Effect of Polyoxin D on *Achlya radiosa*

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INTRODUCTION

The oomycetes have been characterized by the presence of cellulose and absence of chitin in their cell walls (Aronson, 1965; Bartnicki-Garcia, 1968; Dick, 1969). Recently, however, the presence of chitin was unequivocally shown in the oomycete *Apodachlya*, order Leptomitales (Lin & Aronson, 1970; Lin, Sicher & Aronson, 1976). Some evidence was also obtained for its presence in 10 isolates belonging to the orders Saprolegniales and Peronosporales (Dietrich, 1973, 1975), by the production of *N*-acetylglucosamine from the walls after treatment with a chitinase-enriched snail digestive juice preparation. However, the latter results have been questioned (Lin *et al.*, 1976) on the grounds that the enzyme preparation used might contain enzymes capable of liberating *N*-acetylglucosamine from substrates other than chitin.

Since it has been proposed that differences in the amounts of the presumed chitinous compound in the walls of oomycetes are important in the phylogeny of this group of fungi (Dietrich, 1973, 1975), further evidence regarding its chemical nature is needed.

Polyoxin D is a specific inhibitor of chitin synthase (Endo, Kakiki & Misato, 1970) and it has been used to investigate the presence and role of chitin in different fungi (Keller & Cabib, 1971; Bartnicki-Garcia & Lippman, 1972; Gooday, 1972). In this paper we report the effect of polyoxin D on the oomycete *Achlya radiosa*.

METHODS

Chemicals. Polyoxin D was a kind gift from Dr Saburo Suzuki (Rikagaku Kenkyusho, Japan). The antibiotic migrated as a single component in the chromatography system described by Isono *et al.* (1967) and had the same R_F as a pure sample of polyoxin D provided by Dr Nobuo Sasaki (Haken Chemical Co., Japan). [G - ^{14}C]Glucose (66 mCi mm $^{-1}$) was from the Département de Biologie, CEA, France; *N*-[1- ^{14}C]-acetyl-D-glucosamine ([^{14}C]GlcNAc; 5.0 mCi mm $^{-1}$) was from ICN Isotope & Nuclear Division, California, U.S.A.

Organism and culture methods. *Achlya radiosa* Maurizio, strain spc30 (= *Achlya pseudoradiosa* Rogers & Benecke) was from the live collection of the Instituto de Botânica de São Paulo. It was maintained and grown in Seymour's MSPS agar medium (Seymour, 1970). Discs of inoculum were transferred either to the centre of Petri dishes of the same medium or to 125 ml Erlenmeyer flasks containing 50 ml of Seymour's MSPS liquid medium (Seymour, 1970). Polyoxin D and other compounds to be added were sterilized separately by membrane filtration (Millipore filters) and mixed with the autoclaved media at 50 to 60 °C. The media were prepared to give the same final concentrations of constituents after the additions. Cultures were incubated at 22 °C in the dark, without agitation.

Growth measurements. Experiments were carried out with three replicates per treatment. The diameter of agar-grown cultures was measured daily to the nearest millimetre, the diameter of the original inoculum being subtracted. For experiments in liquid medium, the cultures were harvested by centrifugation at 1000 g for 20 min in the cold and washed by centrifugation in cold distilled water three times. The washed mycelia were freeze-dried and weighed, the dry weight of the initial inoculum (mean of 10 discs) being subtracted.

Colonies of *A. radiosa* on agar plates and on agar-coated slides were used to examine the effects of polyoxin D on hyphal morphology. The plates and slides were prepared with the same medium as used for growth analysis. The slides were covered with coverglasses; the inoculum was placed on the periphery of the coverglass and the whole system was maintained in a moist atmosphere during the growth period. Polyoxin D

Table 1. *Effect of polyoxin D on growth and wall composition in A. radiosa*

Treatment	Hyphal diameter* ± s.d. (μm)	Mycelial dry wt† ± s.d. (mg)	Wall composition‡ (%, w/w, of total)	
			Reducing sugars	Hexosamines
Control	19.7 ± 2.2	18.2 ± 1.8	64.8	2.7
+ Polyoxin D (100 μg ml ⁻¹)	24.9 ± 3.0	23.2 ± 2.2	68.4	1.3
Inhibition by polyoxin D (%)	—	—	—	51.9

* Measured 0.5 mm from tips of main hyphae grown in agar medium; mean of 25 determinations.

