Volume 255, number 2, 455–459 FEB 07656 September 1989

Isolation and expression in *Saccharomyces cerevisiae* of a gene encoding an α-amylase from *Schwanniomyces castellii*

Dolores Abarca, María Fernández-Lobato, Manuel Gonzalo Claros and Antonio Jiménez

Centro de Biología Molecular (CSIC-UAM), Universidad Autónoma, Canto Blanco, 28049 Madrid, Spain

Received 14 August 1989

A gene (*SWA1*) encoding an α-amylase activity from *Schwanniomyces castellii* has been cloned and expressed, via yeast cloning vector YEp13, in *Saccharomyces cerevisiae*. By using a riboprobe which is internal to the *SWA1* gene, a 1.55 kb transcript was detected in the poly(A)$^+$ RNA from both *Sw. castellii* and a *S. cerevisiae* clone harboring the *SWA1* gene. This transcript should, therefore, correspond to the *SWA1* gene. In addition, the DNA strand determining the α-amylase activity has been defined. Transcription of the *SWA1* gene appears to be highly regulated in *Sw. castellii*, whereas it is constitutive in the *S. cerevisiae* harboring this gene.

Amylase; Cloning; Expression; Yeast; Starch; *(Schwanniomyces castellii)*

1. INTRODUCTION

The yeast genus *Schwanniomyces*, which was first described by Kloecker in 1909 [1], has recently attracted scientific interest because of the high amylolytic and protein secretory capability of several of its species. *Sw. castellii* and *Sw. alluvius* [2] produce α-amylase, glucoamylase and debranching enzymes. These activities have been purified and characterized by several groups (for review, see [3]). They correspond to thermolabile enzymes, with optimum activity at slightly acidic pH and 40–60°C.

Numerous yeasts, beside *Schwanniomyces* spp., produce glucoamylases and/or α-amylases [4]. Of these, the genes encoding the glucoamylase [5] and the α-amylase [6] from *Saccharomycopsis fibuligera* have been isolated and expressed in *Saccharomyces cerevisiae*. In addition, certain amylolytic varieties of *S. cerevisiae* (previously known as *S. diastaticus*) carry any of the *STA1*, *STA2* or *STA3* genes which code for the same extracellular glucoamylase [7]. These three genes have been cloned and are known to be regulated negatively at the transcriptional level by the STA10 gene of *S. cerevisiae* [8–10]. The presence of this repressor gene in certain industrial yeasts would make it more difficult to express STA genes in brewing yeast strains. In contrast, the amylase genes from *Schwanniomyces* spp. are not expected to be repressed in *S. cerevisiae*. Moreover, assuming that their promoters are functional, they may be constitutionally expressed in *S. cerevisiae*. Two genes from *Sw. occidentalis*, *ODC* [11], and *ADE2* [12] have been cloned in *S. cerevisiae* and their promoters have been found to be active in this yeast.

The present work reports the cloning and expression in *S. cerevisiae* of a gene (*SWA1*) encoding an α-amylase from *Sw. castellii*.

Correspondence address: A. Jiménez, Centro de Biología Molecular (CSIC-UAM), Universidad Autónoma, Canto Blanco, 28049 Madrid, Spain

2. MATERIALS AND METHODS

Schwanniomyces castellii ATCC 26077 was obtained from Dr M.A. Delgado, Cruz Campo S.A., Sevilla, Spain. *Saccharomyces cerevisiae* strains MCCX1-5d (a, *leu2-3 2-112*, *his4*, *sta°*, *sta10*), SPX3-6b (α, *leu2-3 2-112*, *his4*, *ade2*, *STA3*, *sta10*) [8] and IM1-8b (a, *leu2-3 2-112*, *his4*, *sta°*, *sta10*, MAL$^+$) were obtained from our stock. *Escherichia coli* DH-5 was used for transformation and plasmid amplification. The *S. cerevisiae–E. coli* shuttle vector YEp13 was described by others [13]. Plasmids pGEM1 and pGEM2, used to prepare riboprobes, were purchased from Promega Biotech (Madison, Wis.).

