Lysis of the Cyanobacterium *Anabaena flos-aquae* by Antibiotic-producing Fungi

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The β -lactam antibiotic cephalosporin C has been extracted and partially purified from liquid cultures of the fungi *Emericellopsis salmosynnemata* and *Acremonium kiliense*. The ability of these and certain other fungi to lyse cyanobacteria is attributable to the production of this antibiotic. This is supported by the similarities, seen in light and electron microscopic preparations, of *Anabaena flos-aquae* cells exposed to pure cephalosporin C and to fungal exudates.

INTRODUCTION

Several types of microbial pathogen that lyse cyanobacteria have been previously isolated and studied, including viruses, actinomycetes and bacteria (Stewart & Daft, 1976, 1977). However, with the exception of the Chytrids (see Canter, 1972), little is known about fungal lysis of cyanobacteria.

In a previous paper (Redhead & Wright, 1978) we described the isolation and characterization of several filamentous fungi that lysed a variety of cyanobacteria. Certain of these fungi, identified as *Emericellopsis minima*, *Em. salmosynnemata*, *Acremonium charticola* and *Acrem. kiliense*, produced heat-stable diffusible extracellular materials which inhibited growth and lysed several species of cyanobacteria. Evidence was presented that the anti-cyanobacterial substance could be a cephalosporin antibiotic.

Cyanobacteria have previously been reported to be lysed by antibiotics or antibiotic-like materials produced by bacteria (Granhall & Berg, 1972; Reim *et al.*, 1974; Burnham *et al.*, 1976), actinomycetes (Safferman & Morris, 1962, 1963) and fungi (Safferman & Morris, 1962). However, in few cases has the identity of the anti-cyanobacterial agent been established. The present study was undertaken to investigate in more detail the lysis of cyanobacteria by filamentous fungi. This paper reports on the lysis of a strain of *Anabaena flos-aquae* by *Acremonium* and *Emericellopsis* isolates and describes the isolation and identification of a lytic agent.

METHODS

Organisms and culture methods. The axenic culture of *Anabaena flos-aquae* (1403/13a) was obtained from the Culture Centre of Algae and Protozoa, Cambridge. Batch liquid cultures of this cyanobacterium (100 ml in 250 ml Erlenmeyer flasks) were grown from 2 ml inocula in mineral medium D plus H₂ micro-elements solutions (K & M medium) of Kratz & Myers (1955). Cultures were incubated at 30 °C on an orbital shaker (90 rev. min⁻¹) and continuously illuminated (1800 lux) by warm-white fluorescent light. For *A. flos-aquae* cultures in Petri dishes, the same medium, incorporating 1.5 % (w/v) purified agar (Oxoid), was uniformly surface-inoculated with 1 ml from batch cultures (7 d) and incubated under the same standard temperature and light regimes. These conditions were also used for maintenance of the cyanobacteria on agar slants.

The fungal cultures used were the cyanobacterial-lysing fungi *Emericellopsis minima* (ALF-6), *Em. salmosynnemata* (ALF-21) and *Acremonium kiliense* (ALF-62), previously isolated (Redhead & Wright,

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1978) and deposited in the American Type Culture Collection with the accession numbers ATCC 36892, 36893 and 36894, respectively. The fungi were maintained at 30 °C on nutrient agar (Oxoid) and also by fortnightly subculture on lawns of *A. flos-aquae* incubated at 30 °C under constant illumination (1800 lux).

Standard fungal inocula were prepared by applying material from fungal plaques on *A. flos-aquae* lawns to the surface of 100 ml malt agar slants in 500 ml flat bottles which were then incubated for 5 d at 30 °C. Fungal growth was removed from the agar by shaking vigorously for 2 min with 20 ml sterile distilled water containing glass beads (4 mm diam.). The resulting suspension was decanted and diluted 10-fold with sterile distilled water for immediate use as a standard inoculum which contained between 150 and 200 µg dry wt fungal material ml⁻¹.

