Expression of an \( \alpha \)-galactosidase gene under control of the homologous inulinase promoter in *Kluyveromyces marxianus*

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Received: 16 February 1993/Received revision: 14 May 1993/Accepted: 19 May 1993

**Abstract.** For expression of the \( \alpha \)-galactosidase gene from *Cyamopsis tetragonoloba* in *Kluyveromyces marxianus* CBS 6556 we have used the promoter of the homologous inulinase-encoding gene (*INU1*). The *INU1* gene has been cloned and sequenced and the coding region shows an identity of 59% with the *Saccharomyces cerevisiae* invertase gene (*SUC2*). In the 5'-flanking region of *INU1* we found a sequence (TAAATCCGGGG) that perfectly matches to the MIG1 binding consensus sequence (WWWWTSYGGGG) of the *S. cerevisiae* GAL1, GAL4 and SUC2 genes. Using the *K. marxianus* INU1 promoter and prepro-signal sequence, we obtained a high \( \alpha \)-galactosidase production level (153 mg/l) and a secretion efficiency of 99%. Both the production level and the secretion efficiency were significantly reduced when the INU1 pro-peptide was deleted. With either the *S. cerevisiae* PGK or GAL7 promoter we could obtain only low \( \alpha \)-galactosidase production levels (2 mg/l).

**Introduction**

Various production problems have been encountered when *Saccharomyces cerevisiae* is used for the industrial production of heterologous proteins (Romanos et al. 1992). Accordingly, alternative host systems more suitable for the production of heterologous proteins have been sought. For this purpose, yeasts of the genus *Kluyveromyces* are especially attractive, since they have been granted GRAS (Generally Recognized As Safe) status. Two species of the genus, *K. lactis* and *K. marxianus*, are well known for their ability to ferment whey and *K. lactis* has already been used for the production of heterologous proteins (van den Berg et al. 1990; Fleer et al. 1991a, b; Rossolini et al. 1992; Bergkamp et al. 1992). However, the growth characteristics of *K. marxianus* are superior to those of *K. lactis*. For instance, *K. marxianus* shows a broader range of substrate utilization, has a much shorter generation time (about 45 min) and grows at much higher temperatures (up to 45\(^\circ\)C) (Rouwenhorst et al. 1988; Steensma et al. 1988). In *K. lactis*, the 1.6-\( \mu \)m plasmid PKD1 (Chen et al. 1986; Falcone et al. 1986) is frequently used as a vector for the expression of (heterologous) genes. Vectors based upon pKD1 are also able to replicate in some *K. marxianus* strains (Chen et al. 1989).

As a model for heterologous gene expression in *K. marxianus* CBS 6556 by means of a pKD1-based vector system, we have chosen the \( \alpha \)-galactosidase gene from the guar plant *Cyamopsis tetragonoloba* (Hughes et al. 1988; Overbeeke et al. 1989). The \( \alpha \)-galactosidase is responsible for the removal of 1-6 linked \( \alpha \)-D-galactopyranosyl units from galactomannan, a storage polysaccharide (Meier and Reid 1982). The guar \( \alpha \)-galactosidase has been expressed in various micro-organisms, including *Bacillus subtilis* (Overbeek et al. 1990), *Hansenula polymorpha* (Fellinger et al. 1991; Sierkstra et al. 1991) and *S. cerevisiae* (Verbakel 1991). We have recently shown that \( \alpha \)-galactosidase can be efficiently produced and secreted in *K. lactis* (Bergkamp et al. 1992), using the *S. cerevisiae* GAL7 promoter and the *S. cerevisiae* SUC2 (invertase) signal sequence.

