Current Genetics (1983) 7:185–190

Current Genetics

Isolation and Characterisation of a Double-Stranded RNA Virus-Like Particle from the Yeast *Yarrowia lipolytica*

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Summary. A virus-like particle (VLP) of 50 nm diameter has been isolated from the hydrocarbon-utilising yeast *Yarrowia (Saccharomycopsis) lipolytica*. The VLP contains a linear, double-stranded RNA molecule of MW 3.8×10^6 and has a major capsid protein of MW 75,700.

Key words: Double-stranded RNA – Virus-like particle – Yeast – *Yarrowia lipolytica*

Introduction

Many fungi contain non-infectious virus-like particles (VLP's), the genomes of which consist of one or more double-stranded RNA (dsRNA) molecules (Lemke 1979; Buck 1980). The VLP of the yeast *Saccharomyces cerevisiae* and its associated killer system has been studied extensively (for reviews, see Wickner 1979; Bussey 1981). There have also been reports of electron-microscopic observations of similar cytoplasmic particles in *Candida tropicalis* (Nesterova 1973; Lemke 1976) and *Candida albicans* (Mehta et al. 1982).

We have been surveying the yeast *Yarrowia (Saccharomycopsis) lipolytica* for the presence of extrachromosomal genetic elements. This yeast is used commercially in the production of citric acid and can use alkane hydrocarbons as its sole carbon source (Bassel and Ogryzdiak, 1978). It also has the useful property of producing truly extracellular enzymes including a number of proteases (Ogryzdiak and Mortimer 1977). *Y. lipolytica* is a natural haploid and methods for performing genetic crosses and meiotic tetrad analysis have been established (Ogryzdiak et al. 1978). In this paper, we report the isolation of a VLP from this yeast and the characterisation of its protein and nucleic acid components.

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Materials and Methods

Organisms and Culture Conditions. The following strains of yeast were used in this study:

Yarrowia lipolytica ATCC 18944 (obtained from the American Type Culture Collection).

Saccharomyces cerevisiae S7, a haploid prototrophic strain containing elevated amounts of L-dsRNA (Oliver et al. 1977).

Saccharomyces cerevisiae Σ 1278B, haploid prototrophic killer strain (obtained from Dr. M. Grenson).

For the preparation of nucleic acids or VLP's, all strains were grown to stationary phase in YEPD medium (1% w/v yeast extract; 2% w/v peptone; 2% w/v glucose) at 30 °C.

Isolation of VLP's. The method of Oliver et al. (1977) was used to isolate VLP's from both *Saccharomyces* strains and *Y. lipolytica*. The composition of the CsCl step gradient was modified for the isolation of VLP's from *Y. lipolytica* in order that the VLP band could be clearly separated from polysaccharide material. The modified gradient had steps of 1.3, 1.4 and 1.6 g cm^{-3} CsCl.

Analysis of VLP Proteins. The analysis of the protein composition of VLP by SDS – polyacrylamide gel electrophoresis was carried out exactly as described in Oliver et al. (1977). Following overnight staining with coomassie brilliant blue (0.02% w/v in 50% v/v methanol, 7.5% w/v acetic acid) the gels were destained for 2 h in 5% v/v methanol, 0.75% w/v acetic acid. The stained gels were scanned for absorbance at 590 nm using a Beckman DU-8 spectrophotometer.

Preparation and Electrophoresis of RNA. Double-stranded RNA was prepared from isolated VLP's by extracting twice with phenol-cresol mixture (Kirby 1965) and then precipitating overnight with 2 volumes of ethanol at −20 °C.

RNA was extracted from whole yeast cells using the freeze-thaw technique described by Bevan et al. (1973).

Ethanol-precipitated nucleic acid samples were dried in a vacuum dessicator (Edwards) and resuspended in sterile loading mix (0.1 *M* EDTA; 0.15 mg ml^{-1} bromophenol blue; 20% Ficoll 400 (Pharmacia)). Electrophoresis was carried out in 1.0% w/v agarose gels run for 3 h at 50 V in TEB buffer (10.8 gl^{-1} Tris; 0.93 gl^{-1} EDTA; 5.5 gl^{-1} boric acid) containing 1 μg ml^{-1} ethidium bromide.

Enzymatic Digestion and Denaturation of RNA. RNA samples were digested with the following enzymes: snake venom phosphodiesterase (Boehringer-Mannheim), calf spleen phosphodiesterase (Boehringer-Mannheim) and ribonuclease T1 (Sigma). The incubation conditions were as described by Barrell (1971).

For snake venom phosphodiesterase, incubations were carried out in 0.01 *M* Tris-HCl, 0.01 *M* $MgCl_2$, pH 8.9 for 2 h at 37 °C. Digestion with calf spleen phosphodiesterase was performed in 0.02 *M* Tris-HCl, 0.02 *M* $MgCl_2$, pH 7.5 for 2 h at 37 °C. The same buffer was used for the ribonuclease T1 digestions but the RNA sample was incubated in the buffer at 37 °C for 15 min before cooling on ice and then adding the enzyme and incubating on ice for a further 10 min.