Published by Elsevier Science Publishers B.V. (Biomedical Division)
00145793/89/$3.50

Yeast cells were grown on YEPD (1% yeast extract, 2% peptone, 2% glucose) or YNB (0.7% yeast nitrogen base w/o amino acids, 2% (w/v) starch, supplemented with 40 μg/ml amino acids and either 0.15% (w/v) glucose or 0.2 (w/v) maltose when required) media.

Halo-forming (SWA^+) phenotype was scored on plates containing YNB medium supplemented with 0.15% glucose (for maltose non-consuming strains) or 0.2% maltose (for maltose consuming strains). Colonies were transferred to this medium and incubated for 7–10 days before starch was precipitated by keeping the plates at 4°C for 1–2 days. A clear halo appeared around the SWA^+ colonies. Liquid medium for amylase production was YNB buffered with 50 mM phosphate buffer, pH 5.5 [14]. L medium [8] was used for culturing bacterial cells and, when appropriate, it was supplemented with 100 μg/ml ampicillin.

Yeast transformation was carried out as described by Burgers et al. [15]. Genomic and plasmid DNA from yeast were isolated as described by Sherman et al. [16]. Bacterial transformation and plasmid isolation were performed as described by Hanahan et al. [17]. $Poly(A)^+$ RNA was prepared by chromatography through oligo (dT)-cellulose of total RNA [18] which was obtained by vortexing yeast cells with glass beads. Southern blots and colony hybridization were performed as described elsewhere [19]. DNA probes were labelled with ^{32}P by nick translation [19]. Northern blots and hybridization with ss-RNA probes were carried out as previously reported [8]. RNA dot blot technique was performed as described by others [20].

A gene library of total DNA from *Sw. castellii* was prepared in plasmid YEp13 as described elsewhere [8]. A total of 22 500 clones were obtained. The DNA inserts from the recombinant plasmids had a mean size of 5.4 kb. Yeast spheroplasts from strain MCCX1-5d (a starch non-fermenting strain) were transformed with this gene bank. LEU^+ and SWA^+ transformants were selected on regeneration agar plus starch plates (0.7% yeast nitrogen base w/o amino acids, 0.4% YEPD, 2% soluble starch, 0.5% glucose, 1 M sorbitol, 3% agar). Plates were incubated 10–15 days at 30°C and then cooled at 4°C for 2–3 days after which starch-fermenting colonies were detected by the clear haloes of solubilized starch.

To assay α-amylase activity, a mixture of 0.5 ml of culture filtrates and 0.5 ml of 50 mM phosphate pH 5.5 buffer, 1% (w/v) soluble starch (American Society of Brewing Chemists, Inc., St. Paul, MN 55121, USA) was incubated at 40°C for 90 min. Reactions were stopped by immersion of the tubes in a boiling bath for 5 min. Samples (25 μl) were then added to 0.1 ml of a water solution containing 0.5% (w/v) KI and 0.15% (w/v) I_2. Hydrolyzed starch was estimated by reading absorbance at 595 nm. One unit of α-amylase activity was defined as a decrease of absorbance of 0.1 under the indicated conditions. This method is a modification of that described in [21].

