization of the vector. This means that no

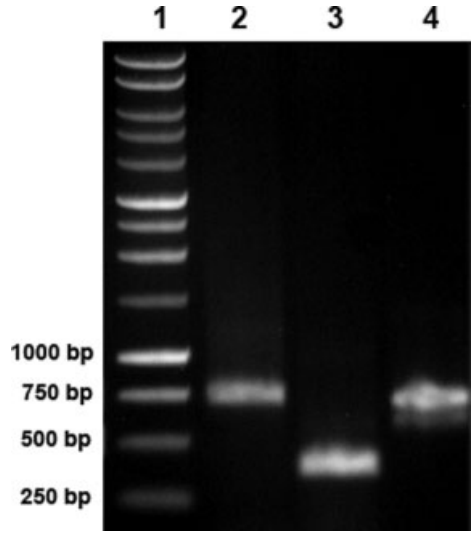

Figure 2. Verification of *JEN2* ligation to CIp20−2 μ and of plasmid integration in the *RPS1* genomic locus by analytical PCR. Correct recombination of *JEN2* in CIp20−2 μ was confirmed by diagnostic PCR with primers CaJEN2A1fwd/CaJEN2A2rev (764 bp) and DRPS10fwd/DCaJEN2Rev (387 bp), as represented in lanes 2 and 3, respectively. Additionally, correct integration of the CIp20−JEN2 in the *RPS1* genomic locus was confirmed with primers HIS1_fw/RPS10_5ORF (672bp) fwd and is represented in lane 4

DOI: 10.1002/yea

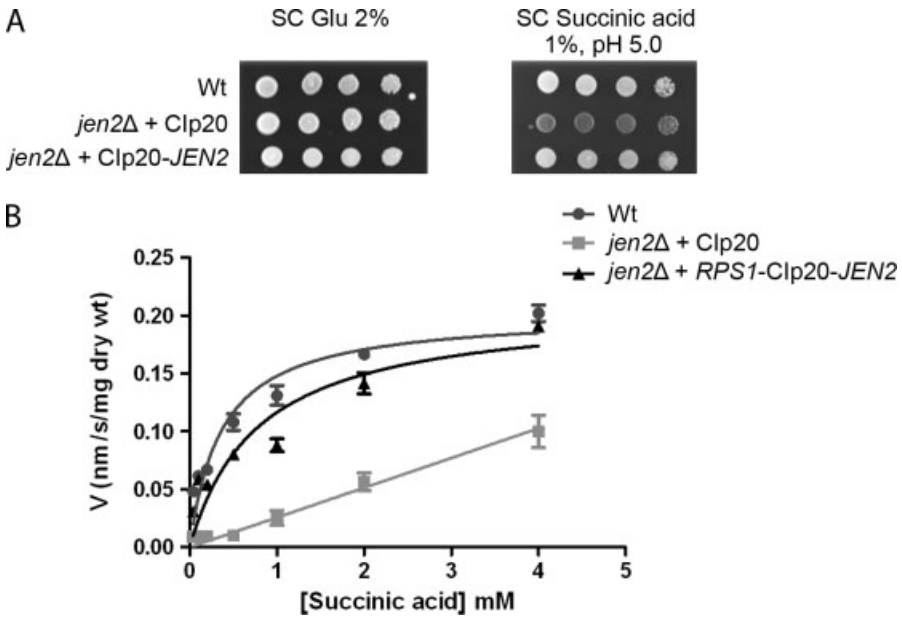

Figure 3. Growth phenotypes and transport of succinic acid in *C. albicans* jen2 mutants. RM1000 (wild-type), CNV3 (*jen2*) and the reintegrant CNF5 (*jen2, RPS1–CIp20–*JEN2). (A) *C. albicans* strains were incubated for 48 h at 37 °C in the following solid media: SC Glu (2%, w/v) and SC succinic acid (1%, w/v, pH 5.0). Cells were serially diluted and 5 μl drops of each dilution were spotted onto the plates: Wt, *C. albicans* RM1000 (*JEN2/JEN2*); *jen2*Δ + CIp20, CNV3 (*jen2/jen2, RPS1–CIp20*); *jen2*Δ + CIp20–JEN2, CNF5 (*jen2/jen2, RPS1–CIp20–*JEN2). (B) Initial uptake rates of (2,3-^{14}C) succinic acid at pH 5.0, as a function of succinic acid concentration after growth in medium containing succinic acid. ●, *C. albicans* RM1000 (*JEN2/JEN2*); ■, CNV3 (*jen2/jen2*); ▲, CNF5 (*jen2/jen2, RPS1–CIp20–*JEN2). Error bars represent SD of experiments carried out in biological triplicates

