Cloning of Autonomously Replicating Sequence from Phaffia rhodozyma

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A Phaffia rhodozyma chromosomal fragment (approximately 3.8 kb) capable of functioning as an origin for the replication of a kanamycin resistance (Km') plasmid in S. cerevisiae was isolated by the use of origin search plasmid, pHN134. In S. cerevisiae, transformation frequencies using the plasmid pHN134 containing an autonomously replicating sequence of P. rhodozyma was 450-580 CFU/μg DNA. The stability of the recombinant plasmid were 16-19%.

Phaffia rhodozyma produces astaxanthin (3,3'-dihydroxy-β,β-carotene 4,4'-dione) as its principal carotenoids (1). Astaxanthin was already known to be responsible for carol formation in salmon and trout as they accumulate it from the producer of the marine environments. Potentially, the excellent anti-oxidant properties of astaxanthin (11) could also be medically and commercially attractive.

One of the limiting factors for wide-scale application of P. rhodozyma as a fish food ingredients turns out to be the low astaxanthin content of this yeast. Though random mutagenesis and cell fusion have been used to achieve some increase in pigmentation (3, 6), the maximum amount of astaxanthin productivity still appears to be limited.

Recombinant DNA technology could probably overcome this bottle-neck in strain improvement. However, this is considerably impeded by the lack of a transformation system in this yeast. To develop this system, the construction of an autonomously replicating vector is essential. Autonomousely replicating sequence (ARS) have been identified from yeasts (2, 5, 9) and fungi (13). Since the transformation system for P. rhodozyma is not established, we could not test the replication of S. cerevisiae ARS or 2 μm origin in the P. rhodozyma. However, most of ARS fragment originating from other organisms (2, 5, 9) may function in S. cerevisiae as well as in themselves. In this paper, we describe the cloning of P. rhodozyma ARS in S. cerevisiae.

E. coli HB101[F, hsdR52[rB-, mB-], recA8, ara-14, proA2, Ispl20[Sm'], xyl-5, mtl-1, lacY1, galK2, supE 44, λ] was used for all bacterial transformation and gene library construction. S. cerevisiae SHY3 (a, ste-VCP, ura3-52, trpl-289, leu2-3, leu2-112. his delta 1, ade1-101, can 1-100) was used as host for the cloning of P. rhodozyma ARS. LB medium with ampicillin was used for cultivation and selection of E. coli harboring plasmid of Ap'. The corresponding medium for yeast was YEPD (1% yeast extract, 2% Bactopeptone, 2% dextrose) with or without the aminoglycoside antibiotic G418. Plasmid DNA isolation and transformation were performed with the method described by Sambrook et al. (10), and P. rhodozyma chromosomal DNA was extracted from the 36 hours grown cells by the method of Nagy et al. (7). Southern blot analysis was carried out with the method described by Park et al. (8).

P. rhodozyma genomic DNA was completely digested with EcoRI, ligated into the corresponding site of plasmid pHN134 (4), an autonomously replicating sequence cloning vector containing kanamycin (G 418) (Sigma Co., USA) resistant gene of Tn903 as a selection marker, and introduced into E. coli HB101. A P. rhodozyma genomic library prepared from a total of 2.5×10^8 independent bacterial colonies was introduced into the shperoplast of the S. cerevisiae.

Two S. cerevisiae transformants were selected as their growth on YEPD containing 200 μg/ml of G418 at the concentration of which S. cerevisiae do not grow. Recombinant plasmids were recovered from these transformants and reintroduced into S. cerevisiae. All S. cerevisiae transformants grow well on YEPD with antibiotic drug. Two recombinant plasmids had approximately 3.7 and 3.8 kb inserts, and they were designated as PAR1 and PAR2, respectively. Two inserts had one KpnI site, one SalI

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colonies on the drug plus plate, was only 16 to 19%. The *S. cerevisiae* transformant with PARS plasmids was rather unstable when compared with the value reported for *S. cerevisiae* transformants with *Candida boidinii* CARS plasmid (9) or ARS-based plasmid (12). Southern blot analysis of *P. rhodozyma* genomic DNA and plasmid recovered from *S. cerevisiae* transformants with PARS1 DNA as probe showed specific bands (Fig. 2), proving that the PARS1 fragment was cloned from the *P. rhodozyma* (lane a of Fig. 2) and maintained in *S. cerevisiae* (circular and nicked form in lane e of Fig. 2). However, further studies are needed to determine the essential region for replication activity of PARS and also to transform *P. rhodozyma* with PARS plasmid. Such studies are under investigation to obtain a better understanding.

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