Light microscopy. In addition to wet mounts containing mixed antagonist and cyanobacterial suspensions, two types of slide culture were used. Thinly-poured K & M agar plates were seeded with cyanobacterial suspension, inoculated with antagonist cultures and pieces of the agar were removed at intervals and placed on microscope slides for immediate examination. Glass chambers (13 × 9 mm × 1 mm deep) made on glass microscope slides were also used. The chamber was filled with molten K & M agar which was allowed to solidify before a mixture of the cyanobacterial and fungal suspensions was placed on the surface and the chamber was enclosed by a coverslip sealed at the edges with paraffin wax. This system could be incubated at 30 °C for up to 5 d without desiccating. Samples were examined at various stages during lysis of the cyanobacteria using a Leitz Wetzlar Orthoplan microscope with phase optics.

Electron microscopy. All samples were fixed in glutaraldehyde (3 %, v/v). Material for scanning electron microscopy was critical-point dried, sputter-coated with gold and examined in a Stereoscan S4 scanning electron microscope (Cambridge Scientific Instruments) at 10 kV.

Material for transmission electron microscopy was treated with osmium tetroxide (2 %, w/v) and embedded in TAAB (Reading) resin. Sections were cut on a Reichert OmU 3 ultramicrotome, stained with uranyl acetate and Reynolds' lead citrate and examined in a Jeol JEM 100 CX transmission electron microscope at 80 kV.

Extraction and partial purification of cephalosporin C. Nutrient broth (100 ml in 250 ml Erlenmeyer flasks) inoculated with fungal suspension (1 ml) was incubated at 30 °C with shaking (100 rev. min⁻¹) for 4 d. Cephalosporin C was extracted from 200 ml batches of such cultures by a method similar to that described by Trown *et al.* (1962) based on column chromatography using Amberlite IRA-400 (acetate form; 100 to 200 mesh), Amberlite CG4B (acetate form; 100 mesh) and Dowex 50 (H⁺ form; X8) resins. This procedure yields a crude preparation of cephalosporin C (sodium salt).

Thin-layer chromatography (t.l.c.). Chromatograms were run on thin-layer plates (Kieselgel HF₂₅₄₊₃₆₆, Typ. 60, Merck, 0.4 mm thick) which were developed using 1-butanol/ethanol/water (4:1:5, by vol.). Cephalosporin C spots were visualized under u.v. light (220 to 300 nm).

RESULTS

Observations on cyanobacterial lysis

Microscopic observations of fungal plaques on *A. flos-aquae* lawn cultures showed an outer region devoid of fungal hyphae in which many cyanobacterial cells had lysed. Nearer to the centre of the plaque, fungal mycelium was intermingled with cyanobacterial cells, most of which were undergoing lysis. The centre of the plaque contained fungal mycelium and conidia with small amounts of cyanobacterial debris.

The sequence of cyanobacterial lysis was also studied using mixtures of *A. flos-aquae* cells and fungal conidia in slide culture. After 2 h incubation at 30 °C many fungal conidia had begun to germinate. Within 6 h some cyanobacterial cells which were not in contact with the fungi began to show lysis. Vegetative cells of cyanobacterial filaments became brown-grey, and lost their distinct outline. After 18 h the germinated conidia had produced hyphae up to 80 µm long. Cells within cyanobacterial trichomes had darkened, the cell walls were indistinct and spaces appeared between adjacent cells. Cyanobacterial filaments then began to break up and after 30 h had lost their linear arrangement, green colour and definition. Heterocysts did not appear to be damaged at this stage, but they eventually lost their contents and appeared as 'ghosts'.

Anabaena flos-aquae cells incubated alone in slide culture were still normal after 30 h. Conidia incubated on K & M agar without cyanobacteria showed comparatively limited germination and hyphal growth.

Material for electron microscopy studies was taken from the edge of the peripheral zone

Fig. 1. Scanning electron micrographs: (a) control *Anabaena flos-aquae* filaments grown on K & M agar; (b) *A. flos-aquae* filaments entangled with ALF-62 (*Acremonium kiliense*) hyphae, from liquid K & M culture; (c) *A. flos-aquae* cells showing pitting of the cell wall (arrowed), from the periphery of an ALF-62 plaque on *A. flos-aquae* lawn culture. All bar markers represent 2 μm .

of ALF-62 (*Acrem. kiliense*) plaques on *A. flos-aquae* lawns, untreated lawns, *A. flos-aquae* cultures (static) inoculated with ALF-62 and control *A. flos-aquae* liquid cultures.