3. RESULTS AND DISCUSSION

3.1. *Isolation of an amylase gene*

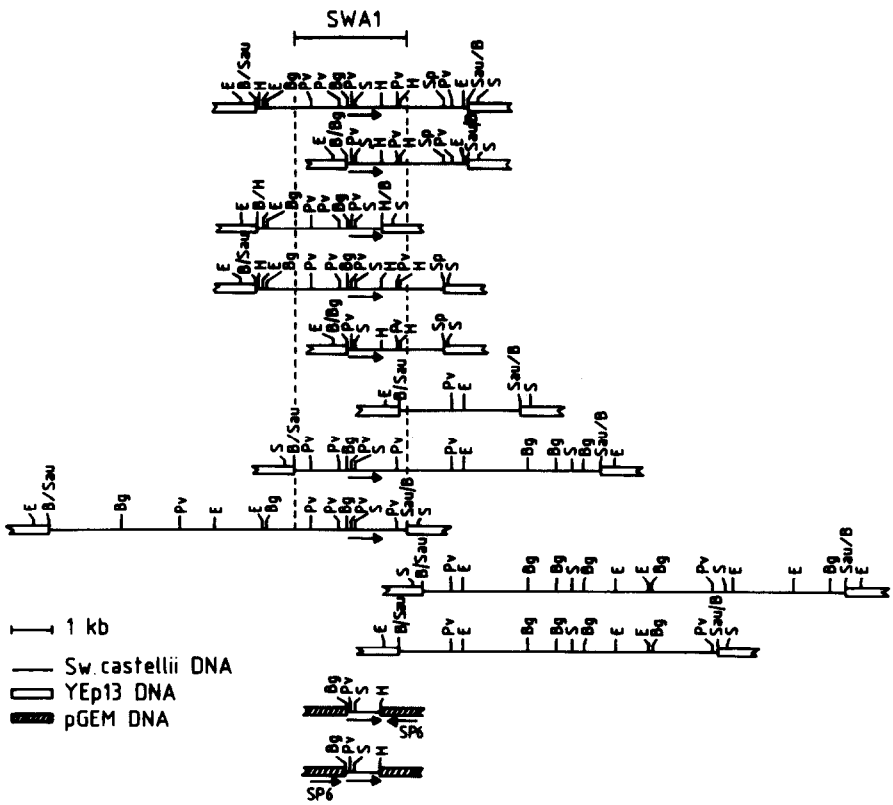
Several halo-forming, LEU^+ *S. cerevisiae* transformants were obtained from the *Sw. castellii* gene library. Plasmid DNA from one of these clones was used to transform *E. coli*. Several transformants were found to carry plasmid pFH4 (fig.1), which consists of plasmid YEp13 with a DNA insert of 5.3 kb. When yeast spheroplasts were transformed with pFH4, all LEU^+ clones were also SWA^+. In the absence of selection, this phenotype had mitotic instability indicating that the SWA^+ character was conferred by a gene (named *SWA1*) which was present in plasmid pFH4. Plasmids pFH4.1 to pFH4.4 (fig.1) were generated by subcloning experiments. Of these, only pFH4.3 gave rise to a SWA^+ phenotype.

The 5 kb *Eco*RI fragment from pFH4 was used as a probe to screen a gene library (see section 2). Plasmids pFH5 to pFH9 (fig.1) were isolated from the relevant hybridizing *E. coli* colonies. Spheroplasts from *S. cerevisiae* MCCX1-5d were transformed with these plasmids and the SWA^+ phenotype was scored in the resulting transformants. All plasmids expressing an SWA^+ phenotype contained the same 2.8 kb DNA fragment (fig.1). Therefore, this sequence must contain the *SWA1* gene.

A comparison of the restriction maps of plasmids pFH4.1, 4.2, 6 and 7 (fig.1) indicates that the 0.85 kb *Bgl*II-*Hin*dIII fragment should be internal to the *SWA1* gene. This fragment was used as a probe in a Southern blot analysis, which showed that the 5 kb *Eco*RI sequence from this plasmid derived from *Sw. castellii* DNA and that no homologous DNA was present in *S. cerevisiae* MCCX1-5d (results not shown).