To obtain a high production and secretion level of \( \alpha \)-galactosidase in *K. marxianus* CBS 6556, we cloned the inulinase-encoding gene (*INU1*) and fused its promoter and secretion signal to the \( \alpha \)-galactosidase gene, since the inulinase enzyme (2,1-\( \beta \)-D-fructan fructanohydrolase, EC 3.2.1.7) is produced at a high level and is secreted well in *K. marxianus* (Rouwenhorst et al. 1988). Furthermore, the synthesis of inulinase can be induced by growing cells on an appropriate carbon source, either sucrose or inulin. We now show that the \( \alpha \)-galactosidase production level in *K. marxianus* is increased about 75 times by using the homologous inulinase promoter instead of the *S. cerevisiae* GAL7 or PGK promoter.
Materials and methods

Strains, media and transformations. Escherichia coli strains JM83 (ara D(lac-proAB), pRPL, 480, lacZAM15) and JM109 (recA1, supE44, endA1, hsdR17, gyrA96, relA1, thi, D(lac-proAB), F' [traD36, proAB+, lacI, lacZAM15]) (Yanisch-Perron et al. 1985) were used as hosts for molecular cloning. E. coli strains were grown in brain heart infusion medium (Oxoid). For transformation of competent E. coli cells the RbCl procedure (Sambrook et al. 1989) was used.

K. marxianus strain CBS 6556 was obtained from the Centraalbureau voor Schimmelcultures, Delft, The Netherlands. K. marxianus strain KMS1 (a leu2- mutant derived from K. marxianus CBS 6556; Bergkamp et al. 1991) was transformed according to the electroporation protocol of Melihoc et al. (1990) with the following modifications: K. marxianus cells were grown at 37°C to a cell density of 3×10⁷/ml. The electroporation apparatus used was the Gene Pulser (Bio-Rad, Richmond, Calif., USA) with 0.4-cm cuvettes. The voltage used was 800 V and the selected capacity was 25 μF.

Yeast transformants were grown in YNB medium (0.67% yeast nitrogen base without amino acids, 2% glucose) or in YPD, YPG or YPS medium (1% yeast extract, 2% bacto-pepitone and 2% glucose, galactose or sucrose, respectively).

Construction of plasmids. Molecular cloning procedures were performed according to Sambrook et al. (1989). The following series of plasmids were constructed:

1. pKMGAL1, pKMGAL2. In the unique EcoRI site of the plasmids pSY9 and pSY32 (Harmsen et al. 1993) we cloned the plasmid pKD1, which was isolated as a 4.7-kb EcoRI fragment from plasmid pE1 (Bianchi et al. 1987), giving plasmids pKMGAL1 and pKMGAL2, respectively (cf. also Fig. 1). Plasmid pSY9 contains an α-galactosidase cassette cloned in the plasmid YIplac128 (Gietz and Sugino 1988). The α-galactosidase cassette contains the S. cerevisiae GAL7 promoter (Nogi and Fukasawa 1983), the S. cerevisiae SAC2 (invertase) signal sequence (Tausig and Carlson 1983), the α-galactosidase gene and the S. cerevisiae PGK terminator. Plasmid pSY32 is identical to pSY9, except for the GAL7 promoter which was replaced by the S. cerevisiae PGK promoter.

2. pPUR2421, pPUR2422. Two sets of degenerate polymerase chain reaction (PCR) primers were derived from amino acid sequences of the N-terminus and an internal fragment of inulinase. With the primers a PCR amplification was performed on total genomic DNA. The reaction generated a specific 290-bp fragment which was used as a [α-³²P]-labelled probe for the construction of a physical map of the inulinase-encoding gene. Results of this chromosomal restriction analysis revealed two positive DNA fragments of about 2.0 kb after EcoRI and 4.0 kb after PstI digestion, respectively. Therefore, chromosomal K. marxianus DNA was digested with EcoRI and PstI and the appropriate fragments were isolated and purified with the GeneClean II kit (Bio 101 Inc). The EcoRI and PstI fragments were cloned into pTZ19 plasmids (Mead et al. 1986) and used to transform E. coli JM109. Positive transformants were identified by colony hybridization using pKMG3 and pKMG4 as probes. The sequence of this oligonucleotide was 5'-CCC AAG CTT ACC TGC CAT GGG CCC TCT 1GT AAT TAA CTG 3'. This latter oligonucleotide contains sequence information for a functional connection between inulinase and α-galactosidase. The primers were used in a PCR with pUR2421 as template. Subsequently, the PCR product was cloned in pTZ19R, giving plasmid pPUR2427. The plasmid pPUR2428 was constructed in a similar way, only in this case an oligonucleotide was used that is partly complementary to the sequence of the signal peptide cleavage site. The sequence of this oligonucleotide was 5'-CCC AAG CTT ACC ATG CGG CCG CAC TGA CTC CTG CCA ATG-3'.

