The Barrier-Ring Plate Technique for Studying Extracellular Enzyme Diffusion and Microbial Growth in Model Soil Environments

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A novel technique has been developed to study complex spatial interactions between micro-organisms, enzymes and their substrates in soil using barriers composed of soil and soil components inserted into agar plates. This has allowed the investigation of extracellular enzyme diffusion and microbial growth through soil-like but carefully controlled environments. Using 'barrier-ring plates' the effects of small quantities of soil and various soil components on endoglucanase and β -D-glucosidase diffusion was shown. Bentonite, with a relatively high unit surface area and a high cation exchange capacity, reduced the distance diffused by both enzymes. Kaolinite, a clay with a relatively low unit surface area and a low cation exchange capacity, had no effect while the colloidal-size ($< 2 \mu\text{m}$) clay-humic fraction separated from a silt loam soil reduced the distance diffused by endoglucanase by an amount intermediate between that of kaolinite and bentonite. The same barrier-ring plate technique was used to demonstrate how soil components differentially affect the radial growth of a cellulolytic *Streptomyces* sp. and *Trichoderma viride*, *T. koningii* and *Botryotrichum piluliferum*.

INTRODUCTION

The many difficulties and inaccuracies involved in measuring micro-organisms and their activities in soils have been well documented (Jenkinson & Ladd, 1981) and the value of such indices as total counts, biomass, ATP, respiration rates and dehydrogenase activity is limited by the methodology adopted and by the heterogeneous nature of soil. Of all the soil components, clays have a dominant influence on microbial activity because of their relatively high cation exchange capacity, extensive surface areas and capacity to retain water and humic matter (Burns, 1983). There have been numerous attempts to quantify the effect of clays on some aspects of microbial growth (Filip, 1973; Marshall, 1976; Sorensen, 1981). In some experiments (Filip *et al.*, 1972) direct microbial contact with the clay surface was avoided by enclosing the clay in a membrane before its immersion in the culture vessel. Other workers have studied the microbial availability of carbon, nitrogen and energy substrates adsorbed to soil particulates (Stotzky, 1980; Marshman & Marshall, 1981) and the activity of microbial enzymes attached to clays (Theng, 1979; Ottow *et al.*, 1983). All approaches are designed to produce a laboratory system that in some way mimics the natural soil environment yet can be manipulated in a predictable and reproducible way.

Cellulolytic soil micro-organisms are faced with a number of problems. The polymeric and physically complex structure of cellulose, together with the presence of lignin, pectins and hemicelluloses, means that individuals or communities of cellulolytic micro-organisms are reliant on a suite of extracellular enzymes for the release of soluble growth and energy substrates (Burns, 1982). However, in general, extracellular enzymes are rapidly inactivated in soil (Zantua & Bremner, 1976) by a combination of adsorption to clays, chemical denaturation and

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Abbreviations: CMC, carboxymethylcellulose; CSF, colloidal-size fraction; RGR, radial growth rate.

proteolysis. Furthermore, substrates in soil are unevenly distributed, both in time and space, and exist in microsites that are physically or chemically unsuitable for enzymic hydrolysis.

Strategies for the successful hydrolysis of exogenous carbon and nitrogen polymers in natural environments have been discussed (Cohen, 1980; Burns, 1983) and it is apparent that mechanisms of extracellular enzyme regulation, revealed by studies of axenic cultures *in vitro* and in the absence of adsorptive surfaces, are inadequate when applied to soil.

In order to improve our understanding of extracellular enzymes in soil, we have modified the radial or agar cup-plate technique first described by Dingle *et al.* (1953). The resulting 'barrier-ring plates' were used to demonstrate how soil and soil components affect (i) the diffusion of extracellular cellulases and the hydrolysis of remote substrate; and (ii) the radial growth of cellulolytic micro-organisms.

