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ScienceDirect
Journal of Biotechnology 131 (2007) 20–26
Journal of BIOTECHNOLOGY
www.elsevier.com/locate/jbiotec

Application of the Cre-*lox*P system for multiple gene disruption in the yeast *Kluyveromyces marxianus*

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Received 10 January 2007; received in revised form 8 May 2007; accepted 25 May 2007

Abstract

The yeast *Kluyveromyces marxianus* presents several interesting features that make this species a promising industrial yeast for the production of several compounds. In order to take full advantage of this yeast and its particular properties, proper tools for gene disruption and metabolic engineering are needed. The Cre-*lox*P system is a very versatile tool that allows for gene marker rescue, resulting in mutant strains free of exogenous selective markers, which is a very important aspect for industrial application. As the Cre-*lox*P system works in some non-conventional yeasts, namely *Kluyveromyces lactis*, we wished to know whether it also works in *K. marxianus*. Here, we report the validation of this system in *K. marxianus* CBS 6556, by disrupting two copies of the *LAC4* gene, which encodes a β-galactosidase activity.
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Keywords: Cre-*lox*P system; *Kluyveromyces marxianus*; Gene disruption; β-Galactosidase; Gene targeting efficiency

1. Introduction

Although the full genome sequence of the non-conventional yeast *Kluyveromyces marxianus* is not available, special attention has been paid to the promising biotechnological aspects of this non-conventional yeast, since it presents the possibility of producing homologous enzymes, such as inulinase (Rouwenhorst et al., 1988) and β-galactosidase (Bojorge et al., 1999; Furlan et al., 2000; Martins et al., 2002), as well as heterologous proteins (Bergkamp et al., 1993a). The high protein production rates usually observed for this species are related to its high capacity of converting substrate into biomass, without significant by-product formation (Fonseca et al., 2007). There is an evidence that *K. marxianus* is even more Crabtree-negative than its close relative *Kluyveromyces lactis* and other commonly studied non-conventional yeasts (Bellaver et al., 2004). Other relevant aspects of *K. marxianus* include the high temperature range in which it can grow (25–45 °C), the high growth rate at elevated temperatures (Steensma et al., 1988) and the capacity of growing on different substrates, including lactose as the sole carbon and energy source.

The availability of disruption tools for use in *K. marxianus* is rather scarce, and in order to circumscribe this limitation, it becomes necessary to create or adapt new methodologies. In addition, there is no information available on the ploidy status of most of *K. marxianus* strains and probably, the majority will not be haploid.

The Cre recombinase has been described as "the universal reagent for genome tailoring" (Nagy, 2000). The Cre-*lox*P system makes use of the site-specific recombinase Cre from the phage P1 that catalyzes the recombination between two of its DNA recognition sites, called *lox*P (Hamilton and Abremski, 1984). The *lox*P site is a 34 bp consensus sequence composed of two 13 bp inverted repeats separated by an asymmetric 8 bp core sequence. Concerning the molecular mechanism of recombination, the Cre protein must locate and bind to the *lox*P site, perform synapsis of DNA at two such sites, and then break and rejoin the DNA to generate a recombinant molecule (Sauer, 1987). Thus, recombination between two directly repeated sites on the same

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doi:10.1016/j.jbiotec.2007.05.027

DNA molecule results in excision of the DNA segment lying between the sites.

The first application of the Cre-*lox*P system in yeasts was described by Sauer (1987) in *Saccharomyces cerevisiae*. Since then, its use has been extended and when necessary adapted, to several other yeast strains, namely *K. lactis* (Steensma and Ter Linde, 2001; Gueldener et al., 2002), *Yarrowia lipolytica* (Fickers et al., 2003), *Candida albicans* (Dennison et al., 2005), *Schizosaccharomyces pombe* (Iwaki and Takegawa, 2004; Hentges et al., 2005) and *Hansenula polymorpha* (Krappmann et al., 2000).

Considering this, the aim of this work was to test the efficiency of Cre-*lox*P system for multiple gene disruption in *K. marxianus*. The validation of the system was done by disruption of all copies of the *K. marxianus LAC4* gene, using a gene disruption cassette that combines the advantages of the *kanMX* gene (which confers resistance to the antibiotic geneticin, G418) with those of the Cre-*lox*P system (Guldener et al., 1996).

