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Presence of double-stranded RNA and virus-like particles in *Phaffia rhodozyma*

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Abstract. Four double-stranded RNA (dsRNA) molecules were isolated from *Phaffia rhodozyma* UCD 67-385. Their molecular sizes were approximately 4.3, 3.1, 0.9 and 0.75 kilobase pairs (kbp) as determined by agarose-gel electrophoresis and they were designated as L, M, S_1 and S_2, respectively. By differential centrifugation in sucrose gradients, these dsRNAs copurified with isometric virus-like particles 36 nm in diameter. A cured strain, UV-S2, lacking the S_2-dsRNA was obtained from *P. rhodozyma* UCD 67-385 by ultraviolet (UV) light treatment. UV-S2 strain contains identical virus-like particles to those from the wild-type strain, as determined by electron microscopy, suggesting that the S_2-dsRNA was not essential for the expression of mycovirus structural polypeptides. On the other hand, both the UCD 67-385 and UV-S2 strains were able to kill *P. rhodozyma* UCD 67-383, a strain without dsRNAs. These results suggest that the dsRNA molecules also encode a killer system. Finally, the UV-S2 strain maintains killer ability, which suggests that S_2-dsRNA is not involved in the killer phenotype expression.

Key words: Double-stranded RNA – *Phaffia rhodozyma* – Killer system – Virus-like particles

Introduction

Extrachromosomal genetic elements of double-stranded RNA (dsRNA) have been described in a wide variety of filamentous fungi and yeasts. They are usually encapsidated into virus-like particles (VLPs) and in a few cases associated with membranous vesicles (Buck 1986; Brown and Finnegan 1989). The presence of dsRNA in fungi can be detected by different immunological and biochemical methods (Adler and Del Vecchio 1979). Since no discernible phenotypic alterations are found in a number of fungi bearing dsRNA or viral infections (Dodds et al. 1984), a first approach to detect these genetic elements is to analyze their total nucleic-acid composition by gel electrophoresis. Nevertheless, there are a few reported cases where a clear phenotype is associated with dsRNAs. These include the virulence in *Rhizoctonia solani* and *Phytophthora infestans*, the debilitation of *Helminthosporium victoriae*, and the hypovirulence in *Cryphonectria parasitica* (Finkler et al. 1985; Ghabrial 1986; Nuss 1987; Nuss and Koltin 1990). In addition, both *Saccharomyces cerevisiae* and *Ustilago maydis* express a killer toxin encoded by dsRNA molecules encapsidated within virus-like particles. The secreted toxins are responsible for the death of sensitive target cells (Tipper and Bostian 1984; Koltin 1986; Peery et al. 1987).

Phaffia rhodozyma is a carotenoid-producing fermentative yeast of the *Deuteromycotina* (*Blastomycetes*), with properties indicative of a basidiomycetous origin (Miller et al. 1976). The major synthesized carotenoid pigments are astaxanthin with a minor proportion of β-carotene and other pigments which are responsible for their orange to salmon-red color (Andrewes et al. 1976; Miller et al. 1976). In the present work, we describe four dsRNA molecules present in the wild-type *P. rhodozyma* UCD 67-385 strain. Furthermore, evidence is provided for the association of these molecules with virus-like particles and with the expression of a killer system.

Materials and methods

Strains and culture conditions. The wild-type *P. rhodozyma* strains used in this study were UCD 67-383 (ATCC 24203) and UCD 67-385 (ATCC 24230). UV-S2 strain lacking S_2-dsRNA was obtained in this study by UV irradiation. Cells were grown in YM medium (An et al. 1989), containing 20 g of glucose per liter, at 22 °C and with orbital shaking at 120 rpm.