Under the scanning electron microscope, control *A. flos-aquae* (Fig. 1a) appeared as long, regular filaments of smooth-walled cells. The entrapment of cyanobacterial filaments by ALF-62 hyphal growth in liquid culture is shown in Fig. 1(b). Scanning electron micrographs of cyanobacteria from the edge of ALF-62 plaques (Fig. 1c) showed fragmentation of the cyanobacterial filaments, with the cells in disarray. The cell walls appeared rough and in some cases were deeply pitted.

Fig. 2. Transmission electron micrographs: (a) partially lysed *Anabaena flos-aquae* cells, from an ALF-62 (*Acremonium kiliense*) plaque on *A. flos-aquae* lawn culture, showing folds of thylakoid membrane (tm); (b) normal *A. flos-aquae* filament; (c) section of a normal *A. flos-aquae* vegetative cell showing L₁, L₂, L₃ and L₄ wall layers. Bar markers represent 1 μ m in (a) and (b), 0.1 μ m in (c).

Transmission electron microscopy showed that ALF-62 had caused loss of the wall of *A. flos-aquae* vegetative cells (Fig. 2a). Normal vegetative cells (Fig. 2b) had a regular appearance, were surrounded by a smooth wall and contained various inclusions. Detail of the wall structure (Fig. 2c) reveals the four layers (L₁, L₂, L₃ and L₄) which are typical of cyanobacteria (Jost, 1965; Lang, 1968). After treatment with ALF-62, the wall lost its definition and the electron-dense L₂ layer could no longer be distinguished. The wall eventually disappeared, leaving protoplasts containing membranous folds surrounded by the plasmalemma (Fig. 2a). These protoplasts were very similar to those produced by

Myxobacter CP-1 attack on *Nostoc piscinale* (Daft & Stewart, 1973). Lysis of the proto-plasts produced folds of membranous material and the release of lipid droplets.

Lysis of *A. flos-aquae* by the fungi in liquid culture was greater under static than shaken conditions. Incubation in darkness and variation in the age of the *A. flos-aquae* cultures (up to 25 d) did not affect their susceptibility to lysis. The extent of lysis decreased with temperature but all three fungi lysed *A. flos-aquae* cultures at temperatures as low as 15 °C and ALF-62 had significant lytic activity even when grown at 10 °C.

Identification of cyanobacterial-lysing agent

Microscopic examination of material from the periphery of clear zones produced by the application of solutions of cephalosporin C (40 µg ml⁻¹) supported earlier evidence (Redhead & Wright, 1978) that this antibiotic could be the anti-cyanobacterial substance produced by the fungal antagonists. Cyanobacterial damage was very similar to that observed at the edge of *Emericellopsis*- and *Acremonium*-induced plaques. Scanning and transmission electron micrographs showed that vegetative cyanobacterial cells treated with cephalosporin C exhibited effects apparently identical to those from the edge of ALF-62 plaques (Figs 1 c and 2 a).

Solutions were produced by immersing 30 agar cores (0.5 cm diam.), removed from 1 cm in front of ALF-62 growth (4d) on nutrient agar plates, in 10 ml of sterile water for 24 h. Samples (0.02 ml) formed clear zones 1 cm in diameter in *A. flos-aquae* lawns after 5d incubation. These active solutions produced faint u.v. quenching spots which co-chromatographed (R_F 0.24) with standard cephalosporin C on t.l.c. plates. These results imply that small amounts of cephalosporin C, originating from the ALF-62 growth, had been extracted from the agar cores. Control solutions, using cores from uninoculated plates, showed no lytic activity or chromatography spots.

Extraction of cephalosporin C from fungal cultures

The final partially purified preparation, obtained after column chromatography of ALF-21 and -62 nutrient broth culture (4d) liquids, formed clear zones when 0.1 ml was applied to *A. flos-aquae* lawns. The average diameters of the zones after 5d incubation were 2.0 cm (ALF-21) and 3.5 cm (ALF-62). Microscopic examination of material from the edge of these plaques showed a cyanobacterial response similar to that caused by ALF-6, -21, -35 and -62 isolates and by authentic cephalosporin C.

Samples of the active preparations co-chromatographed with authentic cephalosporin C (R_F 0.24). It can therefore be concluded that ALF-21 (*Em. salmosynnemata*) and ALF-62 (*Acrem. kiliense*) produced cephalosporin C when grown in pure culture in nutrient broth and probably also on nutrient agar and when grown in the presence of cyanobacteria, such as *A. flos-aquae*, on agar plates, in slide culture and liquid culture.

