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Variability and inheritance of double-stranded RNA viruses in *Phaffia rhodozyma*

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Abstract The present survey demonstrates polymorphism in both the length and the number of double-stranded RNAs (dsRNAs) among six *Phaffia rhodozyma* strains. Strains with one-, three- and four-types of dsRNA molecules were found, while two strains proved to be dsRNA-free. Elongated icosahedral virus-like particles (VLPs) 34 × 26 nm in size were detected in strains carrying four- or three-types of dsRNAs. One 3.7-kb dsRNA molecule was found not to form part of the VLP genome. Transmission of the VLPs of strain ATCC 24203 was followed through the basidiospores during the sexual cycle. Cytoplasmic inheritance was observed.

Key words *Phaffia rhodozyma* · Double-stranded RNA · Virus-like particle · Virus transmission

Introduction

Cytoplasmic virus-like particles (VLPs) with double-stranded RNA (dsRNA) genomes have been isolated from some yeast species (Lemke 1979; Buck 1986). Morphologically, they can either be of the bacteriophage type (Kozlova 1973) or isometric (Wickner 1991), although non-encapsidated (Esteban et al. 1993) and retrovirus-like (Boeke and Sandmeyer 1991) elements have also been observed. These VLPs are usually cryptic, i.e. their presence or absence does not cause any phenotypic effect. Nevertheless, in *Saccharomyces cerevisiae* (Wickner 1991) and *Ustilago maydis* (Koltin and Steinlauf 1980; Koltin et al. 1980) they possess a well-defined function, conferring killer activity to the host cell. A similar VLP-associated killer phenomenon has been described in *Phaffia rhodozyma* (Castillo and Cifuentes 1994).

P. rhodozyma is a red-pigmented, fermentative yeast species isolated from exudates of deciduous trees in cold climatic areas (Miller et al. 1976). The characteristics of its sexual life cycle indicate that this species belongs in the section *Basidiomycetes* as currently presented (Golubev 1995). The existence of VLPs in *P. rhodozyma* strain UCD 67–385 was recently reported (Castillo and Cifuentes 1994). The present paper surveys the dsRNA and VLP contents of different *P. rhodozyma* strains and provides reliable evidence on the efficient transmission of the VLPs via basidiospores during the sexual life cycle.

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Materials and methods

Strains. P. rhodozyma strains CBS 5905, CBS 5908, CBS 6938, ATCC 24203, ATCC 24229 and ATCC 24261, strains CBS 6938 Leu$^-$ and ATCC 24203 Lys$^-$, yellow, and *S. cerevisiae* strains T 158C (killer) and S6 (sensitive) were used in our experiments.

Culture conditions. Strains were grown in 2 × YPD medium (2% yeast extract, 2% peptone and 2% glucose) at 20 °C, with shaking at 200 rpm.

Purification of dsRNAs (mini-lysate method). Five-day-old cells were collected by centrifugation, re-suspended in 1.5 ml of enzyme solution (10^7 cells ml^{-1} in 0.5% snail gut enzyme, 0.25% NovoZym and 1.2 M KCl) and incubated at room temperature for 3 h. The spheroplasts were pelleted by centrifugation and, after washing with 1.2 M KCl, were re-suspended in 54 µl of 50 mM Tris/HCl pH 8, 5 mM EDTA, 0.3% SDS and 50 µg ml^{-1} proteinase K. The solution was further incubated at 65 °C for 1 h. The cells and cell debris were removed by low-speed centrifugation. Ten microliters of the supernatant were mixed with 3 µl of bromophenol blue and loaded onto agarose gels. Electrophoresis was carried out as described by Maniatis et al. (1982). The molecular weights of the dsRNA molecules were calculated from that of the marker λ *Eco*T14 I, with

correction for the difference between the mobilities of dsRNA and dsDNA (Livshits et al. 1990).

Enzymatic treatment. The samples were digested with ribonuclease A (2 $\mu g\,\mu l^{-1}$) in TE buffer (low salt) or in 2 × SSC (high salt concentration) at 37 °C for 30 min and with S1 nuclease (10 $U\,\mu l^{-1}$) at 37 °C for 30 min in the recommended buffer (Maniatis et al. 1982).

Isolation and purification of VLPs. VLPs were purified by the method of Oliver et al. (1977).

