Lytic Plaque Formation and Variation in Virus Titre among Strains of *Penicillium chrysogenum*

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SUMMARY

Strains of *Penicillium chrysogenum* derived from a virus-infected culture have been investigated for reduction in virus titre. These strains were obtained following treatment with either heat (74 °C) or specific antimetabolites. Virus titres were measured with a combination of immunological and radiochemical assays. One strain was cured of the mycophage and exhibited an increased growth rate and stability. Other strains, which show an apparent reduction in intracellular virus titre, actually released increased levels of virus from cells. Lytic plaques developed when infected strains were grown on an unbuffered, high lactose-containing medium, and virus titres could be correlated with plaque formation. All strains, regardless of virus titre, retained the ability to synthesize penicillin.

INTRODUCTION

The fungus *Penicillium chrysogenum* is infected with a virus containing double-stranded ribonucleic acid (Banks *et al.* 1969; Lemke & Ness, 1970). Biochemical and biophysical characterization of this mycophage and its component nucleic acid has been pursued in several laboratories (Buck, Chain & Himmelweit, 1971; Wood & Bozarth, 1972; Nash *et al.* 1973). The biology of this virus has not been extensively studied, yet the biological implications of a virus in *P. chrysogenum* are many. Strains of *P. chrysogenum* are known to vary spontaneously with regard to growth rate, sporulation efficiency, and antibiotic productivity (Backus & Stauffer, 1955). Such instability among cultures of *P. chrysogenum* is perhaps related, at least in part, to variation in virus titre. *Penicillium chrysogenum* is a haploid organism and has been adapted for formal genetic analysis (Sermonti, 1957). This fungus should, therefore, provide a model experimental system for extensive biological characterization of a virus in a eukaryotic cell. In this paper we report that virus content in *P. chrysogenum* is variable. Strains heavily infected with virus are shown to exhibit lysis, and a stable strain cured of virus is described.

METHODS

Strains and growth media. A mutant culture of *Penicillium chrysogenum* (E-15), derived from NRRL 1951 (Elander, 1967), was used as the control strain for this investigation. The E-15 culture and strains obtained from it were maintained on a minimal sporulation medium containing purified lactose, 12 g; NaNO_3 , 3 g; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.1 g; KH_2PO_4 , 60 mg; $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$, 2 mg; $\text{Fe}(\text{NH}_4)_2(\text{SO}_4)_2 \cdot 6\text{H}_2\text{O}$, 20 mg; NaCl , 10 g; agar, 25 g; distilled water, 1000 ml. This medium was used as a routine plating medium. All cultures were grown at 25 °C. Spore inocula were grown either in a complex vegetative medium (Vanderhaeghe, Claesen, Vlietinck & Parmentier, 1968) or in a minimal vegetative medium (Lemke & Ness, 1970). Either spores (10^7 cells) or vegetative cells (mycelia after 72 h of growth in complex vegetative medium) were used to inoculate 500 ml Erlenmeyer flasks containing 100 ml of a complex fermentation medium described by Moss & Cole (1964). Growth in this medium proceeded for 3 to 8 days at 25 °C on a rotary shaker (250 rev./min).

Antiviral treatment. In order to reduce virus titre, spores from the control culture (E-15) were either exposed to high temperature or grown in the presence of selected compounds. Heat treatment was conducted in the following manner. Spores were collected in distilled water containing 0.1 % polyoxyethylene sorbitan mono-oleate (Sigma Chemical Co., St. Louis, Missouri, U.S.A., P1754), adjusted to a titre of 10^7 spores/ml, and incubated at 74 °C in a gyratory (25 rev./min) water-bath shaker (New Brunswick Scientific, New Jersey, U.S.A., G176). Samples (1 ml) were removed at 10 min intervals up to 2 h and plated without dilution. Survivors of treatment at the highest dosage were subcultured on to sporulation medium and grown for 2 weeks at 25 °C. Spores were again collected and treated a second time. After three successive heat treatments, strains were examined for reduction in virus titre.

A series of antimetabolites and antiviral compounds were tested for their toxicity to *Penicillium chrysogenum*. The sensitivity of the control culture to a particular compound was determined according to procedures described previously (Lemke, 1969). Spores (10^6 /ml) were incubated at 27 °C for 48 h in minimal vegetative medium containing a sub-lethal concentration of a given compound (see below). Mycelia were collected by centrifugation (1000 g) and washed twice with 10 ml distilled water. These cells were macerated for 90 s in a Waring blender at high speed. The macerate was plated with serial dilution on to sporulation medium. Spores from isolated colonies were subcultured, and strains were again treated with the same compound before evaluating virus titre. Compounds surveyed include aranotin (Dr N. Neuss, Lilly Research Laboratories), 5-azacytidine (Calbiochem, La Jolla, California, U.S.A.), 6-azauridine (Nutritional Biochemicals Corporation, Cleveland, Ohio, U.S.A.), cycloheximide (Sigma Chemical Co.), ethidium bromide (Calbiochem), mycophenolic acid (Imperial Chemical Industry), and pyrazomycin (Dr K. Gerzon, Lilly Research Laboratories).