DISCUSSION

Evidence was presented previously (Redhead & Wright, 1978) suggesting that the extra-cellular cyanobacterial-lysing activity of the *Emericellopsis* and *Acremonium* isolates is associated with the antibiotic cephalosporin C. The similarities between the effects of pure cephalosporin C and the exudates of *Acrem. kiliense* (ALF-62) on *A. flos-aquae* cells also support this suggestion. The extraction of lytic substances from agar cores containing fungal exudates which co-chromatographed with cephalosporin C, together with the extraction and partial purification of cephalosporin C from *Em. salmosynnemata* and *Acrem. kiliense* culture liquids, have confirmed that these fungi produce cephalosporin C. There seems little doubt that the lytic and inhibitory actions of these fungi on cyanobacteria are caused by this antibiotic.

The lysis of cyanobacteria by micro-organisms, other than streptomycetes, which produce

antibiotic-like materials has been previously noted. Granhall & Berg (1972) reported that two *Cellvibrio* strains produced antibiotic-like substances that caused lysis of *Anabaena inaequalis* vegetative cells and Reim *et al.* (1974) attributed the cyanobacterial-lysing activity of their *Bacillus brevis* isolate to the production of an antibiotic, probably gramicidin S.

Cephalosporin C is known to be produced by several *Emericellopsis* species (Elander *et al.*, 1960; Korzybski *et al.*, 1967) and by strains of *Acremonium chrysogenum* (Trown *et al.*, 1962). It is believed to act in the same way as other β -lactam antibiotics by inhibiting the enzymes involved in the final stage of peptidoglycan biosynthesis, the attachment of nascent peptidoglycan to pre-existing peptidoglycan by cross-linking between the peptide side-chains. Spratt (1978) has summarized the available information on the mechanism of action of the β -lactam antibiotic penicillin on bacteria. β -Lactam antibiotics may cause changes in cell morphology, inhibition of cell division or a reduction in the peptidoglycan layer. Most of these effects eventually lead to the formation of sphaeroplasts or protoplasts which lyse in normal growth media.

β -Lactam antibiotics, such as cephalosporin C, can be expected to act similarly on enzymes involved in the final stages of biosynthesis of peptidoglycan in the L_2 layer of the cell wall in cyanobacteria. As this layer is the main rigid structural component of the cyanobacterial cell wall, the action of such antibiotics would presumably cause some variation in cell shape, and probably the production of sphaeroplasts or protoplasts. Such effects were evident in electron micrographs of *A. flos-aquae* cells exposed to both cephalosporin C solutions and to *Acrem. kiliense* (ALF-62) exudates.

Cyanobacteria are known to be very sensitive to penicillin, $0.02 \mu\text{g ml}^{-1}$ being sufficient to inhibit the growth of *Anacystis nidulans* (Kumar, 1964). The production of clear zones in lawns of *A. flos-aquae* by the application of 0.02 ml of a $10 \mu\text{g ml}^{-1}$ cephalosporin C solution (Redhead & Wright, 1978) further establishes the sensitivity of cyanobacteria to β -lactam antibiotics.

The *Emericellopsis* and *Acremonium* isolates probably produce small quantities of cephalosporin C, which affect cyanobacterial cells only when they are in close proximity to the fungus, as occurs in the aggregates produced in agitated liquid culture. Several cyanobacteria, including *Anabaena* spp., produce sheaths of complex polysaccharides. It is possible that cephalosporin C produced by fungi in contact with or close to the sheath is concentrated to antagonistic levels by diffusion into this layer. The discovery that some cyanobacteria produce β -lactamase enzymes (Kushner & Breuil, 1977) which inactivate β -lactam antibiotics suggests that these cyanobacteria may have naturally encountered β -lactam antibiotics.

Most of the experimental results in this study were obtained *in vitro*. However, it appears that certain *Emericellopsis* and *Acremonium* species may be present in natural cyanobacterial habitats (Redhead & Wright, 1978) and the cephalosporin C produced by these fungi could lyse the cyanobacteria. The contribution of these fungi to the rapid disappearance of cyanobacterial blooms warrants further study.

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