Nucleic acids isolation and purification. For total nucleic acid isolation the cultures (40 ml) were grown at 22 °C to near-stationary phase. The cells were harvested by centrifugation for 7 min at 5 000 rpm in a Sorvall SS34 rotor, washed twice with TE (Tris-HCl 10 m*M*, EDTA 1 m*M* pH 8.0) and resuspended in 3 ml of TE. The harvested cells were disrupted using glass beads (0.5 mm diameter, Sig-

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ma) by mixing equal volumes of suspended cells and beads and vortexing four times during 30 s with cooling on ice between vortexing. Then, 1 vol of phenol (pH 8.0; water-saturated phenol equilibrated first with 1 *M* and then with 0.05 *M* Tris-HCl pH 8.0) : chloroform : isoamyl alcohol (25 : 24 : 1) was added, vortexed for 1 min and centrifuged for 10 min at 10 000 *g*. The aqueous phase was transferred to a sterile tube and extracted twice with 3 ml of the phenolic mixture. Afterwards, the aqueous phase was extracted twice with 3 ml of chloroform : isoamyl alcohol (24 : 1). Nucleic acids were precipitated with 1/10 vol of 3 *M* sodium acetate and 2.5 vol of ethanol at –20 °C overnight. The ethanol-precipitated nucleic acids were resuspended in TE and mixed with an equal volume of 4 *M* LiCl to precipitate the single-stranded RNAs (Baltimore 1966). The mixture remained at 4 °C overnight and was then centrifuged at 20 000 *g* for 15 min. The supernatant fraction was dialyzed against TE and finally re-precipited with ethanol as described above. Total dsRNAs were purified by CF11 cellulose chromatography as described by Franklin (1966).

Enzymatic digestions. The reaction conditions of DNase I, RNase H, and S1 nuclease were those described by Sambrook et al. (1989) and Muthukrishnan and Shatkin (1975). The RNase A digestions were carried out in the following high- and low-ionic strength buffers: 2×SSC (0.3 *M* NaCl, 0.03 *M* sodium citrate, pH 7.0) and 0.01×SSC, respectively (Pryor and Boelen 1987).

Nucleic acids analysis. Nucleic acids were resuspended in TE and analyzed by gel electrophoresis in 1.2% (w/v) agarose. Ethidium bromide, 0.5 µg/ml, was used to stain the gels (Sambrook et al. 1989).

Isolation of virus-like particles. This was achieved by the method of Dickinson and Pryor (1989), with minor modifications. Cells (10 *g* wet weight) were disrupted in 50 ml of phosphate buffer (100 m*M* sodium phosphate pH 7.5, 10 m*M* $MgCl_2$) by vortexing with glass beads. Cellular debris was removed by centrifugation at 10 000 *g* for 10 min at 4 °C. The supernatant was adjusted to 9% (w/v) PEG 8000 (Sigma), 2.5% (w/v) NaCl and maintained with gentle agitation overnight at 4 °C. After centrifugation at 10 000 *g* for 15 min the pellet was resuspended in phosphate buffer and re-centrifuged at 100 000 *g* for 3 h to sediment the particulate material. The pellet obtained was resuspended in a minimum volume of phosphate buffer and layered onto a 10–50% (w/v) linear sucrose gradient and centrifuged at 30 000 rpm for 90 min in a swinging bucket rotor RPS 40 (Hitachi). The UV absorbance profile at 260 nm was determined and peaks of UV absorption were pooled and dialysed against phosphate buffer. Virus-like particles were recovered by pelleting at 35 000 rpm for 4 h in a RP 40 rotor (Hitachi).

Electron microscopy. Drops of particulate suspensions were negatively stained with 2% (w/v) potassium phosphotungstate, pH 7.0, on Formvar and carbon-coated 200 mesh cooper grids. The observations were made with a Phillips 300 microscope.

UV treatment. An exponential-phase culture of *P. rhodozyma* UCD 67-385 was irradiated with ultraviolet light using a General Electric germicidal 15 W lamp type G8T5, which emitted radiation primarily at 254 nm. The flux of the UV source was 0.6 W/m^2. The cells were spread on YM solid medium and grown at 22 °C until the appearance of colonies; then their total nucleic acids were extracted and analyzed by agarose-gel electrophoresis.

Assay for killing activity. UCD 67-385 and UV-S2 strains were tested for killing activity. Around 10^7 cells of the sensitive strain UCD 67-383 were mixed with 10 ml of 0.7% (w/v) agar YM medium, buffered at pH 4.6 with citrate/phosphate buffer (Gomori 1955) containing 0.003% (w/v) methylene blue to stain dead cells, and then layered onto a Petri dish. Streaks of a killer and nonkiller strain were applied on the surface and the plate was incubated for at least 3 days at 22 °C. After this time clear zones were observed only around the killer strains.