Preparation of extracts. Forty grams of mycelia from 72 h cultures grown in the complex fermentation medium were washed twice with cold distilled water, suspended in 20 ml tris buffer (0.02 M, pH 8.0) containing 0.01 M- MgCl_2 and disrupted in a French pressure cell at 20000 lb/in². Cellular debris was removed by centrifugation for 15 min at 30000 g. Virus particles were pelleted from the supernatant by centrifugation (Beckman L3-50) for 4 h at 100000 g. This pellet was resuspended in 0.5 ml of tris buffer. The presence of virus was determined by the Ouchterlony double-diffusion method.

Immunological assay. Virus was isolated from the control culture and purified by isopycnic zonal centrifugation in sucrose and in caesium chloride (Nash *et al.* 1973). Purified virus

was adjusted with phosphate buffer, 0.02 M, pH 7.0, containing 0.01 M-MgCl₂ to a titre of 1.4 mg virus/ml buffer. This stock suspension of purified mycophage ($E_{260} = 10$) served as the antigen (AgM).

Rabbit antiserum to purified mycophage (Ab_{RM}) was prepared by injecting a single animal intramuscularly with four 10 mg doses of virus over a 20-day period. Each dose was combined with an equal volume of complete Freund's adjuvant (Difco, Detroit, Michigan, U.S.A.) and the rabbit was sacrificed on the 30th day.

Specificity of antiviral antiserum was determined by the Ouchterlony double-diffusion assay (Horwitz & Scharff, 1969), with Kallestad immunodiffusion plates. No serological reactions occurred with normal rabbit serum or serum from rabbits injected with complete Freund's adjuvant.

Virus titre in fungal extracts was estimated either by the agar immunodiffusion assay or by direct immunological precipitation of radioactively labelled virus. In this study, virus was labelled with tritium by the addition of uracil-6-³H (Amersham/Searle, Arlington Heights, Illinois, U.S.A., 1000 mCi/mmole) to fermentation medium at a concentration of 1 μ Ci/2 ml. Mycelia were harvested by centrifugation and washed with tris buffer. Extracts were prepared as mentioned above. Labelled virus from these extracts was precipitated directly with antiserum (0.2 ml Ab_{RM}/ml extract). Virus from broth samples was precipitated either by the addition of rabbit antiviral serum (0.2 ml Ab_{RM}/ml broth) or by addition of an equal volume of 10 % trichloroacetic acid. All precipitates were washed repeatedly with phosphate buffer (0.02 M, pH 7.0). Radioactivity was then measured with a Packard Tri-Carb liquid scintillation spectrophotometer (Model 3375) with an external standard. Aquasol (New England Nuclear, Boston, Massachusetts, U.S.A.) was used as a scintillation medium.

Biological assay. In order to test for lytic plaque formation, spores of *Penicillium chrysogenum* were plated densely (10^5 to 10^6 /plate) on to medium containing purified lactose, 180 g; peptone, 10 g; agar (Baltimore Biological Laboratories, Cockeysville, Maryland, U.S.A.), 20 g; and distilled water, 1000 ml. Cultures of *P. chrysogenum* were incubated at 25 °C. On this medium, devised by Borré and co-workers (1971), viral plaques were formed in two other species of *Penicillium*, *P. citrinum* and *P. variable*.

Assay for antibiotic. Culture broths were assayed for penicillin by reaction with hydroxylamine (Boxer & Everett, 1949). This assay was modified for the Technicon autoanalyser and had a precision of ± 1 %.

RESULTS

In order to reduce virus titre among strains of *Penicillium chrysogenum*, spores of this organism were exposed to heat or germinated in the presence of specific compounds. The sensitivity of a virus-infected culture to heat (74 °C) or to a series of compounds (5-azacytidine, 100 μ g/ml; cycloheximide, 200 μ g/ml; ethidium bromide, 1000 μ g/ml; pyrazomycin, 200 μ g/ml) was first determined. Four other compounds, aranotin, 6-azauridine, mycophenolic acid and rifampicin, were also tested but were non-toxic to *P. chrysogenum*. Priority was given to treatment with compounds toxic to the host organism.

