Cloning and Nucleotide Sequence of a Linear DNA Plasmid from *Xanthophyllomyces dendrorhous* (Phaffia rhodozyma)

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ABSTRACT. Extrachromosomal elements were found in a strain of *X. dendrorhous*, and were characterized as linear DNA forming two well defined groups, pPh1 with 3 high-copy-number molecules, pPh11 (6.9 kb), pPh12 (5.7), pPh13 (4.7), and pPh2 with 2 low-copy-number molecules, pPH21 (3.6 kb), pPH22 (3.0). A 4077 bp fragment from pPh13 was cloned in pUC18 (pDK1) and sequenced (accession no. AJ 278424). Seven putative ORF and some possible regulator sequences were defined.

*Xanthophyllomyces dendrorhous* (Golubev 1995; Slaninová et al. 2000), previously called *Phaffia rhodozyma*, is a moderately psychrophilic yeast, with a maximum growth temperature of ≈27°C (Miller et al. 1976). Wild-type strains produce astaxanthin (3,3'-dihydroxy-β,β-carotene-4,4'-dione), a potent antioxidant, whose activity against peroxide radical-mediated phospholipid peroxidation may be stronger than that of β-carotene (Lim et al. 1992). Presently the main demand for astaxanthin comes from aquaculture, where it is used to supplement the food source in order to achieve a normal pigmentation of the fish flesh in salmon and other economically important species (Johnson et al. 1977).

*X. dendrorhous* belongs to the basidiomycetes. Because of its economic potential, efforts to improve the astaxanthin yield have so far been focused on culture conditions and the generation of hyperproducing mutants by classical mutation techniques (Ducrey Santopietro et al. 1998a,b; Rubinstein 1998). As for basidiomycetes in general there is no extensive literature on the genetics of *X. dendrorhous*. Uracil-requiring mutants were isolated (Adrio et al. 1993) and a high copy number plasmid was constructed (Wery et al. 1997), based on kanamycin resistance, which also carries sequences of ribosomal DNA and integrates into the genome. A successful transformation of *X. dendrorhous* was achieved using the spheroplast method but with a low efficiency (Adrio and Veiga 1995); besides, an electroporation protocol with high efficiency has been also reported (Rubinstein et al. 1997).

Four double-stranded (dsRNA) molecules were isolated from *X. dendrorhous* UCD 67-385 (Castillo and Cifuentes 1993) and found to be related to a killer phenotype of this strain against *X. dendrorhous* strains lacking these molecules. Other killer toxins in yeasts were demonstrated to be coded by linear dsDNA plasmids, located in the cytoplasm, e.g. in *Kluyveromyces lactis* (Gunge and Sakaguchi 1981) and *Pichia acaciae* (McCracken et al. 1994).

Two major types of terminal structures in linear DNA species are known, one represented by some linear mtDNAs and eukaryotic chromosomes, which is a hairpin structure. The other class has a terminal protein at the 5'-end, like adenovirus DNA, the pSLA1, and 2 plasmids from *Streptomycyes rochei* and the pGKL plasmids from *K. lactis*. Another linear mtDNA of the yeast, *Hansenula mrakii*, has a quite different 5'-end, which can be directly end-labelled without any previous treatment (Węsolo-wski and Fukuhara 1981). In an extensive screening among 1800 yeast strains, Fukuhara (1995) found that 28 yeast strains contained linear DNA plasmids, indicating that <2% of the known yeast species contain linear DNA plasmids.

We found extrachromosomal DNA molecules in *X. dendrorhous*, exhibiting some characteristic features of linear plasmids. We cloned one linear plasmid and its sequence determined and analyzed.

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MATERIALS AND METHODS

Strains. X. dendrorhous NRRL Y-17269 was obtained from the NRRL collection in Peoria (IL, USA) by courtesy of Dr. C.P. Kurtzman. Saccharomyces cerevisiae was from our culture collection.

E. coli XL1 Blue was purchased from Stratagene (Heidelberg, Germany).

Cultures conditions. X. dendrorhous was grown at 22 °C on a YM medium containing (in %, W/V): glucose 1, peptone 0.5, yeast extract 0.3, malt extract 0.3; pH 4.5 - 5.5. S. cerevisiae was grown at 30 °C on YPD medium containing (in %): glucose 2, peptone 2, yeast extract 1; pH 4.5 - 5.5. E. coli was grown at 37 °C on LB medium (in %: tryptone 1, yeast extract 0.5, NaCl 0.5; pH 7.5) containing ampicillin (100 mg/L) when required. LB agar was made by addition of Difco Bacto-agar (1.5 %).