Results

Detection of extrachromosomal genetic elements

Total nucleic acids were isolated from two wild-type strains of *P. rhodozyma* (UCD 67-383 and UCD 67-385) and analyzed by 1.2% (w/v) agarose-gel electrophoresis (Fig. 1). The electrophoretic profile revealed four sharp bands migrating faster than chromosomal DNA in the UCD 67-385 strain (lane 2), but not in the UCD 67-383 strain (lane 3) which showed only chromosomal DNA. The molecular sizes of the genetic elements present in the UCD 67-385 strain were estimated using a mixture of λDNA/*Hin*dIII and ΦX174 DNA/*Hae*III fragments as molecular size markers. They were named as L, M, S_1, and S_2, with molecular sizes of 4.3, 3.1, 0.9 and 0.75 kilobase pairs (kbp), respectively (Fig. 1). The approximate molecular weight of these molecules was 2.95×10^6, 2.13×10^6, 6.18×10^5 and 5.15×10^5 daltons, considering 687 daltons per base pair (bp) for dsRNA (Field et al. 1983).

Chemical nature of extrachromosomal genetic elements

The extrachromosomal genetic elements of *P. rhodozyma* UCD 67-385 were completely degraded by incubation in alkaline media (Holnes and Atfield 1971) and by treatment with RNase A in a low-salt concentration buffer (0.01×SSC) (Fig. 2, lane 3). However, they were resistant to both pancreatic DNase I and *Eco*RI restriction endonuclease treatment, whereas the chromosomal DNA was totally (Fig. 2, lane 4) and partially (Fig. 2, lane 9) digested. These results suggest that these genetic elements corre-

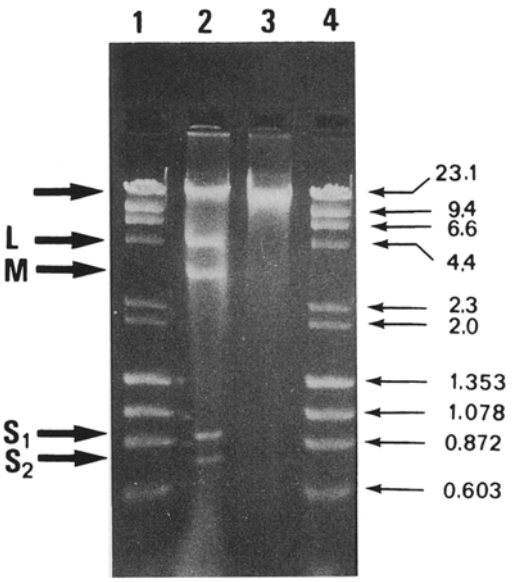

Fig. 1. Agarose-gel electrophoresis of total nucleic acids from wild-type strains of *P. rhodozyma*. *Lanes 1 and 4*, mixture of λ DNA/*Hin*dIII and φx174 DNA/*Hae*III fragments; *lane 2*, total nucleic acids from the UCD 67-385 strain; *lane 3*, total nucleic acids from the UCD 67-383 strain. The *numbers* on the right side indicate molecular sizes, expressed in kilo-base pairs. The *arrows* on the left indicate the position of genomic DNA and dsRNAs