Survivors of treatment were selected at random and compared with the control culture for reduction in virus titre. Fungal extracts were initially screened with an immunodiffusion assay (Fig. 1). In addition, all strains were compared with the control culture for ability to synthesize penicillin. Cultural characteristics such as relative growth and colony colouration were recorded for each isolate (Table 1). All strains retained the ability to synthesize antibiotic, although considerable variation was observed among isolates with regards to net accumulation of penicillin and the pH profile during fermentation (Table 1). Growth rate

Fig. 1. Ouchterlony double-diffusion assay of *Penicillium chrysogenum* mycophage: rabbit antiserum to purified virus (centre), purified virus (1), control strain (2), C6 strain (3), P1 strain (4), and $\Delta 2$ strain (5).

Table 1. *Strains of Penicillium chrysogenum* derived from a virus-infected culture

Strain	Treatment	Immunodiffusion assay viral titre	Relative growth and colony colouration	Penicillin production % control		pH	
				3 days	7 days	3 days	7 days
Control (E-15)		Control	Control; green	100	100	6.0	6.1
Δ1	Heat	Reduced	Fast; dark green	106	76	6.1	5.8
Δ2		Absent	Fast; white	38	12	6.1	8.2
Δ3		Absent	Fast; green	41	25	6.1	6.0
Δ4		Absent	Fast; beige	49	11	6.3	8.0
Δ5		Absent	Fast; dark green	89	71	6.4	5.8
Δ6		Absent	Fast; beige	51	12	5.7	7.9
Δ7		Reduced	Fast; green	82	—	5.7	—
Δ8		Reduced	Control; sectorcd	132	—	5.8	—
Δ9		Reduced	Fast; green	94	—	5.7	—
Δ10		Reduced	Slow; light green	103	—	5.8	—
Δ11		Reduced	Slow; white	57	—	5.7	—
Δ12		Reduced	Fast; white	57	—	5.7	—
A1	5-Azacytidine	Control	Slow; pale green	20	1	6.4	7.2
A2		Control	Control; green	87	94	6.0	6.2
A3		Control	Control; green	99	99	6.0	5.9
A4		Control	Slow; white	77	22	6.2	6.0
A5		Control	Fast; green	78	59	5.9	5.7
A6		Control	Fast; green	85	60	6.7	6.2
C1	Cycloheximide	Reduced	Control; sectorcd	114	88	6.2	6.0
C2		Reduced	Fast; white	120	95	6.2	6.0
C3		Control	Fast; dark green	135	81	6.0	6.5
C4		Control	Fast; dark green	117	99	6.1	6.1
C5		Reduced	Fast; sectorcd	180	75	6.1	6.2
C6		Reduced	Slow; sectorcd	124	25	5.9	5.9
EB1	Ethidium bromide	Control	Control; green	101	—	—	—
EB2		Control	Control; green	102	—	—	—
EB3		Control	Control; green	117	—	—	—
EB4		Control	Control; green	113	—	—	—
EB5		Control	Control; green	113	—	—	—
EB6		Control	Control; green	89	—	—	—
P1	Pyrazomycin	Reduced	Control; green	84	66	6.2	6.0
P2		Control	Slow; white	70	97	6.1	6.4
P3		Control	Slow; sectorcd	70	101	6.3	6.4
P4		Control	Fast; dark green	76	95	6.2	5.8
P5		Control	Fast; dark green	86	98	6.3	6.0
P6		Control	Fast; green	85	93	6.2	6.0

and colony colouration varied considerably among isolates (Fig. 2). Such variation is known to occur spontaneously among isolates of *Penicillium chrysogenum* (Backus & Stauffer, 1955; Lemke & Ness, 1970). However, a series of spontaneous variants of this organism assayed for virus by the immunodiffusion method proved to be uniformly infected. Only with successive heat treatments or following repeated treatments with either pyrazomycin or cycloheximide was an apparent reduction in intracellular virus titre observed (Table 1, Fig. 1).

Several strains were selected for further study. Extracellular as well as intracellular virus titres were measured with an immunochemical assay. In this assay viral nucleic acid was labelled with tritiated uracil, and the virus was selectively precipitated with rabbit anti-serum to mycophage. This assay proved to be extremely sensitive, and with it three types of strains were recognized with regard to virus titre (Table 2). One strain type (i.e. Δ2) appeared

Fig. 2. Cultural characteristics of *Penicillium chrysogenum*: (a) control strain, green; (b) c6 strain, sectored; (c) $\Delta 1$ strain, dark green; (d) $\Delta 2$ strain, white. All cultures were photographed after 7 days of incubation and at the same magnification.

Fig. 3. Release of *Penicillium chrysogenum* mycophage during fermentation.

Table 2. *Virus titre of broth and fungal extracts in four strains of Penicillium chrysogenum**

Strain	Broth + Ab _{RM} †	Fungal extract + Ab _{RM}
Δ1	Not tested	756
Δ2	Not tested	0
C6	597	5332
Control	496	29053

* All values represent d.p.m./0.5 ml of either broth or fungal extract.