METHODS

Micro-organisms and growth conditions. Three cellulolytic fungi, *Trichoderma viride* Pers. ex. S. F. Gray aggr., *T. koningii* Oud aggr. (Rifai, 1969) and *Botryotrichum piluliferum* Sacc. March (Commonwealth Mycological Institute) and an unidentified cellulolytic *Streptomyces* species were isolated from a silt loam soil (Hamble Series; Soil Survey Record no. 14) and maintained at 4 °C on minimal medium containing 1% (w/v) FMC Corporation Avicel PH105 (Honeywell & Stein, Wallington, UK) as the sole carbon source. The minimal medium was that of Sternberg (1976) for *T. viride*, *T. koningii* and *B. piluliferum* and that of Berg *et al.* (1972) for the *Streptomyces* sp.

Cellulase enzyme preparations. The sources of crude 'cellulase' preparations were a *T. viride* commercial 'cellulase' (BDH; 1 mg ml⁻¹ in 0.1 M-acetate buffer pH 5.0), and culture filtrate from the *Streptomyces* sp. Both preparations contained endoglucanase and β -D-glucosidase activities. The *Streptomyces* sp. was grown in medium (Ishaque & Kluepfel, 1980) containing 1% (w/v) carboxymethylcellulose 7 L (CMC; Hercules Inc., Wilmington, Del., USA) on a rotary shaker at 30 °C. The cultures were harvested after 48 h by centrifugation (38000 g, 10 min) and the supernatant liquid was filtered through a HT-200 Tuffryn 0.2 μ m bacteriological filter (Gelman). The β -D-glucosidase was a commercial preparation from sweet almonds (Koch-Light; 1000 units mg⁻¹).

Enzyme assays. Activity against CMC (endoglucanase, EC 3.2.1.4) was measured at 40 °C for 30 min by shaking samples (0.2 ml) of crude enzyme preparation with 2 ml of a 2% (w/v) solution of CMC in 0.1 M-acetate, buffered to the pH of maximum activity (i.e. pH 5.0 for *T. viride*; pH 5.75 for the *Streptomyces* sp.). The reaction was terminated by addition of 3 ml dinitrosalicylic acid reagent and heating to 100 °C, as described by Miller *et al.* (1960). Under these conditions, the endoglucanase activity of *T. viride* was 35 μ mol reducing sugars h⁻¹ ml⁻¹ and of *Streptomyces* sp. 23 μ mol reducing sugars h⁻¹ ml⁻¹.

β -D-Glucosidase (EC 3.2.1.21) activity was measured at 30 °C by incubating enzyme samples (0.5 ml) with 1 ml 25 mM-*p*-nitrophenyl- β -D-glucopyranoside (*p*NPG) and 1 ml 0.1 M-acetate buffer (pH 5.0 for *T. viride* and pH 5.75 for the *Streptomyces* sp.) for 4 h. The reaction was terminated by the addition of 2 ml 0.5 M-Tris pH 10.5 and the *p*-nitrophenol (*p*NP) product was determined spectrophotometrically at 400 nm. Under these conditions the β -D-glucosidase activity of *T. viride* was 1.04 μ mol *p*NP h⁻¹ ml⁻¹ and of the *Streptomyces* sp. 0.026 μ mol *p*NP h⁻¹ ml⁻¹.

Barrier-ring plates. Plates for measuring the unimpeded diffusion of endoglucanase through agar were prepared using 20 ml of the appropriate minimal medium containing 0.5% (v/v) CMC 9M8F and 1% (w/v) purified agar (Oxoid). The CMC agar was pipetted at 48 °C into standard 9 cm Petri dishes and, after setting, dried for 15 min in a laminar airflow cabinet. A central well (10 mm diameter) was made using a sterile cork borer. The 'cellulase' solution (100 μ l) was added to the well and the plates were incubated in a humid atmosphere at 25 °C for 72 or 120 h. The extent of diffusion of endoglucanase from the central well through the solid medium was detected by precipitating any unhydrolysed CMC with 1% (w/v) cetyltrimethylammonium bromide for 10 min (Hankin & Anagnostakis, 1977) and averaging the diameters of the cleared zones measured in two directions at right angles to each other. Three replica plates were prepared for each treatment.

Both *p*NPG and *p*NP diffuse rapidly through agar gels. Therefore to determine the extent of β -D-glucosidase diffusion, CMC agar plates were brushed with 25 mM-*p*NPG after 120 h and incubated at 30 °C for an additional hour, after which the pH was raised by flooding with 0.5 ml 0.5 M-NaOH and the diameter of the yellow (*p*NP-containing) zone measured.