Electron microscopy. VLPs purified by gradient centrifugation were stained with 2% uranyl acetate and examined with a Zeiss OPTON EM902 electron microscope.

Separation of VLPs by gel electrophoresis. Electrophoresis was carried out according to a previously described method (Goodwin and Dahlberg 1982) in composite gels containing 0.375% agarose and 2.5% acrylamide in 1 × TBE buffer at 200 V, at 4 °C for 6 h. The gels were immersed in the fixing solution containing 45% methanol and 10% acetic acid for 1 h, then stained in a solution of 0.05% Coomassie Brillant Blue R-250 in 45% methanol and 10% acetic acid overnight. The gels were washed several times in a mixture of 5% methanol and 7.5% acetic acid.

Transmission of VLPs. For analysis of the transmission of VLPs, different auxotrophic and colour mutants were pre-cultivated in 2 × YPD medium, mixed in a 1:1 ratio and subjected to zygote formation and sporulation on YNB ribitol agar medium (Golubev 1995). Basidiospores of the tetrads were isolated with a Jena Zeiss micromanipulator and their phenotypes were determined by replica plating (Kucsera et al. 1995). Where the nuclear markers segregated in a 2:2 ratio, the dsRNA content of the subline of the spores was analyzed.

Testing of killer activity. The killer activities of *P. rhodozyma* strains were tested against each other and against *S. cerevisiae* strain S6. The procedures were carried out according to a previously described method (Phillipskirk and Young 1975).

Results

dsRNA polymorphism

Agarose-gel electrophoresis of the mini-lysates of the six *P. rhodozyma* strains revealed the existence of several RNA bands (Fig. 1) in addition to the nuclear DNA and DNA plasmids (Wilber and Proffitt 1987). These bands proved to be double-stranded, as evidenced by their sensitivity to RNase in low salt buffer and their resistance to RNase at high salt concentration, and to S1 nuclease, which specifically degrades single-stranded nucleic acids. In two of the six strains (CBS 5908 and ATCC 24261) we observed four different dsRNA molecules, with estimated sizes of 0.77, 0.97, 3.7 and 4.3 kb, while strain ATCC 24203 contained three different dsRNA molecules with sizes of 0.97, 3.7 and 4.3 kb. Only one type of dsRNA (3.7 kb) was found in strain ATCC 24229, and no dsRNA could be detected in strains CBS 5905 and CBS 6938.

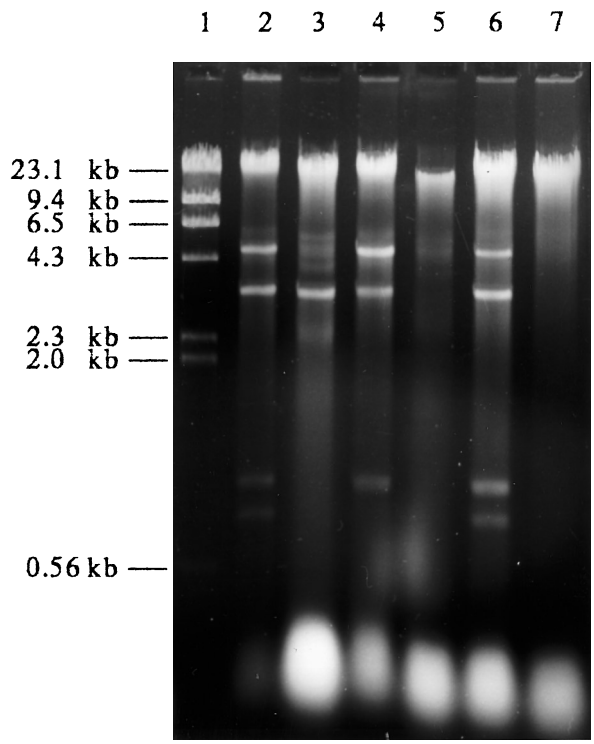

Fig. 1 dsRNA polymorphism of *P. rhodozyma* strains. *Lane 1 Hind* III-digested λ DNA; *lane 2* ATCC 24261; *lane 3* ATCC 24229; *lane 4* ATCC 24203; *lane 5* CBS 6938; *lane 6* CBS 5908; *lane 7* CBS 5905

Characterization of VLPs

Both electron microscopy (Fig. 2) and gel electrophoresis (Fig. 3) revealed the existence of one type of VLP in strain ATCC 24203. This VLP appeared as an elongated icosahedron under the electron microscope, with a size of about 34 × 26 nm. VLPs with the same morphology and size were isolated from strain ATCC 24261, while no VLP was found in strain ATCC 24229.