S. cerevisiae sequences are transferred to *C. albicans* and the resulting integrated construct is not affected by the strategy. These vectors facilitate the cloning and integration of genes that are toxic in *E. coli* in *C. albicans*. A schematic view of the strategy is shown in Figure 1.

The gene *JEN2* encodes a *C. albicans* dicarboxylate transporter, the deletion of which from *C. albicans* impairs the mediated transport of succinic and malic acid and leads to a growth defect on these substrates (Vieira *et al*., 2009). We have previously tried unsuccessfully to clone this gene in *E. coli* by ligating a *JEN2* PCR product into a linearized CIp20 vector (Vieira *et al*., 2009). Exhaustive attempts to clone *JEN2* in *E. coli* using alternative strategies were also unsuccessful in our hands (unpublished). In the current study we amplified the same *JEN2* fragment with primers adding a short stretch of homology to each side of the cloning site in CIp20–2 μ. The CIp20–2 μ and the PCR product were assembled *in vivo* and given the name CIp20–2 μ–*JEN2*. CIp20–2 μ–*JEN2* was amplified by long PCR from *S. cerevisiae*, digested and integrated in a *C. albicans jen2/jen2* double mutant, as described in Materials and methods. The correct integration was confirmed by colony PCR (Figure 2).

The growth phenotypes of *C. albicans* RM1000 (wild-type), CNV3 (*jen2/jen2*) and the reintegrant CNF5 (*jen2/jen2, RPS1–CIp20* –JEN2) were evaluated on both SC glucose 2% and SC succinic acid 1% solid media. No growth defect was found in SC glucose medium (Figure 3A, left panel), as expected. A growth defect was observed on succinic acid for *jen2/jen2* cells, and the insertion of CIp20–2 μ–*JEN2* fully restored the ability to grow on this carbon source (Figure 3A, right panel). In addition, we measured initial uptake rates of labelled succinic acid, pH 5.0, in cells grown on succinic acid. Like wild-type *JEN2/JEN2* cells, the *jen2/jen2/JEN2* strain displayed the ability to transport succinate by a mediated mechanism, indicating that the reintegration of *JEN2* had suppressed this phenotype of the *jen2/jen2* mutant.

DOI: 10.1002/yea

This *in vivo* gap repair strategy is not limited to the three vectors described here but can, in principle, be applied to any integrative *C. albicans* vectors, provided that the enzyme used to linearize the vector is added to each side of the 2 μ sequence before ligation to the vector. The plasmid isolation of the assembled vectors by PCR from *S. cerevisiae* could be done more efficiently using primers annealing immediately up- and downstream of the 2 μ sequences, so that the PCR product is devoid of *S. cerevisiae* sequences and no subsequent digestion is necessary. We chose to use the 2my_4936_fwd and 2my_4057_rev primers, which meant that the extraneous *S. cerevisiae* sequences had to be removed by *Stu*I digestion, since this avoided the synthesis of new oligonucleotides. This novel strategy, which has proved to be a very useful tool for the construction of an integrative vector of a toxic gene, can be used for the reintegration of any gene in *C. albicans*.

Acknowledgements

This study was supported by FEDER, Portugal (Grant No. POCI/BIA-BCM/57812/2004; Eixo 2, Medida 45 2.3, QCAIII–FEDER). N.V. received a FCT PhD fellowship (Grant No. SFRH/BD/23503/2005). F.P. and B.J. were supported by European Union project NILE (Grant No. FP6-019882). AJPB was funded by the BBSRC (Grant Nos BB/F000111/1, BB/D009308/1, BB/F010826/1 and BB/FO0513X/1) and the European Commission (Grant Nos PITN-GA-2008-214004 and ERC-2009-AdG- 249793).

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