† Mycelium grown for 4 d in liquid medium, transferred and grown for 48 h in fresh medium with or without polyoxin D; mean of 5 determinations.

‡ After hydrolysis with 1 M-HCl for reducing sugars and 6 M-HCl for hexosamine determinations. Standard chitin produced 97.8 % hexosamine under the latter conditions.

Table 2. *Effect of polyoxin D on [¹⁴C]GlcNAc incorporation and GlcNAc content in walls of A. radiosa*

Mycelium was grown for 72 h in MSPS containing [¹⁴C]GlcNAc with or without glucose in the presence of polyoxin D (100 μg ml⁻¹). Data are expressed as a percentage of the values obtained in controls grown under the same conditions but without polyoxin D.

Medium	Incorporation of [¹⁴ C]GlcNAc (% of control)		Wall GlcNAc content* (% of control)	
	Whole mycelium	Wall	[¹⁴ C]GlcNAc	Total GlcNAc
MSPS complete	71	49.2	10	56
MSPS without glucose	63	47	50	50

* Measured after wall hydrolysis with a chitinase preparation [specific activity: 0.5 mg GlcNAc liberated (mg protein)⁻¹ (24 h)⁻¹].

(final concentration 100 μg ml⁻¹) was either added to the medium prior to inoculation or applied to small areas on the periphery of the colony. Measurements of hyphae were made with a stage micrometer slide.

Growth in the presence of radioactive substrates. Duplicate cultures were grown for 4 d in liquid medium, harvested and washed under aseptic conditions, and then transferred to fresh medium with the appropriate additions of polyoxin D, [¹⁴C]glucose or [¹⁴C]GlcNAc. The cultures were grown in these media for different periods of time and then harvested. The [¹⁴C]glucose-grown cultures were washed twice with a 0.3 % (w/v) solution of unlabelled glucose and the [¹⁴C]GlcNAc-grown cultures were washed twice with a 0.3 % (w/v) solution of unlabelled GlcNAc before washing with cold distilled water. The disappearance of radioactivity from the medium and the amount found in the mycelium were measured.

Extraction and analysis of the cell walls. The washed liquid-grown mycelia were frozen and homogenized in a VirTis homogenizer at high speed and then subjected to sonic treatment for 5 min intermittently at 20 kHz. In tracer experiments, small portions of the resulting suspensions were rapidly pipetted to scintillation vials containing 10 ml dioxan scintillation fluid (5 g 2,5-diphenyloxazole, 100 g naphthalene, and 1,4-dioxan to 1 litre) and counted in a Beckman model LS 100 scintillation counter. The suspensions were centrifuged at 1000 g for 20 min, the precipitate was resuspended in 20 ml water and the whole procedure was repeated until the precipitate consisted exclusively of wall fragments, as indicated by cotton blue staining (Dietrich, 1975). Total insoluble protein (Lowry *et al.*, 1951) and total carbohydrate (Umbreit, Burris & Stauffer, 1964) were measured in the washed wall preparations. Radioactivity in labelled walls was determined by counting 2 mg dried walls suspended in 10 ml toluene scintillation fluid [4 g 2,5-diphenyloxazole, 0.05 g 1,4-di-2-(5-phenyloxazolyl)benzene, and toluene to 1 litre].