2. Materials and methods

2.1. Strains and plasmids

Escherichia coli Top10 (Invitrogen, Carlsbad, CA) was used for plasmid maintenance and construction.

Strain *K. marxianus* CBS 6556 was obtained from Prof. Marcos Morais (Federal University of Pernambuco, Recife, Brazil) on YPD-agar medium. Cells from a single colony were transferred to YPD liquid medium and grown until late exponential phase. Glycerol was added to the culture (15%, w/w final) and 2 mL aliquots were stocked at −80 °C.

The *LAC4* gene deletion cassette was cloned into pGEM®-T Easy vector from Promega (Madison, WI).

2.2. Culture media

LB medium supplemented with 100 μg ampicillin mL^{-1} was used for *E. coli* cultivation. YPD medium supplemented with 200 μg mL^{-1} filter-sterilized G418 or with 50 μg mL^{-1} ClonNat (active substance, aminoglycoside nourseothricin) was used for the selection and maintenance of the yeast transformants harbouring the G418 resistance gene or the pKlNatCre plasmid, respectively. For selection of transformants after electroporation, selective plates were supplemented with 1 M sorbitol. To detect β-galactosidase mutants, solid YPD medium was supplemented with 40 μg mL^{-1} 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-gal). White colonies represent cells with no β-galactosidase activity while blue colonies indicate β-galactosidase producing cells.

2.3. DNA manipulations

All restriction enzymes were purchased from Fermentas (Ontario, Canada). T4 DNA ligase and shrimp alkaline phosphatase were purchased from Promega. DNA fragments were purified from agarose gels using the Qiaquick Gel Extraction Kit (QIAgen).

Large-scale plasmid DNA preparation from *E. coli* was carried out using the Midi kit from Q-Biogen (Irvine, CA).

2.4. Construction of the gene deletion cassette

The region +211 to +2204 of *LAC4* ORF (accession number AY526090) with 3077 bp full length was amplified by PCR from *K. marxianus* genomic DNA, using the primers Lac4FW GCACCATTTCTGTGCCATCCCATTG and Lac4RV GGGTACCTTCAATGGAAGTTCAGC. The PCR conditions were 95 °C for 4 min, 30 cycles of 95 °C for 30 s, 57 °C for 30 s, 72 °C for 2 min and a final step at 72 °C for 15 min. The 1.9 kb PCR product was gel purified and cloned into pGEM®-T Easy vector.

The *kan*MX cassette was amplified from pUG6 (Guldener et al., 1996) using the following primers Kan1 GCTGTACA-CAGCTGAAGCTTCGTACGC and Kan2 GCTGTACAGC-ATAGGCCACTAGTGGATCTG, creating *Bsp*1407I restriction sites (underlined). The PCR conditions were 95 °C for 4 min, 30 cycles of 95 °C for 30 s, 63 °C for 30 s, 72 °C for 2 min and a final step of 72 °C for 15 min. The PCR product was gel purified and digested with *Bsp*1407I. The 1.9 kb LAC4 fragment that had previously been cloned into the pGEM®-T Easy vector was also digested with *Bsp*1407I, which cuts at positions 974 and 1532 of the *LAC4* gene. To avoid recircularization of the vector, it was dephosphorilated with Shrimp Alkaline Phosphatase following the manufacturer instructions. After this, the *kan*MX cassette was ligated to the digested and dephosphorilated vector. In this way, a disruption cassette with long flanking homology regions of 723 and 712 bp was obtained (Fig. 1).

2.5. Yeast transformation

K. marxianus integrative transformation was performed by electroporation. The preparation of electrocompetent yeast cells was conducted as described elsewhere (Ausubel et al., 1997). An appropriate amount of yeast cells (50 μL) were gently mixed with 5 μg of *LAC4* disruption cassette and transferred into the electroporation cuvettes (0.2 cm electrode, Bio-Rad). An electric pulse of 1.5 kV, 200 Ω was applied, with the capacitance of 25 μF, by using the Gene Pulse X-Cell (Bio-Rad). After electric shock, 1 mL of YPD supplemented with 1 M sorbitol was added to the cell suspension and the mixture was incubated for 6–16 h at 30 °C and 85 rpm. Following incubation, the cells were plated on selective YPD-G418-X-gal plates.

