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Effects of Junlon and Hostacerin on the Electrokinetic Properties of Spores of *Aspergillus niger*, *Phanerochaete chrysosporium* and *Geotrichum candidum*

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The electrophoretic behaviour of freshly harvested spores of *Aspergillus niger*, *Phanerochaete chrysosporium* and *Geotrichum candidum* was determined in solution at various pH values. Freshly harvested spores of all three species lacked positive mobility at low pH values, suggesting a preponderance of acidic surface groups. Spores of the 'non-aggregating' fungus, *G. candidum*, had a pH–mobility curve (peak of negative mobility between pH 3 and 4) which was quite different to those of spores of the 'aggregating' fungi, *A. niger* and *P. chrysosporium*. The pH–mobility curve of swollen spores of *A. niger* which had been incubated in medium differed from that of freshly harvested spores, suggesting that changes in the wall that occur during germination alter the electrophoretic properties of the spores; swollen spores of *A. niger*, unlike freshly harvested spores, had a negative mobility maximum at pH 5·0.

After treatment with Junlon-110 (a polyacrylic acid), spores of all three species had similar pH–mobility curves and all had peaks of negative mobility at pH 4·0. The electrophoretic mobility of spores of *P. chrysosporium* at pH 6·5 increased linearly with the concentration (0·01–0·4%, w/v) of Junlon used in the pre-treatment; electrophoretic mobility after pre-treatment with 0·005–0·1% (w/v) Hostacerin (sodium polyacrylate) increased only up to 0·01% (w/v) Hostacerin. The results obtained show that Junlon-110 and Hostacerin bind to fungal walls, and it is possible that spore and hyphal aggregation is reduced by these compounds because of repulsion between particles resulting from ionized carboxyl groups on the polymer.

INTRODUCTION

Moulds grow in submerged, shake-flask culture either as dispersed, filamentous mycelia (Trinci, 1972) or as mycelial aggregates which may take the form of spherical colonies known as pellets (Burkholder & Sinnott, 1945). In the former case, all hyphae are exposed to the medium and when nutrients are present in excess, growth is exponential. However, growth of cultures containing pellets usually follows cube-root growth kinetics (Marshall & Alexander, 1960) because nutrients (including oxygen) only diffuse into large (>2 mm diam.) pellets fast enough to maintain growth of the peripheral shell of the pellet (Pirt, 1966). In some fungi a pellet may be formed from a single spore, but in most fungi, pellets are formed from a number of spores which aggregate together, usually prior to germ-tube emergence (Galbraith & Smith, 1969; Trinci, 1970). Hyphae (Burkholder & Sinnott, 1945) and even small pellets (Clark, 1962) may also aggregate to form large pellets, and shake-flask cultures of some fungi, e.g. *Neurospora crassa*, end with the formation of a single, large mycelial aggregate. The aggregation phenomena described above explain why only a small number of pellets is formed even when cultures are inoculated with large numbers of spores (Trinci, 1970).

It is important to develop methods to control culture morphology since it can affect product formation. For example, *Aspergillus niger* grows faster and produces more amylase when grown as filamentous, dispersed mycelia than when grown as pellets (Elmayergi & Scharer, 1973).

Elmayergi *et al.* (1973) showed that spore aggregation in *A. niger* was reduced by the anionic polymer Carbopol-934 (carboxypolymethylene). Carbopol was adsorbed to the walls of spores and hyphae during growth and Elmayergi *et al.* (1973) suggested that aggregation was prevented because ionized carboxyl groups on the polymer induced electrostatic repulsion between spores and hyphae. Similarly, Trinci (1983) found that aggregation of spores and hyphae of *A. niger* was reduced when the medium contained the anionic polymers Junlon-110 or Junlon-111 (cross-linked polyacrylic acids) and he also found that these polymers were bound to the biomass.

The technique of particulate electrophoresis has provided valuable information on the properties of cell surfaces. The 'surface' studied by this technique is a region, within a few ångstroms of the actual surface, defined in terms of those ions at the surface or those ionogenic components of the peripheral zone of the cells which contribute to the electrokinetic properties of the cell (James, 1979). In the particulate micro-electrophoretic technique, the rate of migration of a cell is measured under a known applied electric field at a fixed temperature. If the hypothesis of Elmayergi *et al.* (1973) is correct, the electrophoretic behaviour of untreated and polymer (Junlon or Carbopol-934) treated spores should differ. The present investigation was therefore made to determine the effect of Junlon-110 and Hostacerin (sodium polyacrylate) on the pH–mobility curves of spores of *A. niger*, *Phanerochaete chrysosporium* and *Geotrichum candidum*; Hostacerin was included in the study because it has an effect on the morphology of cultures of *A. niger* which is similar to that of Junlon-110.