The freeze-dried walls were subjected to acid hydrolysis as previously described (Dietrich, 1973, 1975) and to enzyme digestion using a chitinase (EC 2.4.1.16) preparation (Sigma) purified by affinity chromatography (Jeuniaux, 1957). This preparation did not release *N*-acetylglucosamine from glycoproteins or from hyaluronic acid, under the experimental conditions used. Walls (5 mg) were incubated with chitinase (10 μg ml⁻¹) at 30 °C for 72 h with continuous shaking. Reducing sugars (Nelson, 1944), hexosamines (Blix, 1948) and

N-acetylglucosamine (Reissig, Strominger & Leloir, 1955) were determined in wall hydrolysates. Products were separated by paper chromatography (Chargaff, Levine & Green, 1948) and thin-layer chromatography (Schimid, Kebrle & Karrer, 1952), followed by staining with silver nitrate reagent (Trevelyan, Procter & Harrison, 1950) or 0.2% (w/v) ninhydrin in acetone at 105 °C. In tracer experiments, paper chromatograms were cut lengthwise into strips (corresponding to each chromatographed sample) and scanned in a Packard scanning apparatus. The regions corresponding to peaks in radioactivity were cut out and counted in toluene scintillation fluid.

RESULTS

Polyoxin D (100 µg ml⁻¹) did not affect the radial growth of *Achlya radios*a in agar medium. Microscopical observations indicated that there was an increase in hyphal diameter (Table 1) along main hyphae, although tip bursting did not occur. The addition of polyoxin D to cultures during growth in liquid medium resulted in an increase in dry weight and a decrease in wall weight of the mycelium. The walls of polyoxin D-treated mycelia produced less glucosamine than the controls (Table 1).

Polyoxin D promoted a slight increase in glucose incorporation from the medium during 24 h exposure of the mycelium to the antibiotic in the presence of [¹⁴C]glucose (not shown). It caused a decrease in the incorporation of [¹⁴C]GlcNAc from the medium and a decrease in the incorporation of this compound into the cell wall (Table 2). Similar effects were observed if unlabelled glucose was present in the growth medium during the treatment.

Enzymic hydrolysis of the [¹⁴C]GlcNAc-labelled walls with chitinase showed that there was considerably less ¹⁴C in walls from polyoxin D-treated mycelium (Table 2). The total amount of GlcNAc liberated by hydrolysis with chitinase was also lower in walls from polyoxin D-treated mycelium, as indicated by colorimetric determination of GlcNAc in these hydrolysates (Table 2). The identity of GlcNAc in wall hydrolysates was determined by paper and thin-layer chromatography, the latter being followed by autoradiography.

DISCUSSION

Polyoxin D promoted enlargement of hyphal diameters in *Achlya radios*a although colony growth and tip integrity were not significantly affected by the antibiotic. Also, cultures grown in the presence of polyoxin D incorporated less *N*-acetylglucosamine in their walls than did untreated cultures.

Polyoxin D is a strong inhibitor of chitin synthesis and acts as a specific competitive inhibitor of chitin synthase in different fungi (Endo *et al.*, 1970; Keller & Cabib, 1971; Bartnicki-Garcia & Lippman, 1972; Gooday, de Rousset-Hall & Hunsley, 1976). The observation that it reduces the synthesis of an *N*-acetylglucosamine-containing compound in the cell walls of *A. radios*a constitutes further evidence for the suggested chitinous nature of this compound (Dietrich, 1973, 1975).

As previously observed by Isono *et al.*, (1967), and shown here, polyoxin D does not inhibit growth of oomycetes to the same extent as that of higher fungi. Since the walls of the oomycetes are composed mostly of glucans (Bartnicki-Garcia, 1968; Sietsma, Eveleigh & Haskins, 1969; Novaes-Ledieu, Jiménez-Martínez & Villanueva, 1967), the inhibition of chitin synthesis by polyoxin D might not cause growth inhibition or the hyphal tip bursting effects observed in fungi known to contain chitin (Bartnicki-Garcia & Lippman, 1972). It could, however, explain the enlargement of hyphal diameter. This effect suggests that the role of chitin in *A. radios*a, and possibly in other oomycetes, may be related to the flexibility of the hyphae.

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