Barrier-rings were introduced to the plates by removing a ring of agar 7.5 mm wide (inner and outer diameters 11.5 mm and 26.5 mm respectively, volume 1.5 ml) surrounding the central well and replacing it with either minimal medium or minimal medium containing bentonite (Fisons; 0.5–5.0%, w/v), kaolinite (Fisons; 0.5–5.0%, w/v) or the colloidal-size fraction (CSF) (i.e. clay and insoluble humic matter) (0.5–18%, w/v) of a silt loam soil (sand, 9%; silt, 72%; clay, 19%; pH 6.4; C, 2%; N, 0.5%; Lethbridge & Burns, 1976). The CSF (diameter < 2 μ m) was separated from a sonicated soil suspension following differential sedimentation of sand (diameter 0.05–0.2 mm) and silt in a 1 litre cylinder of water (Avery & Bascomb, 1974). After 14 h 52 min the CSF was syphoned

off and collected by centrifugation (10000 g, 30 min). Plates containing barrier-rings of sterilized sand (about 2 g) or silt loam soil (about 1 g) were prepared by filling the ring with the autoclaved or gamma-irradiated (10 MRad) sand or soil, and then adding molten minimal medium to ensure continuity. Bentonite (cation exchange capacity 600 $\mu\text{equiv. g}^{-1}$) and kaolinite (cation exchange capacity 20 $\mu\text{equiv. g}^{-1}$) were chosen as representing the range of adsorptive potentials present in soil clays. Carbonate and organic matter were removed from the CSF (cation exchange capacity 148 $\mu\text{equiv. g}^{-1}$) using sodium acetate and H_2O_2 (Avery & Bascomb, 1974).

Radial growth and radial growth rates of the fungal and actinomycete isolates were studied on plates, with and without barrier-rings, from which the core of agar in the centre had not been removed. The centres of the plates were inoculated with either a 10 μl drop of a *Streptomyces* sp. spore suspension which was prepared by flooding a culture slope with 5 ml sterilized minimal medium, or a small core (diameter 3 mm) taken from just behind the margin of an advancing fungal colony. Plates were incubated at 25 °C in a humid atmosphere. Three replicate plates for each treatment were used and radial growth was recorded at intervals by measuring the colony diameter in two directions at right angles to each other and taking the mean of the total. Radial growth rates, defined as the increase in colony diameter per unit time, were calculated using linear regression analysis.

RESULTS AND DISCUSSION

Enzyme diffusion in barrier-ring plates

For the 'cellulase' preparations from both *Trichoderma viride* and *Streptomyces* sp. the diameter of the hydrolysis zones produced by the endoglucanase enzymes in the CMC diffusion plates after 72 h was proportional to the log concentration of the initial crude enzyme preparations added to the central well. The range of enzyme concentrations tested was 100–50000 $\mu\text{g ml}^{-1}$ ($\equiv$ 10–5000 μg per 100 μl in the well) for the *T. viride* 'cellulase' preparation (Fig. 1) while for the *Streptomyces* sp. the concentration range was from undiluted filtrate to a dilution of 1×10^{-2} . Not surprisingly, temperature, depth of agar and volume of enzyme sample added to the well influenced the rate of diffusion of endoglucanase and the diameter of the hydrolysis zone after 72 h. The presence of a barrier-ring containing CMC-free minimal medium caused a small increase (about 4%) in the diameter of the hydrolysis zone after 72 h, although this increase was only significant ($t_{0.975}$) when more than three replica plates were used.

Kaolinite (up to 5%, w/v) in the barrier-ring of a CMC diffusion plate did not significantly affect the diffusion of either of the endoglucanase preparations (Fig. 2). In contrast, bentonite (0.5–5%, w/v) caused a reduction in the diameter of the hydrolysis zone produced by both endoglucanase preparations (Fig. 3). At 0.5% (w/v) bentonite the diameter of the hydrolysis zone due to the *Streptomyces* sp. endoglucanase was reduced by 25% whilst that of the *T. viride* endoglucanase was reduced by 11%. This difference may be due to the different molecular sizes and diffusibility of the endoglucanases, the associated impurities (proteins and others) in the crude preparations or the different adsorptive properties of the enzymes in relation to the clay. At the higher concentrations of bentonite (i.e. 2.5 and 5.0%, w/v) a small clearing zone, 2–3 mm wide, was observed around the edge of the barrier ring even in the absence of enzyme; this was probably due to the adsorption of CMC substrate by bentonite.