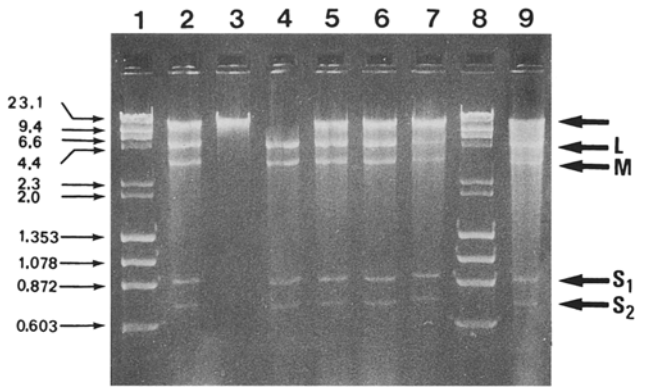

Fig. 2. Agarose-gel electrophoresis of total nucleic acids from *P. rhodozyma* UCD 67-385 after different treatments. *Lanes 1 and 8*, mixture of λ DNA/*Hin*dIII and ϕx174 DNA/*Hae*III fragments. Total nucleic acids treated with: *lane 2*, without treatment; *lane 3*, RNase A (0.1 μg/ml) in a low-strength ionic buffer (0.01×SSC: 1.5 m*M* NaCl, 0.15 mM sodium citrate, pH 7.0); *lane 4*, DNase I (1 U/μg of total nucleic acids); *lane 5*, S1 nuclease (1 U/μg of total nucleic acids); *lane 6*, RNase H (1 U/μg of total nucleic acids); *lane 7*, RNase A (0.1 μg/ml) in a high-strength ionic buffer (2×SSC); *lane 9*, *Eco*RI (1 U/μg of total nucleic acids). The *numbers* on the left side indicate molecular sizes, expressed in kilobase pairs. The *arrows* on the right indicate the position of genomic DNA and dsRNAs

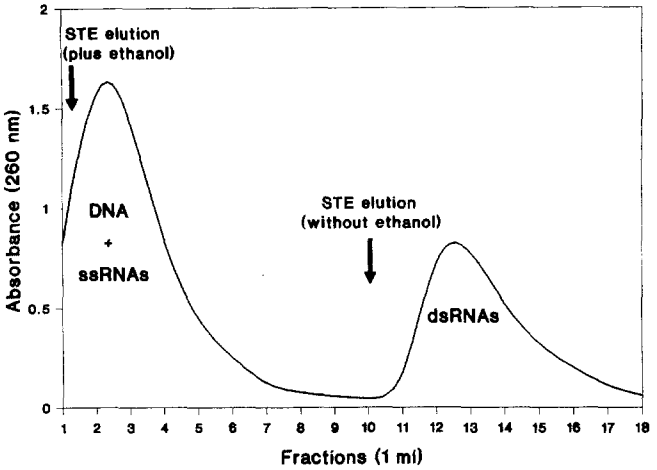

Fig. 3. DsRNAs purification by CF11-cellulose chromatography. The total nucleic acids of *P. rhodozyma* UCD 67-385 were dissolved in STE buffer (100 m*M* NaCl, 1 m*M* EDTA, and 50 m*M* Tris-HCl pH 7.0) containing 16% ethanol and applied to a column equilibrated with the same buffer. The elution with this buffer, proceeded until complete DNA and single-stranded RNAs recuperation. Then, dsRNAs were eluted with STE without ethanol as indicated in the figure

sponded to RNA molecules. On the other hand, neither S1 nuclease (Fig. 2, lane 5) nor RNase H (Fig. 2, lane 6) altered the electrophoretic migration patterns of these molecules, indicating that they correspond to dsRNA without apparently either single-stranded RNA sequences or hybrid DNA:RNA regions. Furthermore, these molecules were resistant to RNase A digestion in a high-salt concentration buffer (2×SSC) (Fig. 2, lane 7) and were specifically retained in CF11 cellulose columns (Fig. 3). When the total nucleic acids were loaded in a column pre-equilibrated with STE (100 m*M* NaCl, 1 m*M* EDTA, and 50 m*M* Tris-Cl pH 7.0) – 16% ethanol and eluted with the same buffer, the elution of only DNA and single-stranded RNAs (ssRNAs) was achieved. The dsRNAs retained in the column were eluted with STE-0% ethanol (Fig. 3). Thus, both their RNase A resistance and their chromatographic behaviour provided a clear confirmation of the double-stranded nature of these RNA genetic elements.

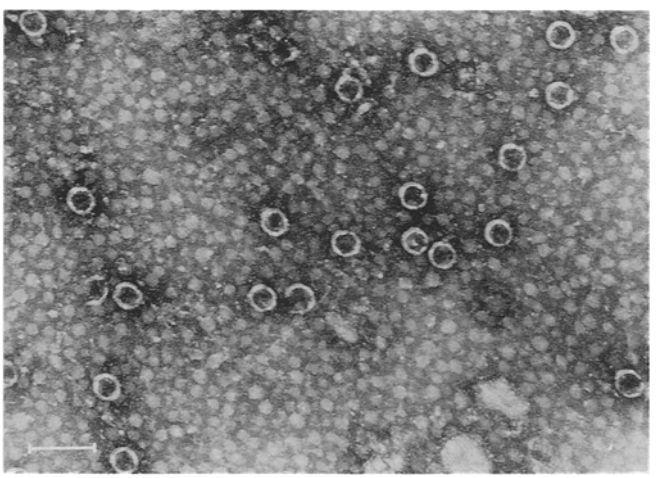

Fig. 4. Electron micrograph of virus-like particles from *P. rhodozyma* UCD 67-385. Negative staining by 2% (w/v) potassium phosphotungstate, pH 7.0. Bar marker represents 100 nm