2.6. Removal of the loxP-kanMX module

After confirmation of cassette integration in the right locus we proceeded with the removal of the *kanMX-loxP* cassette following the protocol described by Steensma and Ter Linde (2001). The mutant strain was transformed with pKlNatCre plasmid and transformants were selected on plates with 50 μg/mL ClonNat (active substance, aminoglycoside nourseothricin). The plasmid pKlNatCre harbours the Cre-recombinase under control of the *GAL1* promoter and the *nat* selective marker, as in pNatCre (plasmid used for *S.*

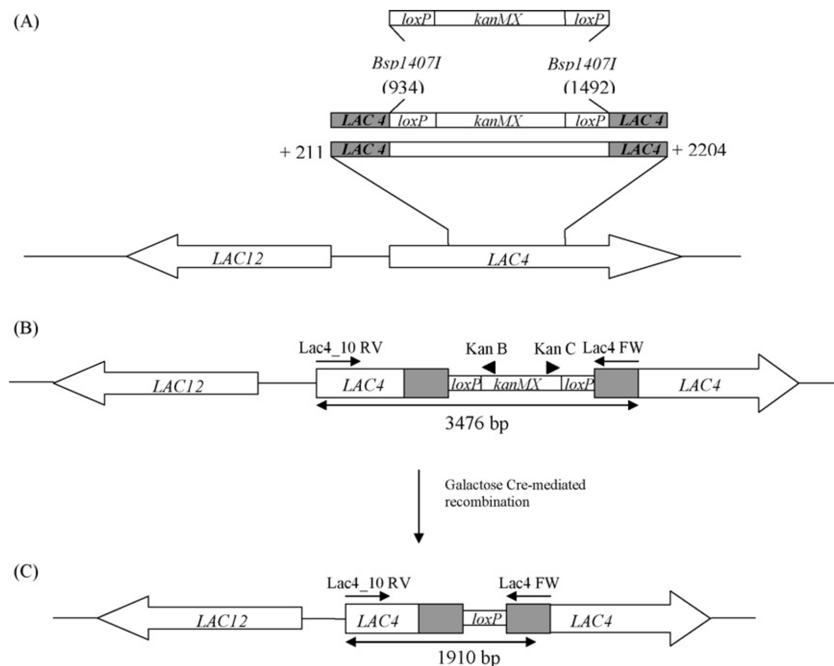

Fig. 1. Schematic representation of the disruption of the *LAC4* gene. After *NotI* excision from pGEM®-T Easy, the deletion cassette comprising a 5′flanking region of 723 bp and a 3′region of 712 bp was integrated into the genome of *K. marxianus*. Gray boxes represent regions of *LAC4* present in the disruption cassette, while empty boxes represent parts of *LAC4* that are not present in the disruption cassette. (A) Construction of the disruption cassette (see Section 2); (B) integration of the disruption cassette into the genome after transformation by electroporation; (C) marker rescue through galactose induction of the Cre-*lox*P recombination system. The primers used for diagnostic PCR are represented by thin arrows (Lac4FW and Lac4_10RV). The size of the PCR product after integration of the cassette in the correct locus is 3476 bp and after removal of the *Kan*MX, a PCR product of 1910 bp is expected, whereas amplification of the wild-type allele should yield a band size of 2434 bp. The primers KanB and KanC used for PCR confirmation are indicated by thick short arrows.

cerevisiae), besides the *ARS* and *CEN2* genes from *K. lactis* (Steensma and Ter Linde, 2001). The complete nucleotide sequence of pKlNatCre plasmid can be found at Leiden University website (http://biology.leidenuniv.nl/ibl/S2/yeast). ClonNat-Resistant colonies were mixed and incubated in YP 2% galactose for 4 h and subsequently plated on YPD. Isolated colonies were restreaked on YPD containing G418 and on YPD containing ClonNat and sensitive colonies (both to G418 and ClonNat) were selected for PCR confirmation.