3.2. *Biochemical studies*

The nature of the enzyme expressed by *SWA1* was examined in culture filtrates of *S. cerevisiae* IM1-8b (pFH4), which showed α-amylase activity (fig.2) and totally lacked any glucoamylase and debranching activities. In addition, analysis by paper chromatography of the products from incubations containing starch and culture filtrates showed the presence of small amounts of maltose and high amounts of maltotriose and higher molecular weight oligosaccharides. No glucose was detected in these reactions (results not shown). Therefore, we concluded that the *SWA1* gene from *Sw. castellii* encodes an α-amylase activity. The production of limited quantities of maltose could explain the low levels of growth, in a starch-based medium lacking any other carbon source, of *S. cerevisiae* IM1-8b (a MAL^+ strain) harboring pFH4. In addition, the values of this α-amylase ac-



PLASMID	INSERT SIZE (kb)	SWA PHENOTYPE
pFH4	5.3	+
pFH4.1	3.0	–
pFH4.2	3.0	–
pFH4.3	4.7	+
pFH4.4	2.4	–
pFH5	3.0	–
pFH6	7.6	+
pFH7	8.9	+
pFH8	10.5	–
pFH9	7.9	–
pSWA1	0.85	ND
pSWA2	0.85	ND

Fig.1. Restriction maps of several plasmids. Only the *Sal*I and *Eco*RI restriction sites from the YEp13 vector are drawn, to indicate the orientation of the DNA inserts. Arrows indicate direction of transcription. SP6, promoter of the RNA polymerase from bacteriophage SP6. B, *Bam*HI; Bg, *Bgl*II; E, *Eco*RI; H, *Hin*dIII; Pv, *Pvu*II; S, *Sal*I; Sau, *Sau*IIIAI; Sp, *Sph*I.

tivity at different pHs or different temperatures paralleled those of purified α-amylase from *Sw. castellii* [21] (results not shown). All these results suggest that the *SWA1* gene encodes the α-amylase which is detected in culture filtrates of *Sw. castellii*.

3.3. *Transcription studies*

The 0.85 kb *Bgl*II-*Hin*dIII DNA fragment from pFH4, which should be internal to the *SWA1* gene, was inserted downstream from the SP6 RNA polymerase promoter of plasmids pGEM1 and pGEM2 to generate plasmids pSWA1 and pSWA2, respectively (fig.1); these differed in the orientation of the insert. These two plasmids were used to prepare riboprobes, which were hybridized to poly(A)$^+$ RNA from *Sw. castellii* and several *S. cerevisiae* strains (fig.3). Hybridization took place only with the riboprobe prepared from plasmid pSWA1, which indicated that the direction of transcription was from the *Bgl*II to the *Hin*dIII site (fig.1). The 1.55 kb transcript which is present in both *Sw. castellii* and *S. cerevisiae* MCCX1-5d (pFH4) should correspond to the SWA1 gene. In addition, a 1.2 kb transcript is present in the *S. cerevisiae* (pFH4) strain. It could result from a second transcription initiation site from *SWA1*, which could be recognized by RNA polymerase II. Multiple transcription initiation sites are often found in yeast genes cloned in *S. cerevisiae* [8,22]. As expected, poly(A)$^+$ RNA from *S. cerevisiae* SPX3-6b (a starch-fermenting strain) and MCCX1-5d (YEp13) did not hybridize with either riboprobe (fig.3).

Both transcription from *SWA1* gene and expression of amylase activity were examined during the growth in liquid culture of *Sw. castellii* and *S. cerevisiae* IM1-8b (pFH4). It appeared that transcription of *SWA1* was regulated in *Sw. castellii*,