Isolation of virus-like particles

To determine if the dsRNA molecules were associated with virus-like particles, a linear sucrose gradient centrifugation of cell-free extracts, obtained by differential centrifugation and PEG 8000 precipitation from *P. rhodozyma* UCD 67-385, was performed (see Materials and methods). The gradient profiles at 260 and 280 nm revealed one major peak that was analyzed by agarose-gel electrophoresis and electron microscopy. The electrophoretic analysis after phenol:chloroform extraction of the major peak, showed the presence of four dsRNA molecules with an electrophoretic mobility identical to those observed in Fig. 1 (data not shown). The electron microscopy observations of the same peak revealed isometric virus-like particles of 36 nm in diameter after negative staining with potassium phosphotungstate (Fig. 4).

Curing the S_2 double-stranded RNA

Several conditions have been described which result in the loss of at least one of the dsRNAs present in fungal cells (Fink and Styles 1972; Wickner 1974; Koltin 1977; Fulbright 1984; Tréton et al. 1987). We used ultraviolet light treatment as a curing procedure on the UCD 67-385 strain. A colony lacking the S_2-dsRNA molecule was obtained and named strain UV-S2 (Fig. 5, lane 3). However, this

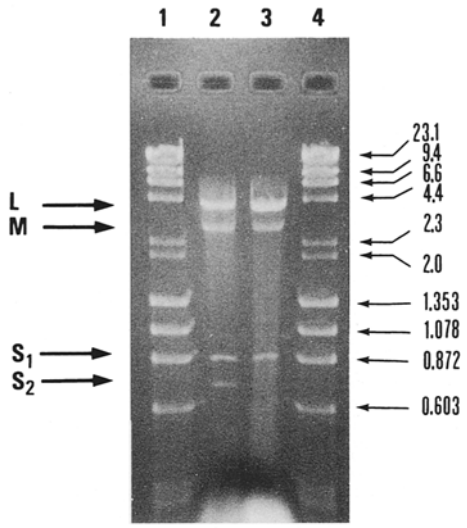

Fig. 5. Agarose-gel electrophoresis of total nucleic acids from cured *P. rhodozyma* strain. *Lanes 1 and 4*, mixture of λ DNA/*Hin*dIII and φx174 DNA/*Hae*III fragments; *lane 2*, UCD 67-385 strain; *lane 3*, UV-S2 strain. The samples were treated with DNase I (1 U/μg of total nucleic acids) to degrade genomic DNA. The *numbers* on the right side indicate molecular sizes, expressed in kilobase pairs. The *arrows* on the left indicate the position of dsRNAs

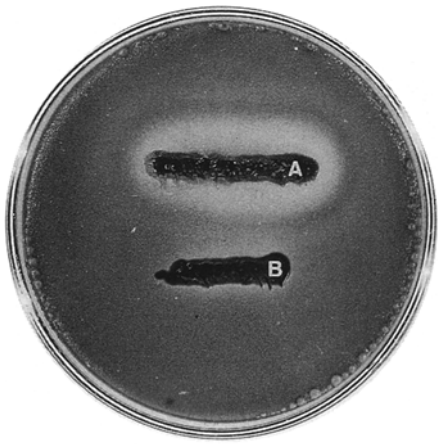

Fig. 6. Assay of killer phenotype. Streaks of the UCD 67-385 (*A*) and UCD 67-383 (*B*) strains onto a lawn of 67-383 strain on YM agar containing 0.003% (w/v) methylene blue

strain contains identical virus-like particles to those of the parental strain (data not shown).