2.7. Analysis of transformants

2.7.1. Polymerase-chain reaction

Confirmation of correct insertion of the transforming DNA was performed by diagnostic PCR, using as template total genomic DNA isolated according to the method of Hofman and Winston (1987), with the modifications referred to in Ausubel et al. (1997). Target gene-specific primers were used, one which anneals to the *LAC4* homologous region within the cassette (Lac4FW, Fig. 1) and another one with homology to the *LAC4* gene, but that does not anneal to the disruption cassette (Lac4_10RV, ATCAGGAGGCTGATATTCG, Fig. 1). This primer set will always lead to PCR amplification; the wild type allele corresponding to a 2434 bp fragment and the correct integration in the *LAC4* locus to a 3476 bp fragment. The removal of the *kan*MX selective marker can also be monitored with this set of primers, yielding a 1910 bp fragment. Other primer combinations were also used to confirm the results, consisting of the target gene-specific primers, either within (Lac4FW and Lac4RV) or out (Lac4_10RV) of the *LAC4* sequence present in the disruption cassette, in combination with disruption cassette-specific primers (KanB GGATGTATGGGCTAAATG and KanC CCTCGACATCATCTGCCC).

2.7.2. Specific β-galactosidase activity

Yeast cells were grown at 30 °C in shake flasks (250 mL flasks containing 100 mL of medium) at 200 rpm on defined medium (Verduyn et al., 1992) with 10 g/L galactose as carbon source to an OD_{600} of 0.5, harvested by centrifugation, and suspended in three packed cell volumes of ice-cold extraction buffer (100 mM sodium phosphate buffer pH 7.0, 10 mM KCl, 1 mM $MgSO_4{\cdot}7H_2O$, 0.28% (v/v) 2-mercaptoethanol, 1 mM EDTA, 0.3 M $(NH_4)_2SO_4$, 5% glycerol, 1 mM DTT, 1 mM phenylmethanesulfonyl fluoride, 0.5× complete EDTA-free protease inhibitor cocktail from Roche). Cells were broken by vortexing (6 cycles of 30 s with 1 min intervals on ice) with 4 packed cell volumes of glass beads, and then diluted with 3 vol of ice-cold buffer Z (100 mM sodium phosphate buffer pH 7.0, 10 mM KCl, 1 mM $MgSO_4{\cdot}7H_2O$, 0.28% (v/v) 2-mercaptoethanol). Cell debris was removed by centrifugation for 15 min at 15,700 × *g* (2 °C) and the supernatant was used for the enzyme assay and determination of protein content.

The beta-galactosidase activity was assayed using p-nitrophenyl-beta-D-galactopyranoside (pNPG) as the substrate (Miller, 1972). Briefly, 200 μL samples of appropriate dilutions of extract in buffer Z (at least 10 times dilution) were transferred

to microplate wells, 50 μL of 4 mg mL^{-1} pNPG were added, the plate was incubated at 30 °C in a microplate reader (BIO-TEK Synergy HT) and the optical density at 405 nm was read over time. At least three different dilutions of each extract were assayed and the standard deviation was <30%. The supernatants from the first centrifugation were assayed for extracellular beta-galactosidase activity: 50 μL of supernatant were mixed with 150 μL of buffer Z and assayed as described above for the diluted extracts.

Protein concentrations in the extracts were measured with Bio-Rad protein reagent (cat. no. 500-0006), using ovalbumine as the standard.

One unit of activity was defined as the amount of enzyme that hydrolysed 1 nmol *p*NPG per minute under the stated conditions. Specific activities were expressed as U per mg of protein.

The percentage of cells without detectable β-galactosidase activity was monitored as white colony-forming units (cfu) on YPD plates containing X-gal.

2.7.3. Shake flask cultivations

A (1–2 days old) colony from a YPD plate culture was transferred to a 100 mL flask containing 10 mL of defined medium (Verduyn et al., 1992) supplemented with 10 g L^{-1} of one of the following sugars: lactose, glucose, galactose, glucose plus lactose (10 g L^{-1} each) or galactose plus lactose (10 g L^{-1} each). The culture was grown at 30 °C and shaking at 200 rpm during 16 h. After this period, about 10% of this exponentially growing culture was inoculated into a 250 mL Erlemeyer flask containing 100 mL of the same medium. Cells were grown under the same conditions, over a period of 30 h, and samples were collected for the determination of cell concentration via absorbance measurements at 600 nm.