Analysis of the nucleic acid contents of the VLPs demonstrated that in strain ATCC 24203 two, and in strain ATCC 24261 three, types of dsRNA were co-purified with the VLPs, while dsRNA with a molecular weight of 3.7 kb was not detected in the VLP fraction in either case (Fig. 4).

Stability of dsRNA during mutagenic treatment

The dsRNA contents of the mutants used for the transmission experiments were analyzed. This proved that, despite extensive mutagenic treatment (UV or γ irradiation, Palágyi et al. 1995), the dsRNA pattern of the auxotrophic and colour mutants of strain ATCC 24203 was identical with that of the wild-type strain. Our attempts to obtain dsRNA-free mutants by chemical treatment (Fink and Styles 1972) were unsuccessful (data not shown).

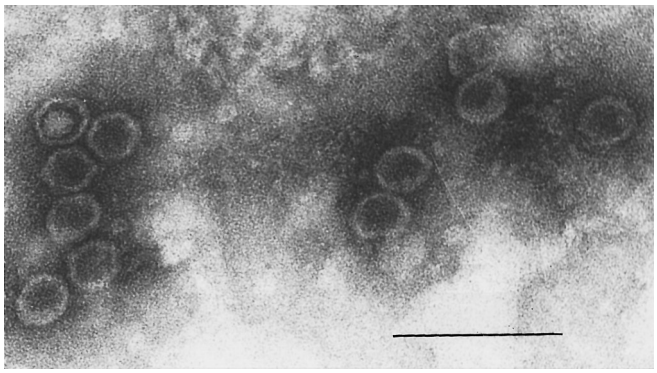

Fig. 2 Electron micrographs of VLPs stained with uranyl acetate. The *bar* represents 100 nm

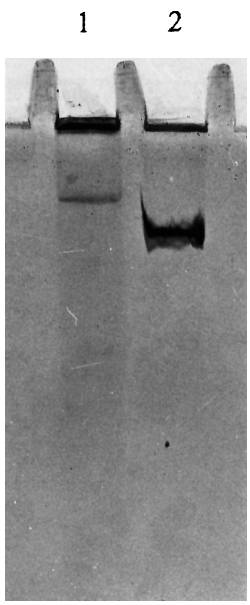

Fig. 3 VLPs separated by gel electrophoresis. *Lane 1* *P. rhodozyma* ATCC 24203; *lane 2 Aspergillus foetidus* CBS 618.78 VLP

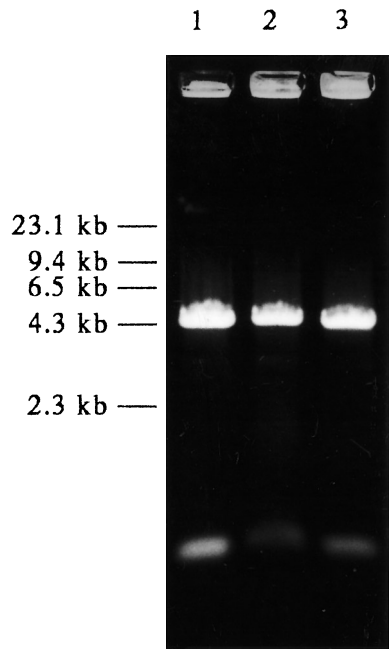

Fig. 4 *Lanes 1–3* dsRNA purified from the VLP fraction of *P. rhodozyma* strain ATCC 24203. The samples derived from three independent isolations