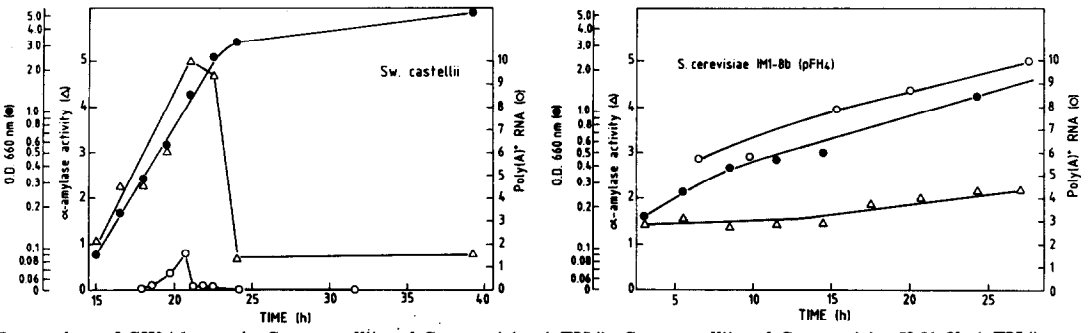


Fig.2. Expression of *SWA1* gene in *Sw. castellii* and *S. cerevisiae* (pFH4). *Sw. castellii* and *S. cerevisiae* IM1-8b (pFH4) were grown in minimal YNB medium supplemented with 0.2% (w/v) maltose. At the indicated times samples were taken to estimate optical density, α-amylase activity and content of poly(A)$^+$ RNA specific for *SWA1* gene. The latter was determined by the dot blot technique using as probe an [α-^{32}P]dCTP-labelled 0.85 kb *Bgl*II-*Hin*dIII fragment from pFH4 (fig.1). Each well received 2 μg of poly(A)$^+$ RNA. The amount of poly(A)$^+$ RNA is given in arbitrary units. The α-amylase activity is given in units as described in section 2.

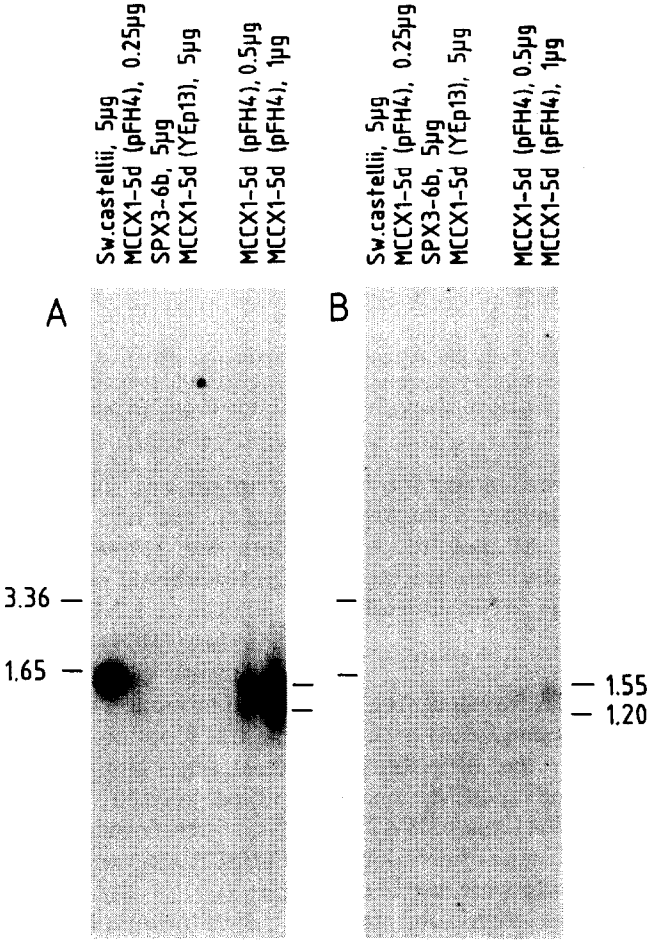


Fig.3. Transcription studies. The indicated amounts of poly(A)$^+$ RNA from *Sw. castellii* and the relevant *S. cerevisiae* strains were developed by denaturing gel electrophoresis and then transferred to a nylon sheat which was then divided into two halves. Each membrane was hybridized to a ^{32}P-labelled riboprobe derived from either plasmid pSWA1 (A) or pSWA2 (B). Numbers indicate the size (in kb) of the RNA standards (left) and transcripts (right).