3. Results and discussion

3.1. Construction of the LAC4 gene disruption cassette

According to Kooistra et al. (2004), the efficiency of gene targeting in *K. lactis* increases with the length of the flanking homology region in the disruption cassette, namely from 0% with 50 bp to 88% with 600 bp flanks. Considering the few gene deletion studies performed in *K. marxianus* (Bergkamp et al., 1991, 1993b; Siekstele et al., 1999), we assumed that the efficiency of homologous recombination in this yeast would be similar to the one observed in *K. lactis*. In a report by Bergkamp et al. (1991), they have constructed a stable *leu*2 mutant in *K. marxianus* by replacing part of the *LEU2* sequence by the bacterial kanamycin resistance gene, using a deletion cassette composed of a 176 and a 331 bp flanking regions. As a result, a targeting efficiency of 5% was obtained. In another report, the use of the resistance gene AUR1-C (encoding resistance to the antibiotic aureobasidin A) as a selection marker for *LEU2* gene disruption resulted in few copies of tandemly integrated plasmids into different non-homologous chromosomal sites (Hashida-Okado et al., 1998). Taken together, these results support the idea that the mechanism of homologous recombination in this yeast has a low efficiency. The *LAC4* gene disruption cassette used in the present work was constructed in such a way that long flanking homologous sequences, namely of 723 and 712 bp, were created (Fig. 1), in order to obtain a high number of transformants with the correct integration of the gene disruption cassette.

3.2. Confirmation of LAC4 disruption and marker rescue by PCR

The correct integration of the gene disruption cassette was confirmed by PCR using the Lac4FW (with homology to both the *LAC4* gene and to the disruption cassette) and the Lac4_10RV (with homology to the *LAC4* gene, but not to the cassette) primer combination (Fig. 1). This primer set leads to the amplification of a 2434 bp fragment for the wild type allele and a 3476 bp fragment for the correctly integrated disruption cassette. Because of their particular position, the combination of these two oligonucleotides will not lead to amplification if the cassette is integrated by non-homologous recombination in a different locus. When analysing the transformants by diagnostic PCR with this set of primers, we observed the presence of the wild type allele in all transformants (Fig. 2A, lane 2) and in some of them, a fragment corresponding to the disruption cassette integrated into the *LAC4* locus was also amplified (Fig. 2A, lanes 3 and 4; Fig. 2B, lane 1). These results indicated that more than one copy of the *LAC4* gene might be present in the genome and that at least a second round of transformation with the disruption cassette was needed, in order to obtain disruption of all *LAC4* alleles.

It is worth noting that when this study started, the ploidy of *K. marxianus* CBS 6556 was not clear. One report indicates that this strain is diploid (De Morais Junior, 2003), while another

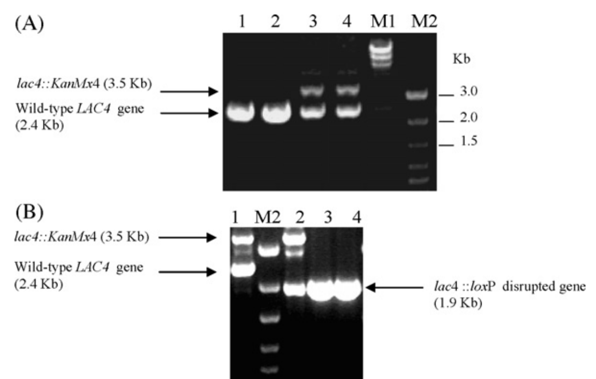

Fig. 2. Confirmation of *lac*4 gene disruption in *K. marxianus* with Lac4FW and Lac4_10RV primers. The PCR was made using as template (A) lane 1, wild type genomic DNA (predicted fragment size 2434 bp); lanes 2, 3 and 4 first round transformant genomic DNA, lane 2 corresponds to cassette integration in the wrong locus and lanes 3 and 4 in the *LAC4* locus (predicted fragment size 3476 bp); (B) lane 1, first round transformant genomic DNA with the disruption cassette integrated in the *LAC4* locus, before marker removal; lane 2 corresponds to the second round transformant with the disruption cassette integrated in the correct locus (band at 3476 bp), the fragment corresponding to the disrupted *LAC4* gene from the first round, after marker removal (1910 bp) can also be observed; lanes 3 and 4 second round transformant genomic DNA after marker removal (predicted fragment size 1910 bp). M1, Gene ruler λ HindIII (Bioron); M2, Gene ruler 100 bp DNA Ladder Plus (Fermentas).