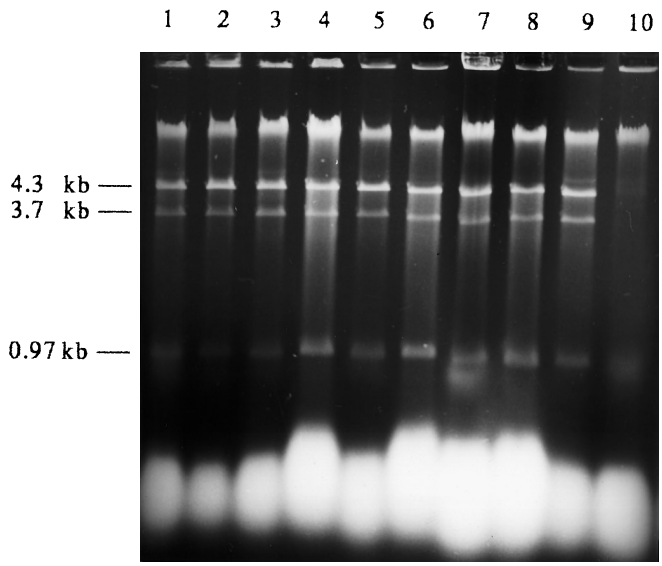

Fig. 5 Inheritance of dsRNAs. *Lanes 1–8* dsRNA from eight basidiospores of two tetrads; *lane 9* ATCC 24203 Lys$^-$, yellow; *lane 10* CBS 6938 Leu$^-$

Transmission of dsRNAs through the basidiospores

To investigate the transmission of the dsRNAs through sexual spores, a dsRNA-free (CBS 6938 Leu$^-$) and a dsRNA-containing strain (ATCC 24203 Lys$^-$, yellow) were allowed to mate and the dsRNA patterns of the four independent sublines of full tetrads were checked. Agarose-gel electrophoresis revealed that each subline examined carried all the types of dsRNAs of the VLP-carrying parental strain (Fig. 5).

Testing of killer activity

As previously published, the presence of VLPs coincides with the killer phenotype of *P. rhodozyma* strain UCD 67–385 (Castillo and Cifuentes 1994). Killer activity was searched for among the descendants of the basidiospores. Despite extensive testing in culture media at different pH values (in the range 4.0–7.2, which includes the pH optima of both *S. cerevisiae* and *K. lactis* killer toxin), no killer activity was detected. Neither, could we demonstrate any killer activity in the wild-type *P. rhodozyma* strains (Fig. 6).

Discussion

Our study revealed the existence of dsRNA molecules of different sizes in four of the six examined *P. rhodozyma*

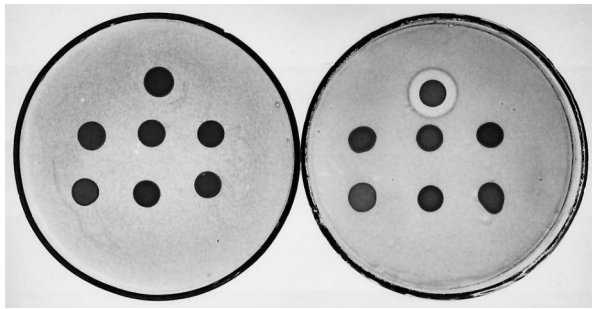

Fig. 6 Killer activity test. Plates seeded with *P. rhodozyma* strain CBS 6938 (left) or *S. cerevisiae* strain S6 (right) were patched with 20 µl of *S. cerevisiae* strain T 158C (upper row), or *P. rhodozyma* strains CBS 5905, CBS 5908, CBS 6938, (middle row), ATCC 24203, ATCC 24229, ATCC 24261 (lower row), and were incubated at 22 °C for 3 days

strains. VLPs, 34×26 nm in size, were isolated only from strains carrying four or three types of dsRNA, while no VLPs were detected in the strain containing only one type of dsRNA. Further study demonstrated that the 3.7-kb dsRNA molecule does not co-purify with the VLP fraction, even in VLP-containing strains. These results suggest that this type of dsRNA does not form part of the VLP genome, and it probably has an organization similar to that of T and W dsRNA in *S. cerevisiae* (Esteban et al. 1993).

Examination of the inheritance of the dsRNAs (VLPs) proved that their transmission through the basidiospores is very efficient, which is in agreement with the results obtained on other fungal species (Schisler et al. 1963; Koltin and Day 1976). Thus the mating process can also be effective in spreading yeast viruses. No phenotypic effect due to the presence of the VLPs could be detected in the descendants of the basidiospores. Furthermore, contrary to the results published earlier (Castillo and Cifuentes 1994), we could not demonstrate any killer activity of the parental strains during extensive testing, suggesting that the VLPs of *P. rhodozyma* have an as-yet unidentified biological function.

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