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NOTE

Cloning of Autonomously Replicating Sequence from *Phaffia rhodzyma*

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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^r) 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). Astaxantin was already known to be responsible for colar 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. Autonomously 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^-, *hsd*S20[rB-, mB-]. *rec*aB. *ara*-14, *pro*A2, *lsp*L20[Sm^r], *xyl*-5, *mtl*-1, *lac*Y1, *gal*K2, *sup*E 44, λ) was used for all bacterial transformation and gene library construction. *S. cerevisiae* SHY3 (a, *ste*-VCP, *ura*3-52, *trp*1-289, *leu*2-3, *leu*2-112. *his* delta 1, *ade*1-101, *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^r. 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 *EcoR*1, 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 2.5×10^4 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 PARS1 and PARS2, respectively. Two inserts had one *Kpn*I site, one *Sal*I

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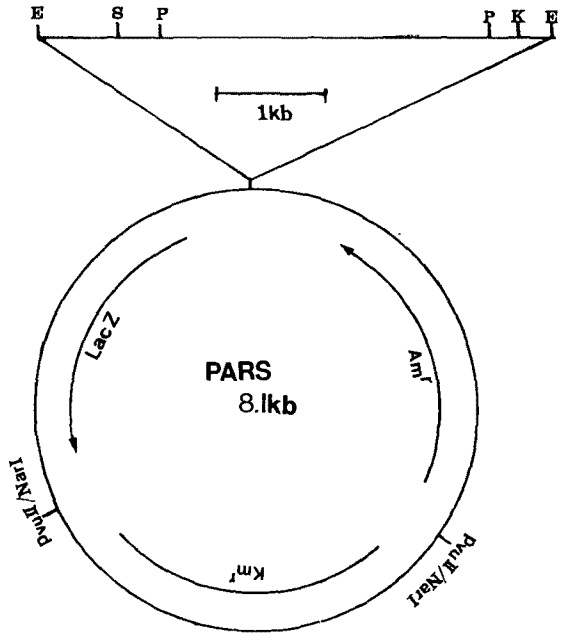

Fig. 1. Restriction map of PARS. The restriction sites for *EcoR*I (E), *Kpn*I (K), *Pst*I (P) and *Sal*I (S) are shown.

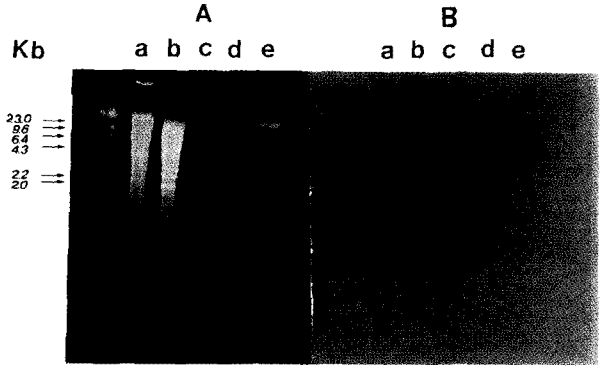

Fig. 2. Southern hybridization of *EcoR*I digests of genomic DNA from *P. rhodozyma* (a) and *S. cerevisiae* (b), PARS2 (c), pHN134 (d), and PARS2 (e) isolated from *S. cerevisiae* transformant. *EcoR*I digests and plasmids were separated on 0.8% agarose gel (panel A) and probed with ^{32}p-labelled 3.8 kb inserts (panel B).

site and two *Pst*I sites. They also exhibited the same restriction map (Fig. 1) and a similar range of transforamtion frequencies (450-580 CFU/μg of DNA), suggesting that the two inserts are identical and also functional in *S. cerevisiae*. The transformation frequencies of PARS plasmids in *S. cerevisiae* were much lower as compared with the corresponding value (4.0×10^4-1.3×10^5) obtained from *Candida boidinii* CARS plasmids (9). The stability for 48 hour-grown cells, expressed as the ratio of the number of colonies on the drug minus plate to the number of 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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