† Ab_{RM} = rabbit antiserum to mycophage.

to be cured of virus, since no label was recovered through precipitation by the immuno-chemical assay. A second strain type (i.e. Δ1) exhibited reduction in virus titre. The third strain type (i.e. C6) exhibited an increased extracellular virus titre.

The extracellular titre of virus during fermentation was compared among strains of *Penicillium chrysogenum* (Fig. 3). Viral nucleic acid was again labelled with tritiated uracil, and broth samples in this instance were precipitated with trichloroacetic acid. The Δ2 strain was consistently negative for the release of virus, and the Δ1 strain, although infected, was reduced in virus titre. Two strains, C6 and A3, exhibited a marked increase in extracellular virus titre. Similar differences in titre were observed when extracellular virus was measured by immunoprecipitation.

Strains of *Penicillium chrysogenum* with increased extracellular virus titre form lytic plaques when grown on an unbuffered, high lactose-containing medium. On this medium erumpent patches of white sterile mycelia developed after 5 days of incubation (Fig. 4). These patches, however, were not formed by the Δ2 culture and were rarely observed with the Δ1 culture. Such patches were formed at a high frequency by other cultures (i.e. C6, P1, A3) and led to

Fig. 4. Early (5-day) plaque formation in *Penicillium chrysogenum*: (a) control strain; (b) heavily infected A3 strain; (c) uninfected Δ2 strain.

Fig. 5. Late plaque formation in *Penicillium chrysogenum*: (a) C6 strain, surface of culture after 2 weeks of incubation; (b) C6 strain, after 4 weeks of incubation, bottom of Petri plate observed by transmitted light; (c) close-up of (b) arrows indicate differences in plaque morphology, i.e. clear (1), turbid with clear centre (2), and turbid (3).

localized plaque formation after 4 weeks of incubation (Fig. 5). Plaques varied in morphology from clear to turbid (Fig. 5c).

DISCUSSION

Mycoviruses have now been reported in several genera representing all of the major taxonomic groups of fungi (Hollings & Stone, 1971 [review]; Day & Ellis, 1971; Lapierre, Albouy, Faivre-Amiot & Molin, 1971; Kazama & Schornstein, 1972; Lapierre, Faivre-Amiot, Kusiak & Molin, 1972; Lhoas, 1972; Tuveson & Peterson, 1972; Koltin, Berick, Stamberg & Ben-Shaul, 1973). Several viruses from imperfect fungi have been studied in considerable detail and, in general, these viruses contain genomes of double-stranded ribonucleic acid. Among the viruses of imperfect fungi, those present in *Penicillium stoloniferum* and *P. chrysogenum* have been characterized most extensively (Banks *et al.* 1968; Buck, Chain & Darbyshire, 1969; Buck & Kempson-Jones, 1970; Bozarth, Wood & Mandelbrot, 1971; Wood & Bozarth, 1972; Nash *et al.* 1973). Certain biological properties characteristic of viruses have now been demonstrated with the viruses of these two fungal species. Viral transmission through heterokaryosis and the native infection of protoplasts by virus have been described for *P. stoloniferum* (Lhoas, 1971*a, b*). An RNA-dependent RNA polymerase activity has been reported for viruses in both *P. stoloniferum* (Lapierre, Astier-Manificier & Cornuet, 1971) and *P. chrysogenum* (Nash *et al.* 1973).

We now report the isolation of strains of *Penicillium chrysogenum* altered with respect to virus titre. Some strains were greatly reduced for virus titre, and this reduction could be demonstrated by an immunodiffusion assay (Fig. 1). One strain was isolated that was apparently freed of virus, since assay for virus by an extremely sensitive radioimmunological method proved negative (Table 2, Fig. 3). This strain can now be utilized in genetic studies of *P. chrysogenum* without the superimposition of a virus. Still other strains of *P. chrysogenum* contained high titres of virus, and these strains were lytic. These results demonstrate that the virus of *P. chrysogenum* was not only variable in titre but was potentially virulent. It remains to be determined if this double-stranded RNA-containing virus can engage in genetic determination beyond its own replication.

Lytic plaques have been reported previously in two species of *Penicillium* infected by virus-like particles (Borré, Morgantini, Ortali & Tonolo, 1971), and transmission of a cytoplasmic factor responsible for cellular breakdown has been observed in *Schizophyllum commune* (Koltin *et al.* 1973) and in a species of *Aspergillus* (Jinks, 1959; Caten, 1972). Our studies with *Penicillium chrysogenum* demonstrate for the first time that the extent of plaque formation in a fungus is related to virus titre.

Antiserum to purified virus of *Penicillium chrysogenum* was prepared with the cooperation of Dr R. E. Bowen, Greenfield Laboratories, Eli Lilly and Company.

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