METHODS

Organisms and media. Phanerochaete chrysosporium Burdsall was obtained from Dr J. M. MacDonald of the University of Manchester Institute of Science and Technology, and *Aspergillus niger* van Tiegham and *Geotrichum candidum* Link ex Persoon were obtained from the culture collection of the Department of Cell and Structural Biology, University of Manchester. The defined medium of Vogel (1956) was used with 10 g glucose l^{-1} as the carbon source instead of sucrose. The medium was sterilized as three separate solutions: Vogel's mineral salts at $\times 50$ final concentration, glucose at $\times 5$ final concentration and (when necessary) a solution of the anionic polymer at $\times 2$ final concentration; the final volume of the medium was adjusted with sterile distilled water. The mineral salts solution was prepared and sterilized as described by Vogel (1956). The glucose and polymer solutions were sterilized by autoclaving at 115 °C for 20 min and the final pH of the medium was adjusted to pH 6·5 with 1 M-NaOH; solutions of Junlon-110, unlike those of Hostacerin, are very acidic. When necessary, medium was solidified with agar (final concentration 15 g l^{-1}; Taiyo, Davis Gelatine). Junlon-110 and Hostacerin were kindly provided by Honeywill & Stein Ltd, 69/73 Manor Road, Wallington, Surrey SM6 0BP, UK.

Spore suspensions. Spores of *A. niger* (conidiospores), *P. chrysosporium* (arthrospores) and *G. candidum* (arthrospores) were harvested with double-distilled water (30 ml per flask) from cultures grown for 5 d at 25 °C in 250 ml conical flasks containing 50 ml volumes of Vogel's agar medium. To minimize the variation in spore age (Fisher & Richmond, 1970), the cultures were inoculated with a spore suspension which was spread over the surface of the agar medium. Spore suspensions were centrifuged at 1250 *g* ($r_{av.}$ 7·5 cm) for 5 min, and, after removing the supernatant, the spores were suspended in 30 ml sterile double-distilled water. This spore suspension was then diluted with sterile double-distilled water until it contained about 3×10^6 spores ml^{-1}. Prior to measurements of electrophoretic mobility, 20 ml spore suspension was centrifuged as before, washed and then suspended in 20 ml of the appropriate buffer. These spores are described as 'freshly harvested'. Electrophoretic mobility is dependent on the ionic strength of the suspending medium, so care was taken to ensure that the ionic strength of each buffer was constant and identical; all buffer solutions had a final ionic strength (I) of 0·05 mol l^{-1}. The buffer solutions used for measuring the electrophoretic mobility of spores were HCl and NaCl for pH 2; NaCl, sodium acetate, sodium barbiturate and HCl for pH 3 to 9; NaCl, sodium acetate, sodium barbiturate and NaOH for pH 10 and 11 (Gittens & James, 1963). All buffers were made up in double-distilled water.

Some freshly harvested spores of *A. niger* were incubated in culture medium prior to measuring their electrophoretic mobility. A 20 ml volume of spore suspension containing about 3×10^6 spores ml^{-1} was centrifuged at 1250 *g* for 5 min, and, after removing the supernatant, the spores were suspended in 20 ml Vogel's medium (pH 6·5). This spore suspension was transferred to a 250 ml conical flask and incubated at 25 °C and 200 r.p.m. on a rotary shaker with a 2·5 cm stroke. The spores were harvested after 7·4 h when about half had formed germ tubes and about half were swollen but had not germinated. The spores were then washed and suspended in buffer as described above. The electrophoretic mobilities of the swollen spores in these preparations were measured.

Other freshly harvested spores of *A. niger* were suspended in 20 ml volumes of Vogel's medium (pH 6·5) containing Junlon-110 or Hostacerin (final concentration about 3×10^6 spores per ml medium) and were then

incubated for 1 h in 250 ml conical flasks at 25 °C and 200 r.p.m. on a rotary shaker. After incubation, each 20 ml volume of spore suspension was diluted with 80 ml sterile double-distilled water and centrifuged at 1250 *g* for 5 min. The spores were then washed and suspended in buffer as described above.