one states that the strain is haploid based on DNA content of the cells (Steensma et al., 1988). Our results give some indication that a diploid status may be possible, since two rounds of transformation were needed to eliminate the *LAC4* gene from the *K. marxianus* genome. Besides this, in the Génolevures 1 initiative (http://cbi.labri.fr/Genolevures/), the only yeast species, among the 13 investigated hemiascomycetous yeasts, for which the ploidy could not be determined, is precisely *K. marxianus*. This again indicates that it is probably not haploid. However, we cannot rule out that the presence of two copies of *LAC4* is due to specific duplication of this gene in a haploid context.

G418-resistant mutants with one disrupted *LAC4* gene were then transformed with the pKlNatCre plasmid (for expression of Cre recombinase) and selected in ClonNat plates. ClonNat-Resistant colonies were then grown in YP containing 2% galactose to induce the removal of the *lox*P-*kanMX* module by expression of the Cre recombinase. After isolation of G418 and ClonNat-sensitive colonies, removal of the module was confirmed by PCR.

After a second round of transformation with the same disruption cassette, the band corresponding to the wild type allele was not amplified by diagnostic PCR (using Lac4Fw and Lac410_RV primers), and two bands were observed, one corresponding to the interrupted gene (3476 bp) and the other to the disrupted allele (1910 bp) from the first round (Fig. 2B, lane 2). After transformation of the null mutants with pKlNatCre, and growth of the cells on galactose containing medium, removal of the *lox*P-*kanMX* module was confirmed by PCR with the same primer set; the 3476 bp band corresponding to the disruption cassette integrated within the *LAC4* locus disappeared and the 1910 bp band corresponding to the disrupted *LAC4* locus was observed (Fig. 2B, lanes 3 and 4).

By using the Cre-*lox*P system, two copies of the *LAC4* gene were disrupted, showing that the *lox*P sequence, left after the first round of transformation and marker removal, does not affect incoming disruptions. The use of Cre-*lox*P proved to be a practical strategy to disrupt two copies of a gene in successive experiments in *K. marxianus*, resulting in a "clean" mutant strain almost free of exogenous DNA (except for the 34 bp *loxP* site left behind).

3.3. Analysis of gene targeting efficiency

After transformation with the disruption cassette, 67 and 65% of white colonies were visualised in YPD-G418-Xgal plates in the first and second round of transformation, respectively (Table 1). However, when streaking the transformants on new plates, for DNA extraction, we could observe that the white colonies turned blue (all white colonies from the first round and 73% of white colonies from the second round). The gene targeting efficiency was calculated based on PCR analysis of 72 colonies of the first round and of all the colonies of the second round, using the Lac4Fw and Lac4_10Rv primer set. In the first round, half of the initially identified as white colonies were not mutants and in the second round, the percentage of false positives was even higher. This can be due to the low permeability of cells to the X-gal dye and/or to events of non-homologous recombination into alternative loci that may indirectly affect β-galactosidase expression.

Table 1
Electroporation results obtained for *K. marxianus* CBS 6556 using the *LAC*4 disruption cassette after the first and the second round of transformation

Color type of colonies	Number of colonies	
	First round	Second round
White	144	44
Light blue	15	23
Dark blue	55	1
Relative percentage of white colonies (%)	67	65
Gene targeting efficiency[a] (%)	34	15

Transformants where plated on YPD + G418 + X-gal plates. The gene targeting efficiency was calculated by PCR analysis.
[a] The number of white colonies that verified the integration of the disruption cassette in a diagnostic PCR using Lac4FW and Lac4_10RV oligonucleotide set.