3. pPUR2427, pPUR2428. For the construction of plasmid pPUR2427 two oligonucleotides were used, one corresponding to position -738 to -719 of the K. marxianus INU1 promoter with the sequence 5'-GGGA ATT CTC AAA CCG AAA TG-3', and one partially complementary to the sequence of the KEX2 protease site and partly complementary to the N-terminal sequence of the gene encoding the mature α-galactosidase. The sequence of this oligonucleotide was 5'-CCC AAG CTT ACC TGC CAT GGG CCC TCT 1GT AAT TAA CTG 3'. This latter oligonucleotide contains sequence information for a functional connection between inulinase and α-galactosidase. The primers were used in a PCR with pUR2421 as template. Subsequently, the PCR product was cloned in pTZ19R, giving plasmid pPUR2427. The plasmid pPUR2428 was constructed in a similar way, only in this case an oligonucleotide was used that is partly complementary to the sequence of the signal peptide cleavage site. The sequence of this oligonucleotide was 5'-CCC AAG CTT ACC ATG CGG CCG CAC TGA CTC CTG CCA ATG-3'.

4. pKMGAL3, pKMGAL4. The inulinase promoter and signal sequence were isolated from pPUR2427 and pPUR2428 as 804-bp and 783-bp EcoRI-BspMI fragments, respectively. These fragments were cloned into pSY9, from which the S. cerevisiae GAL7 promoter and Suc2 signal sequence were deleted by digestion with EcoRI and EagI, giving plasmids pKMG3 and pKMG4. In the unique EcoRI site of the plasmids pKMG3 and pKMG4 we cloned the 4.7-kb pKD1 vector, giving plasmids pKMGL3 and pKMGL4, respectively (cf. also Fig. 1).

Plasmid copy-number analysis of transformants. Transformants were grown for 24 h in selective medium. Total DNA was isolated (Pedersen 1983) and digested with a restriction endonuclease. Southern hybridization (Southern 1975) was performed with a radioactively labelled fragment, which can hybridize with a genomic band and the plasmid band. Plasmid copy numbers were determined by comparing the intensities of these two bands by laser scanning densitometry (LKB UltrascanXL, Pharmacia, Sweden).

To analyse the stability during long-term culturing under non-selective conditions, cultures were, after growth for 24 h in selective medium, diluted 1:10 in YPS. When the cultures reached an optical density at 550 nm (OD₅₅₀) of between 2 and 3 they were diluted to an OD₅₅₀ of 0.1. This procedure was repeated in this way each time the culture reached the exponential growth phase. Cell samples were collected after about 5, 35 and 70 generations of growth. Total DNA was isolated and used for copy-number determination.

Analysis of α-galactosidase production. Transformants were grown during 24 h in YNB medium, diluted 1:10 in YPD, YPG or YPS medium and grown for 48 h. Subsequently, the intracellular and extracellular α-galactosidase activity was determined as described before (Bergkamp et al. 1992).

Western blot analysis of culture supernatants was performed according to Harmsen et al. (1993).

Amino-terminal sequencing of secreted α-galactosidase was carried out as described by Fellinger et al. (1991).

Determination of transcription initiation site. K. marxianus cells were grown as described (Roewenhorst et al. 1990) under induction or glucose-repression conditions. From each 100 ml culture, cells were harvested and total RNA was isolated (Köller and Domdey 1991). For each experiment about 10 μg RNA and 100 pg of primer were dissolved in 40 μl hybridisation buffer, containing 50 mM TRIS-HCl (pH 8.3), 75 mM KCl, 3 mM MgCl₂, 10 mM dithiothreitol (DTT) and 40 units (U) RNase inhibitor (Boehringer, Mannheim, Germany). The mixture was incubated at 65°C for 5' and slowly cooled to room temperature. Then 1 μl [α-³²P]-dCTP (Amersham International) or [α-³²P]-dATP, 1 μl (25 U) reverse transcriptase (Boehringer, Mannheim, Germany). The mixture was precipitated with ethanol and subsequently loaded onto a 5% polyacrylamide gel together with the sequence reactions containing the same primers. Two primers were used: p21T is complementary to position +18 to +48 according to the sequence in Fig. 1; p16T is complementary to position −98 to −84.