whereas both processes were constitutive in the yeast transformant (fig.2). In addition, the α-amylase activity from *Sw. castellii* decreased dramatically, but did not totally disappear at the late stages of cell growth. In contrast, the α-amylase from the cultures of the *S. cerevisiae* clone was totally stable. The reason for this difference in stability is unknown, but it could be due to the presence of proteolytic activity(ies) secreted by *Sw. castellii*. Interestingly, transcription from the *SWA1* gene was more active in *S. cerevisiae* (pFH4) than in *Sw. castellii*, whereas the opposite was found with the levels of amylase activity (fig.2). These findings could be due to either different efficiencies of translation of the *SWA1* mRNA in the two strains or to a reduced secretion capacity of the α-amylase in the *S. cerevisiae* clone relative to that in *Sw. castellii*.

Acknowledgements: We are grateful to Dr M.A. Delgado for his interest and comments during the course of this work and to Mrs Asunción Martín for expert technical assistance. This research was supported by grants from Plan Concertado CAYCIT-Cruz Campo (64/84) and the European Community (BAP 0394.E(JR)) and by institutional grants from Fundación Ramón Areces and FIS to the Centro de Biología Molecular.

REFERENCES

[1] Kloecker, A. (1909) Compt. Rend. Trav. Lab. Carlsberg 7, 273–278.
[2] Sills, A.M. and Stewart, G.G. (1982) J. Inst. Brew. 88, 313–316.
[3] Ingledew, W.M. (1987) CRC Crit. Rev. Biotechnol. 5, 159–176.
[4] McCann, A.K. and Barnett, J.A. (1986) Yeast 2, 109–115.
[5] Itoh, T., Ohtsuki, I., Yamashita, I. and Fukui, S. (1987) J. Bacteriol. 169, 4171–4176.
[6] Itoh, T., Yamashita, I. and Fukui, S. (1987) FEBS Lett. 219, 339–342.
[7] Tamaki, H. (1978) Mol. Gen. Genet. 164, 205–209.
[8] Pardo, J.M., Polaina, J. and Jiménez, A. (1986) Nucleic Acids Res. 14, 4701–4718.
[9] Yamashita, I., Naemura, E., Hatano, T. and Fukui, S. (1985) J. Bacteriol. 161, 572–582.
[10] Pretorius, I.S., Chow, T. and Marmur, J. (1986) Mol. Gen. Genet. 203, 36–41.
[11] Klein, R.D. and Roof, L.L. (1988) Curr. Genet. 13, 29–35.
[12] Klein, R.D. and Favreau, M.A. (1988) J. Bacteriol. 170, 5572–5578.
[13] Broach, J.R., Strathern, J.N. and Hicks, J.B. (1979) Gene 8, 121–133.
[14] Oteng-Gyang, G., Moulin, G. and Galzy, P. (1980) Eur. J. Appl. Microbiol. Biotechnol. 9, 129–132.
[15] Burgers, P.M.J. and Percival, K.J. (1987) Anal. Biochem. 163, 391–397.
[16] Sherman, F., Fink, G.R. and Hicks, J.B. (1986) Methods in Yeast Genetics, Laboratory Manual, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
[17] Hanahan, D. (1983) J. Mol. Biol. 166, 557–580.
[18] Aviv, H. and Leder, P. (1972) Proc. Natl. Acad. Sci. USA 69, 1408–1412.
[19] Maniatis, T., Fritsch, E.F. and Sambrook, K.J. (1982) Molecular Cloning, A Laboratory Manual, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
[20] Constanze, C. and Gillespie, D. (1987) Methods Enzymol. 152, 582–587.
[21] Simoes-Mendes, B. (1984) Can. J. Microbiol. 30, 1163–1170.
[22] Pardo, J.M., Iáñez, E., Zalacaín, M., Claros, M.G. and Jiménez, A. (1988) FEBS Lett. 239, 179–184.