Presence of a killer system in P. rhodozyma UCD 67-385

The presence of dsRNA molecules could confer a characteristic phenotype on *P. rhodozyma* UCD 67-385. We attempted to detect the presence of a killer phenotype using *P. rhodozyma* UCD 67-383, which lacks dsRNAs, as a sensitive strain. After streaks of UCD 67-385 were grown on a lawn of a sensitive strain and incubated at 22 °C for 3–7 days, a clear zone of growth inhibition surrounding the streak was observed. Furthermore, this zone was bounded by a dark-blue ring of dead cells (Fig. 6). This preliminary result constitutes evidence for the existence of such a killer system. Optimal activity conditions of the apparent killer toxin were pH 4.6 and 22 °C.

Discussion

The *P. rhodozyma* UCD 67-385 genetic extrachromosomal elements detected in this work were identified as dsRNA molecules. A clear confirmation of their chemical nature was obtained by their resistance a DNase I, RNase A in a high-salt-concentration buffer, and S1 nuclease digestion, as well as their specific binding to cellulose CF11, a chromatographic resin used to separate single-stranded RNA (ssRNA) from dsRNA (Franklin 1966), and their total sensitivity to RNase A in a low-ionic-strength buffer. These are standard tests used to characterize the dsRNA molecules of other fungi (Koltin 1986; Pryor and Boelen 1987). Sucrose gradient-centrifugation analysis suggested that the dsRNAs were associated with VLPs, since both copurified in the gradient. The dsRNAs probably constitute the genetic material of these particles. However, it has not yet been possible to determine whether these dsRNA molecules are separately encapsidated or not (Buck 1986). For such a determination, it is necessary to use high-resolution techniques, such as CsCl equilibrium density gradients, a methodology used to demonstrate the differential encapsidation of dsRNAs in *S. cerevisiae* (Esteban and Wickner 1986). The size and morphological characteristics of VLPs from *P. rhodozyma* UCD 67-385 are similar to those described for *S. cerevisiae* (Herring and Bevan 1974); that is, they are isometric particles of 36 and 39 nm diameter for *P. rhodozyma* and *S. cerevisiae*, respectively. In both cases some broken particles were also observed and most of them appear to be empty. However, the particles with a darkened center do not necessarily correspond to empty particles, because both full RNA capsids and truly empty capsids are usually penetrated by the stain (Bozarth 1979).

The curing experiments with UV light yielded one strain lacking the smaller dsRNA (S_2-dsRNA). This strain, named UV-S2, contains identical VLPs that those detected in the wild-type strain, suggesting that S_2-dsRNA does not encode structural polypeptides of the viral particles.

Finally, the experiments to detect whether the dsRNAs could confer some special phenotypic characteristic on *P. rhodozyma* UCD 67-385, revealed the presence of a killer system. This system was responsible for the death of a sensitive strain, such as *P. rhodozyma* UCD 67-383, that does not contain dsRNAs. For the optimal visualization of the killer effect a minimum incubation of 72 h was required, whereas killer yeasts of other genera have maximal effect after 24 h incubation (Young 1987). The slower growth rate of *P. rhodozyma* UCD 67-385 compared to *S. cerevisiae*, for example, could explain this difference. On the other hand, the UV-S2 strain presents the same killer activity as UCD 67-385, indicating that the S_2-dsRNA is not important for killer toxin expression. In most fungi that contain dsRNAs it has not been possible to detect a killer system. A probable explanation for this phenomenon is

that the toxins are extremely unstable and are rapidly inactivated in inadequate pH and temperature conditions. Therefore, it is probable that a large number of killer strains have not been detected because inadequate assay conditions have been used.

To demonstrate in which dsRNA molecule the killer toxin is encoded, we are attempting to obtain cured strains in different dsRNA molecules by treatment of UCD 67-385 with sub-lethal concentrations of cyclohexymide and then to determine the retention or loss of killer activity. Up to this time it has been possible to obtain only three additional strains that lack the S_2-dsRNA, all of which retain the killer phenotype. In *S. cerevisiae* the L-dsRNA is the molecule responsible from the expression of the major coat protein of the viral particles, M-dsRNA encodes the toxin-immunity precursor protein and S-dsRNAs are internal deletion products of M (Lee et al. 1986; Wickner 1989). Similar identification of the products encoded by the different dsRNAs isolated from *P. rhodozyma* is now under investigation.

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