TRANSFORMATION OF THE ASTAXANTHIN-PRODUCING YEAST *Phaffia rhodozyma*

J.L. Adrio and M. Veiga*

Departamento de Microbiología, Facultad de Farmacia, Universidad de Santiago, 15706 Santiago de Compostela, Spain.

SUMMARY

This paper describes the genetic transformation of the astaxanthin-producing yeast *Phaffia rhodozyma* with the cloning vector pGH-1. The plasmid replicates autonomously in this yeast, and the selection of transformants was possible by using both, the *URA3* marker from *Saccharomyces cerevisiae*, and the kanamycin resistance (*KmR*) determinant from the bacterial transposon Tn903.

INTRODUCTION

There is an increasing interest in the utilization of *P. rhodozyma* as a microbial source of astaxanthin for the aquaculture industry (Johnson and An, 1991). However, the use of this yeast in the feed industry is limited by the low content of astaxanthin in wild strains. Owing to its cell wall composition and other structural characteristics, as well as its restrictive growth conditions, *P. rhodozyma* is considered to be a fastidious yeast difficult to be genetically manipulated. Some successful attempts have been carried out to improve the astaxanthin production (An et al., 1989; Chun et al., 1992). Recently, the *P. rhodozyma* karyotype has also been presented (Adrio et al., 1995), but transformation of this yeast has not been achieved hitherto. Plasmidic transformation of *P. rhodozyma*, reported for the first time in this paper, will allow in a near future the cloning of genes involved in the astaxanthin biosynthetic pathway which could lead to improve the pigment production.

MATERIALS AND METHODS

**Microorganisms and culture conditions:** *P. rhodozyma* mutants defective in oroticin monophosphate decarboxylase were previously obtained (Adrio et al., 1993). Two of these *ura3* mutants, FOA-4 (ATCC 96220) and FOA-7 (ATCC 96221), which have a reversion frequency < 1 x 10⁻⁸, and the wild type strain ATCC 24230 were used in the transformation assays.

**Plasmid:** The construction of pGH-1 has been previously described (Ernst and Chan, 1985); besides DNA from pBR322, which enables *Escherichia coli* propagation, this vector contains *URA3* and *KmR* markers, an ARS chromosomal origin of replication, and the origin of replication and the STB sequence from 2-μm circle.
Transformation: Yeast transformations were made by using the spheroplast method (Hinnen et al., 1978). Yeast cells were grown at 24°C in 100 ml YPD medium (2% glucose, 2% bactopeptone, 1% yeast extract) to an A600 = 0.6-0.8. Cells were centrifugated and washed once with water and suspended in 3 ml of sorbitol-S (1.2M sorbitol, 10mM Tris-HCl, pH 7.0). Spheroplasting was made by adding Lysing Enzymes from Streptomyces S-35 (strain isolated in our laboratory with strong lytic activity towards P. rhodozyma cell wall) and incubating at 24°C. The treatment was stopped when spheroplasting was ≥90%. A similar treatment using Lysing Enzymes from Trichoderma harzianum (Sigma Chemical Co., Mo. USA) can also be used to obtain the spheroplasts. To facilitate expression of the selective genes, a post-transformation incubation was done in YPD-S (YPD in 1.2M sorbitol) or SOS medium (1 M sorbitol, 6.5 mM CaCl2, 1/3 YPD strength and 5 μg/ml of leucine, uracil and triptophan) before plating on selective media. YPD-S containing 100 μg of antibiotic G418 per ml was used for KmR transformants selection and MM-S medium (2% glucose, 0.67% yeast nitrogen base without aminoacids, 1.2M sorbitol) was used for URA3 transformants selection. Transformants appeared after 5-7 days of incubation at 24°C.

RESULTS AND DISCUSSION

Table 1 shows the results of the transformation experiments. When the transformants selection is done by G418 resistance, it seems that a lag phase is required prior to administration of the antibiotic to achieve maximal transformation frequency, probably because the antibiotic inhibits protein synthesis before the KmR is expressed and confers resistance (Das et al., 1984). Thus, no transformants to antibiotic G418 resistance were obtained when cells were plated directly (0 h) or after being incubated for less than 18 h. The maximal transformation frequency was obtained after an incubation of 24 to 48 h; since the mitotic stability is fairly high (see below), the decline in the frequency of transformation observed after 48 h probably results from the killing of the spheroplasts in the expression medium rather than to the vector loss due to the lack of selective pressure.

Similar low frequencies of transformation were obtained using Kanamycin resistance as the genetic marker in transformation assays carried out with another yeasts (Das et al., 1984; Webster and Dickson, 1983). These authors have suggested that the low frequency of transformation using the KmR as the selective marker could be due to the own antibiotic G418 selection. In any case, with P. rhodozyma, the transformation frequency using the URA3 marker was also low and not significantly different from that obtained with the KmR marker, indicating the existence of other causes for the low transformation efficiency. The auxotrophic strains showed a transformation efficiency lower than the wild-type strain, reflecting perhaps their lower growing rate.

Although the frequency of transformation was low, pGH-1 replicates autonomously in P. rhodozyma. The autonomous presence of the plasmid in the transformed cells was confirmed by plasmid rescuing: quick plasmid DNA preparations from P. rhodozyma transformants were obtained by Strathem and Higgins's method (1991) and used to transform E. coli DH5α to AmpR. Only 2 out of 26 P. rhodozyma transformants tested failed to produce E. coli AmpR retrotransformants. In these cases, the plasmid rescuing failure might well have been due either, to integration of the plasmid in the yeast genome, or to a plasmid DNA rearrangement affecting the bacterial replication origin and/or the AmpR gene. Plasmid DNA analysis from the E. coli retrotransformed clones showed that these DNA