The gene targeting efficiency, *i.e.*, the number of white colonies that verified the integration of the disruption cassette in a diagnostic PCR using Lac4FW and Lac4_10RV oligonucleotide set, was 34% for the first and 15% for the second round of transformation. These values obtained with a disruption cassette with long flanking homologous sequences, namely 723 and 712 bp, are much lower than the one expected for *K. lactis* (88% for 600 bp flanking homologous sequences, Kooistra et al., 2004). In fact, high non-homologous recombination events have been described in *K. marxianus* (Pecota et al., 2007).

3.4. Specific β-galactosidase produced by wild type and mutant strains

In cell extracts prepared from exponentially growing cultures on defined medium (Verduyn et al., 1992) supplemented with galactose, the *Kmlac4* mutant containing one copy of the intact *LAC4* gene showed an activity value corresponding roughly to half of the original value (Fig. 3). As expected, the *Kmlac4* null mutant (obtained after the second round of transformation

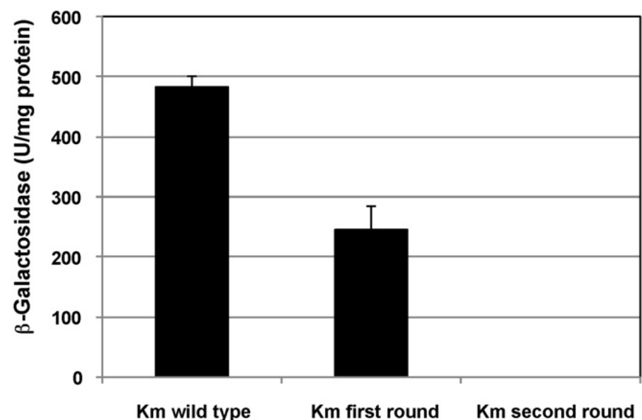

Fig. 3. Average β-galactosidase activities and standard deviations obtained from three independent assays from: *K. marxianus* wild type strain (Km wild type), *K. marxianus* with one copy of the *LAC4* gene disrupted (Km first round) and *K. marxianus lac4* mutants, *i.e.*, with two copies of the *LAC4* gene disrupted (Km second round).