Sand in the barrier-ring did not reduce the diameter of the hydrolysis zone although the standard deviation of measurements from replica plates was increased from 0.5 mm to 1.5 mm. The effect of silt loam soil in the barrier-ring was difficult to quantify initially because diffusion of coloured humic compounds from the soil into the surrounding agar masked any hydrolysis zones which may have been produced. This problem was reduced by washing the soil with distilled water to remove soluble humates. Nevertheless, by increasing the enzyme concentrations added to the central well, so that the clearing zone in the control plates (containing no soil) was of a greater diameter than the zones coloured by humic compounds on the soil plates, it was shown that soil reduced the distance diffused by both endoglucanase preparations. When the CSF separated from soil was incorporated into the barrier-ring (1–18%, w/v), the diameter of the hydrolysis zone was reduced. The size of the hydrolysis zone decreased with increasing concentrations of CSF such that in plates containing 5% (w/v) and 18% (w/v) CSF the diameter of the hydrolysis zone produced by the endoglucanase preparation from *Streptomyces* sp. was reduced by 7% and 21% respectively when compared to the control

Fig. 1. Relationship between concentration of *Trichoderma viride* 'cellulase' in the well and the diameter of the cleared zone in CMC agar after 72 h. Clearing was due to endoglucanase diffusion.

Fig. 2. Diffusion of *Streptomyces* sp. endoglucanase after 120 h in CMC barrier-ring plates containing kaolinite. The dark zone outside the barrier-ring indicates CMC hydrolysis. 1, Control plate (CMC in barrier-ring); 2, no substrate (basal medium in barrier-ring); 3, kaolinite 0.1% (w/v); 4, kaolinite 0.5% (w/v); 5, kaolinite 1% (w/v); 6, kaolinite 5% (w/v).

(containing no CSF) plates. In plates containing 12.5% (w/v) CSF (equivalent to the amount of CSF in 1 g soil), the diameter of the hydrolysis zone was 37.5 mm. This reduction is equivalent to that caused by 0.3% (w/v) bentonite.

For β -D-glucosidase the diameter of the diffusion zone was again proportional to the log of the enzyme concentration added to the central well for the range of enzyme concentrations 25–1000 $\mu\text{g ml}^{-1}$. Bentonite (1%, w/v) when present in the barrier-ring significantly reduced the distance diffused by the enzyme (Fig. 4). No diffusion at all occurred at concentrations below 250 $\mu\text{g ml}^{-1}$. A similar reduction in the diameter of the yellow zone of released product *p*NP was seen when soil (1 g) was incorporated in the barrier-ring.

Fig. 3. Effect of bentonite (0.5–5.0%, w/v) on diffusion of endoglucanase of *Trichoderma viride* (●) and *Streptomyces* sp. (▲) after 120 h. Broken line signifies outer limit of barrier-ring.

Fig. 4

Fig. 5

Fig. 4. Effect of bentonite (1%, w/v) on diffusion of β -D-glucosidase after 120 h. ○, Control plates (CMC in barrier-ring); ●, bentonite. Broken line signifies outer limit of barrier-ring.

Fig. 5. Effect of bentonite on radial growth of *Streptomyces* sp. ○, Control plates (no bentonite); ▲, bentonite 0.5% (w/v); ●, bentonite 5% (w/v). Broken line signifies outer limit of barrier-ring.