Enzymes. The cell wall lytic enzyme Novozym 234 was purchased from Sigma-Aldrich (Deisenhofen, Germany). Restriction endonucleases and other DNA-modifying enzymes were purchased from Amersham Pharmacia Biotech (Freiburg, Germany) and were used according to instructions provided by the manufacturers. Proteinase K was obtained from Roche Diagnostics (Mannheim, Germany). RNAase A digestion was done in 10 mmol/L TE (10 mmol/L Tris-HCl, pH 7.4; 1 mmol/L EDTA, pH 8.0) as a low-salt and in 2 × SSC (0.3 mol/L NaCl, 30 mmol/L trisodium citrate, pH 7.0) as a high-salt concentration buffer.

DNA isolation. Total nucleic acid was isolated from 30 mL culture of X. dendrorhous grown at 22 °C and of S. cerevisiae at 30 °C to near stationary phase. Spheroplast formation was carried out by methods of Ducrey (1995). The spheroplasts were pelleted by centrifugation and, after washing with 1 mol/L KCl, resuspended in 5 mL of 50 mmol/L TE (50 mmol/L Tris-HCl, 20 mmol/L EDTA, pH 8.0) with 1 % SDS. The solution was incubated at 65 °C for ½ h. Then 1.5 mL of 3 mol/L potassium acetate was added and incubation continued on ice for 1 h. The sample was centrifuged (12,000 g, 10 min, 4 °C). The supernatant was recovered and DNA precipitated with 1 volume of 2-propanol pre-cooled to −20 °C and centrifuged (6000 g, 15 min, 4 °C). The pellet was washed with 4 mL 50 % (V/V) ethanol and recentrifuged (12,000 g, 10 min, 4 °C), dried and finally suspended in 3 mL 10 mmol/L TE (pH 7.4). RNAase (50 μg/mL) was added and incubated for ½ h at 37 °C. Then proteinase K was added to a final concentration of 100 μg/mL and incubation continued for ¼ h at 37 °C. The sample was extracted once with an equal volume of phenol–chloroform–3-methylbutanol (25:24:1) and once with chloroform–3-methylbutanol (24:1). The DNA was reprecipitated, washed and finally after drying, resuspended into 1 mL 10 mmol/L TE (pH 7.4) and stored at 4 °C.

Agarose gel electrophoresis was carried out in horizontal slab gels in TAE buffer (40 mmol/L Tris-acetate, 1 mmol/L EDTA) at 10 V/cm for normal gels and in alkaline buffer (0.3 mol/L NaOH, 20 mmol/L EDTA) at 2 V/cm for denaturing gels. DNA was recovered from gels using a commercial kit (Macherey-Nagel, Duren, Germany). λ-HindIII, 1 kb ladder (MBI Fermentas, St. Leon-Rot, Germany) and molar mass IV markers (Roche Diagnostics) were used for calibration.

Construction of a pDK1 and related plasmids. After separation of undigested total DNA on a 0.7 % agarose gel, the band corresponding to pPH13 was eluted from the gel, incubated (0.5 μg) with the Klenow fragment of DNA polymerase I and phosphorylated with polynucleotide kinase according to the manufacturer’s instructions (SureClon kit, Amersham Pharmacia Biotech, Freiburg, Germany). Ligation was performed into the dephosphorylated Smal site of the pUC18 vector and competent cells (Maniatis et al. 1982) of E. coli XL1 Blue were transformed. The plasmids from positive clones (blue colonies on X-gal/IPTG LB-amp plates) were isolated using the alkaline lysis procedure (Maniatis et al. 1982) and electrophoretically analyzed after restriction with PstI.

Related plasmids from restricted and modified fragments of pDK1 were constructed and recovered in the same way.

DNA sequence analysis. Sequencing was done on subclones from the pDK1 by the dyeoxy chain termination method with a Taq Dye Primer Cycle Sequencing Core Kit (Applied Biosystems Inc., Foster City, CA, USA) and an A.L.F. DNA sequencer (Pharmacia LKB, Sweden). The nucleotide sequence was assembled with the DNASIS sequence analysis program (Hitachi Software Engineering Co., Yokohama, Japan). Database searching was performed with the Advanced BLAST2 Search Server http://www.bork.embl-heidelberg.de/Blast2/ (Heidelberg, Germany) (Yan et al. 1998).