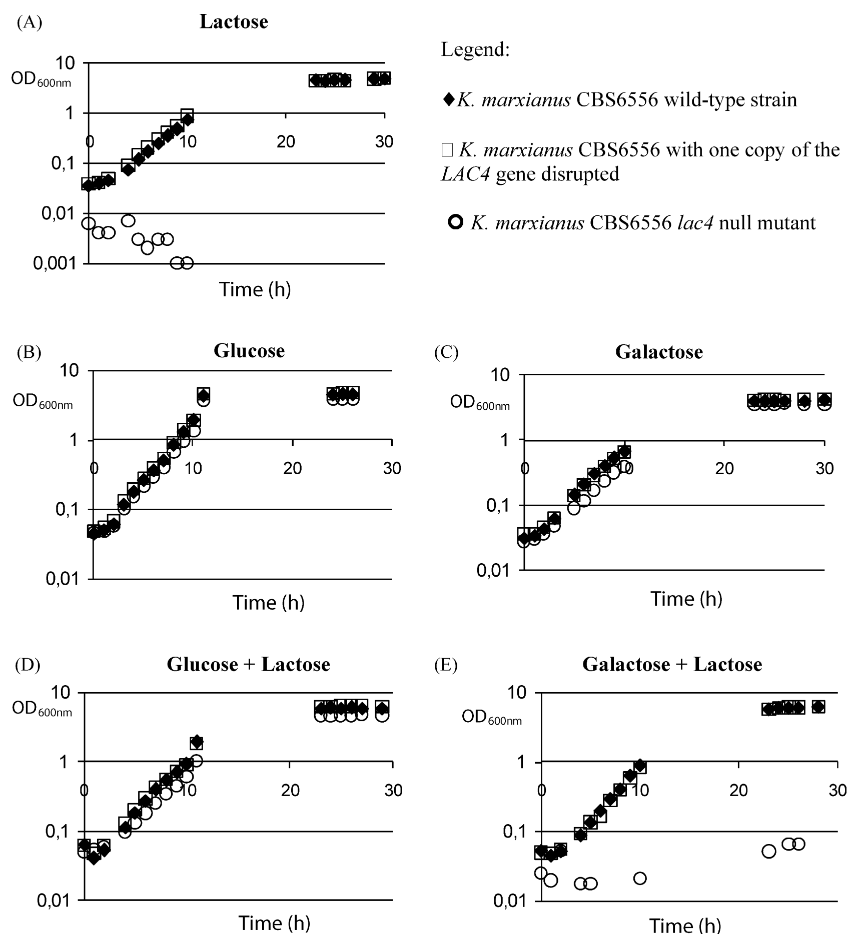

Fig. 4. Growth curves on mineral medium containing different carbon sources. The results shown are the mean of duplicated independent assays.

and marker removal) showed no β-galactosidase activity. The absence of activity in *lac4* mutants confirms that all copies of the β-galactosidase coding gene were disrupted.

3.5. Growth on different carbon sources

The growth rate of *K. marxianus* wild-type strain and its isogenic *lac*4 mutant was evaluated in a yeast-defined medium (Verduyn et al., 1992), containing either glucose, galactose, lactose, glucose plus lactose or galactose plus lactose as carbon source.

On lactose, the wild-type strain and the mutant containing one disrupted *LAC4* gene showed a duplication time of 2 h and, as expected, the *lac*4 null mutants showed no growth on lactose as the sole C-source (Fig. 4A), which confirms once again that the desired gene disruption was successful.

As can be observed in Fig. 4B and C, the growth kinetics of the mutant containing one disrupted *LAC4* gene and the null mutant was not different from the corresponding wild-type strain, on media containing either glucose or galactose as the sole C-source. The final biomass concentration was slightly lower (15%) for the null mutant when compared with the wild type strain.

In the case of medium containing galactose plus lactose, while the mutant with just one-disrupted *LAC4* gene copy presented the same growth kinetics and final biomass concentration as the wild type strain, the *lac4* null mutant was unable to grow (Fig. 4E). The inability of the null mutant to grow on galactose in the presence of lactose might be related with the functionality of the *LAC12* gene, which encodes a lactose permease. Lactose enters the cells and, due to the inability of this mutant to hydrolyze lactose, this compound accumulates in the cells creating a high intracellular osmotic pressure becoming toxic. This effect has previously been shown in *K. lactis lac4* mutants (Lodi and Donnini, 2005).

In the case of medium containing glucose plus lactose, the growth kinetics for the three strains is similar and the final biomass concentration is 20% lower for the null mutant (Fig. 4D). In this case, glucose is probably repressing *LAC12* expression and thus lactose will only enter the cells after glucose exhaustion. In some, but not all strains of *K. lactis*, glucose represses expression of the lactose–galactose regulon (Breunig, 1989).

4. Conclusion

Besides testing the application of the Cre-*lox*P system in *K. marxianus*, one of the purposes of this work was to construct a disruption cassette that offered a high percentage of correct transformants. Even using a disruption cassette with long

flanking homologous sequences (723 bp upstream and 712 bp downstream), the gene targeting efficiency in *LAC4* loci was very low, 34 and 15% for the first and second round of transformation, respectively. This indicates that it is necessary to construct gene disruption cassettes with long-flanking homology regions, when *K. marxianus* is the organism under investigation.

In spite of its wide use, to our knowledge this is the first report in which the Cre-*lox*P system has been applied to *K. marxianus*, opening new perspectives for metabolic engineering of this yeast with several promising applications in biotechnology. Having demonstrated the applicability of the Cre-*lox*P system in this strain, the next step will be the deletion of genes involved in specific metabolic pathways, in order to contribute to the physiological characterization of this yet poorly characterized yeast.

Acknowledgements

We thank Carla Oliveira for helping with the electroporation experiments. A.K. Gombert acknowledges a grant obtained from Coordenação de Aperfeiçoamento de Pessoal de Nível Superio (CAPES), Brazil. O. Ribeiro was financed by Agência de Inovação with the grant UMINHO/POCI-Zimlac/BI/2/05, Portugal. We would also like to acknowledge one of the anonymous reviewers of this manuscript, for her/his careful revision.

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