Radial growth of soil isolates on barrier-ring plates

No difference in radial growth after 5 weeks was observed when the *Streptomyces* sp. was grown over a substrate (CMC)-free barrier-ring or over a barrier-ring containing kaolinite (0.5–5.0%, w/v). However, a reduction of 4.5, 12.0 and 18.0% in colony diameter after 5 weeks was seen when bentonite (0.5, 1.0 and 5.0%, w/v respectively) was incorporated in the barrier-ring. The radial growth rates (RGR) of colonies subsequent to their emergence from the barrier-rings were not further affected by the constituents of the barrier-rings (Fig. 5). By growing the *Streptomyces* sp. on plates in which the barrier-ring was moved outwards (i.e. inner and outer diameters 19.5 mm and 38.0 mm compared with 11.5 mm and 26.5 mm), it was possible to measure RGR prior to the colony traversing the barrier-ring and to confirm that the reduction in RGR occurred during passage through the ring and not before. The reduction in RGR is due not

Fig. 6

Fig. 6. Effect of CSF extracted from soil on radial growth of *Streptomyces* sp. ○, Control plates (no CSF); ▲, CSF 0.5% (w/v); ●, CSF 10% (w/v).

Fig. 7

Fig. 7. Effect of CSF without organic matter (CSF-O) extracted from soil on radial growth of *Streptomyces* sp. ○, Control plates (no CSF-O); ▲, CSF-O 0.5% (w/v); ●, CSF-O 10% (w/v).

only to bentonite restricting enzyme diffusion and the physical impedance of filamentous growth but also the adsorption of the products of CMC hydrolysis (Greenland, 1956) and thus the decline in available carbon. When silt loam soil was added to the barrier-ring, colonies after 5 weeks were slightly but consistently larger (about 4%) than the controls and this stimulation appeared to be independent of the method of soil sterilization. The nutritional value of soil organic matter to soil micro-organisms is well documented (Burns, 1982) and an increase (5.2–22.8%) in the average RGR (as determined by the slope of the regression line fitted to colony diameter measurements vs time) of the *Streptomyces* sp. over the whole plate was observed when the CSF separated from soil was incorporated (0.5–5.0%, w/v) in the barrier-ring (Fig. 6). However, when carbonate and organic matter were removed from the CSF prior to its incorporation (10%, w/v) in the barrier-ring, a temporary reduction in RGR was observed as the *Streptomyces* sp. traversed the ring although the RGR of the colonies after emergence from the ring were the same (i.e. the regression lines fitted to the points $x = 28$ –56 d in Fig. 7 were parallel but not identical). These two ways in which the components in the barrier-ring can influence radial growth, one being localized and the other disseminated, require that the data be analysed in two distinct ways and illustrate the difficulty in interpreting RGR data when growth is significantly affected in both ways at the same time, as appears to be the case with *T. viride*.

Bentonite and sand, when present in the barrier ring, caused a reduction in radial growth of the *T. viride* isolate, while on plates containing barrier-rings of substrate (CMC)-free medium, kaolinite or silt loam soil, a small increase in radial growth was observed. On plates containing barrier-rings of silt loam soil a marked increase in hyphal density was observed when compared to the control plates. Two other cellulolytic soil isolates, *T. koningii* and *Botryotrichum piluliferum* were similarly grown on barrier-ring plates. The behaviour of *T. koningii* was similar to that of *T. viride* while the radial growth of *B. piluliferum* was actually stimulated by the presence of bentonite in the barrier-ring. The hyphal density of the fungal colonies was significantly affected by some of the additions, e.g. whole soil, to the barrier-ring. It is not possible to explain these observations as the relationship between substrate and metabolites and radial growth is complicated (Trinci, 1969; Robinson, 1978), and the environmental and genetic factors which influence colony morphology are only poorly understood.

The difference between the barrier-ring technique used in this work and conventional adsorption studies involving soil or clays on the one hand and micro-organisms or enzymes on the other is that the experiments have a vectorial dimension and are more closely related to the

spatial interactions occurring between enzymes, micro-organisms and substrates within the microenvironment of the soil (Stotzky & Burns, 1982). For example, in addition to the adsorptive and desorptive properties of the soil or soil components, the number, size and continuity of the pores in the barrier will influence the diffusion of enzyme and the growth of micro-organisms.

In conclusion, barrier-ring plates provide a versatile system which has some of the characteristics of soil microenvironments and could be adapted to investigate many other spatial interactions between microbes and exogenous substrates. Possible applications presently under consideration include the influence of soil components on the chemotactic responses of specific groups of rhizosphere micro-organisms such as *Rhizobium* spp. and phytopathogens (inoculated outside the barrier-ring) to plant root exudates (added to the central well).

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