In order to measure the hyperchromic shift in UV absorption following denaturation, RNA samples in 1 x SSC buffer (0.15 *M* NaCl, 0.015 *M* Na citrate) were heated to 100 °C for 5 min followed by rapid cooling on ice for 5 min. The absorbance of the sample at 260 nm was measured before and after the shift using a Cecil CE 292 spectrophotometer.

Agarose gel electrophoresis of denatured RNA samples was carried out essentially as described by Maniatas et al. (1982). RNA precipitates were dissolved in sterile distilled water and mixed with an equal volume of 2 x loading buffer (25 μl CH_3HgOH; 500 μl 4 x running buffer; 200 μl glycerol; 175 μl sterile distilled water; 100 μl 2% w/v bromophenol blue). The samples were then loaded into 1% (w/v agarose gels and run overnight at 55 V in 1 x running buffer (50 *mM* boric acid; 5 *mM* sodium borate; 10 *mM* Na_2SO_4). The buffer was constantly recirculated and stirred.

Following electrophoresis, the gel was stained for 1 h in 0.5 μg/ml ethidium bromide in 0.5 *M* ammonium acetate. The ammonium salt is included to complex the methyl mercury and enhance the binding of the dye to RNA.

Gels were photographed on a short wavelength U.V. transilluminator (Chromato-Vue 0-63) using a Polaroid MP4 land camera. Photographic negatives were scanned for absorbance at 590 nm using a Beckman DU-8 spectrophotometer.

CF-11 Chromatography. Cellulose CF-11 was purchased from Whatman Ltd. and nucleic acid chromatography performed as described in Franklin (1966).

Electron Microscopy of VLP's and dsRNA Molecules. Purified VLP's were suspended in VPE buffer (0.15 *M* NaCl; 0.01 *M* EDTA; 0.03 *M* phosphate buffer pH 7.5) and the suspension spread on carbon-coated grids. Staining was carried out in 0.3% w/v uranyl acetate for 30 s. The grids were then washed in distilled water and dried in air before being examined in an AEI EM6B electron microscope. RNA samples were spread with cytochrome C onto pyroxyllin coated grids using the procedure of Kleinschmidt et al. (1964); the hypo and hyperphases being 0.1 *M* and 1 *M* ammonium sulphate respectively. Contrast was enhanced by staining with uranyl acetate (Davis 1971) followed by rotary shadowing with platinum/palladium using a Polaron E6000 coating unit. Photographs of grids co-spread with *Y. lipolytica* and M dsRNA were taken in the AEI EM6B electron microscope. Contour lengths of 42 M and 63 *Y. lipolytica* molecules were determined using the computerised pen and graphics tablet of an Apple microcomputer.

Results

Isolation of the VLP

A 100,000 g pellet from a cell lysate of *Y. lipolytica* 18944 was resuspended in 3.5 ml of 1.3 g cm^{-3} CsCl in VPE buffer and layered on top of equal volumes of 1.4 g cm^{-3} and 1.6 g cm^{-3} CsCl in a cellulose nitrate centrifuge tube (Beckman). The gradients were centrifuged overnight at 29,000 rpm in a SW41Ti rotor and the bands visualised by illuminating the tubes from below. A major band of polysaccharide material (probably glycogen) was seen at P = 1.385 g cm^{-3} with the smaller VLP band above it at P = 1.335 g cm^{-3}. The pellet contained ribosomal core-particles.

The VLP band was withdrawn from the gradient by puncturing the side of the tube with a hypodermic syringe. The material was then prepared for electron-microscopy using the negative staining technique described in Methods. Isometric particles with a diameter of ca. 50 nm were found (Fig. 1). In some preparations, square or cuboid particles could be seen. However, it was possible to generate these deliberately by improper handling of the electron microscope grids and we consider them to be artefacts.

Analysis of VLP Proteins

SDS-polyacrylamide gel electrophoresis was used to analyse the protein components of the VLP. A major band, which we assume to represent the major capsid protein of the *Y. lipolytica* VLP, was found together with a number of minor bands whose presence and proportion varied from preparation to preparation (track B, Fig. 2). The apparent molecular weight of this band was determined using a calibration curve generated by the co-electrophoresis of 6 proteins of known molecular weight (Fig. 3) and was found to be 75,700. At the same time, we estimated the molecular weight of the major capsid protein of the VLP isolated from *S. cerevisiae* S7 (track A, Fig. 2) and this was found to be 92,000 (Fig. 3). This is an increase on our previous estimate (Oliver et al. 1977) and reflects the use of more high molecular weight protein standards to establish the calibration curve.

The Y. lipolytica VLP Contains a Linear dsRNA Molecule

Nucleic acid was extracted from purified *Y. lipolytica* VLP's and subjected to digestion by a number of nucleases. Bacteriophage λ DNA, tRNA and L dsRNA from *S. cerevisiae* were also digested as controls. The results (Table 1) showed that the nucleic acid extracted from