Measurement of spore mobility. The cytopherometer (47 75 00) and current regulator (39 25 47) were obtained from Zeiss. The cytopherometer had platinum electrodes and a phase-contrast optical system, and the electrophoresis cell was maintained at 25 °C. The technique is described by James (1979). Measurements of the time taken for spores to traverse 32 μm were made in both directions after current reversal and each mean mobility (μm s^{-1}) was obtained from at least 20 observations. The voltage was varied according to spore mobility and was taken into account when calculating the electrophoretic mobility. The conductivity of buffered spore suspension was measured at 25 °C in a stoppered conductivity cell using a Wayne-Kerr B 221 bridge. Electrophoretic mobilities are expressed as 10^{-8} m^2 V^{-1} s^{-1} (SI units) and are equivalent to μm cm V^{-1} s^{-1}.

RESULTS AND DISCUSSION

Fig. 1 shows the electrophoretic behaviour of freshly harvested spores of *A. niger*, *P. chrysosporium* and *G. candidum*. The pH–mobility curve of freshly harvested spores of *A. niger* was similar to that of *A. niger* spores that had been incubated at pH 4·5 for 12 h (Seviour & Read, 1985), and freshly harvested spores of *P. chrysosporium* had a pH–mobility curve which was similar to that of conidia of *Penicillium expansum* (Fisher & Richmond, 1969). However, freshly harvested spores of *G. candidum* had a peak of negative spore mobility (i.e. they moved towards the anode) between pH 3 and pH 4 and the pH–mobility curve of this species was unlike the pH–mobility curves of all other fungal spores so far studied in showing such a sharp peak (Douglas *et al.*, 1959; Fisher, 1973; Richmond & Fisher, 1971). However, the pH–mobility curve of *G. candidum* resembled that of a methicillin-sensitive strain of *Staphylococcus aureus* (Hill & James, 1972).

Spores of *A. niger* and *P. chrysosporium* had isoelectric points (no mobility) at a buffer pH of 2·0, and the lack of positive mobility of the spores of all three species at low pH values suggests a preponderance of acidic groups on the spore surface. Seviour & Read (1985) interpreted their results, on the basis of the known surface architecture of *A. niger* conidia, as suggesting that the predominant ionizable groups on the spore surface were possibly phosphate or carboxyl. Spores of *Penicillium expansum* also had an isoelectric point at pH 2·0 and Fisher & Richmond (1969) suggested that their surface contained highly acidic phosphate groups.

Eddy & Rudin (1958) developed a method of analysing pH–mobility curves of yeast and suggested that at least three types of ionizable groups contributed to mobility. These were: A, positive and effective below pH 4; B, negative and effective above pH 4; and C, negative, but with a constant effect on mobility through the range pH 3–9. They suggested that the type C surface charge was due to phosphate groups and, tentatively, that A and B represented charges due to proteins in the surface layers of the wall. The phosphates were presumed to provide a negative charge over a wide pH range, so charged phosphates could well be responsible for the bulk of the negative charge on the spore mobilities illustrated in Fig. 1. The unusual peak in the *G. candidum* mobility curve around pH 3–4 may indicate a population of particular carboxyl groups on the spore surface which became ionized in this pH range.

Freshly harvested spores and swollen spores (spores incubated for 7·4 h prior to making mobility measurements) of *A. niger* had different pH–mobility curves (Figs 1 and 2). The peak of negative mobility of swollen spores of *A. niger* at pH 5·0 (Fig. 2) was not observed by Seviour & Read (1985) in their studies of *A. niger* spores pre-incubated in media at pH values from 2 to 4·5. Changes in wall composition occur during spore germination and these are presumably responsible for the difference in the pH–mobility curves of freshly harvested and swollen conidia of *A. niger*. In the light of the previous discussion, it is possible that the spore swelling process exposes additional carboxyl groups.

Treating spores of *A. niger*, *P. chrysosporium* and *G. candidum* with 0·2% (w/v) Junlon-110 in Vogel's medium changed their electrophoretic behaviour (Figs 1 and 3), indicating that the polymer is bound to the spore surface and is not removed by the washing treatment. The pH–mobility curve of all Junlon-110 treated spores showed peaks of negative mobility at pH 4·0 and the mobilities of the spores of the three species at this pH were very similar

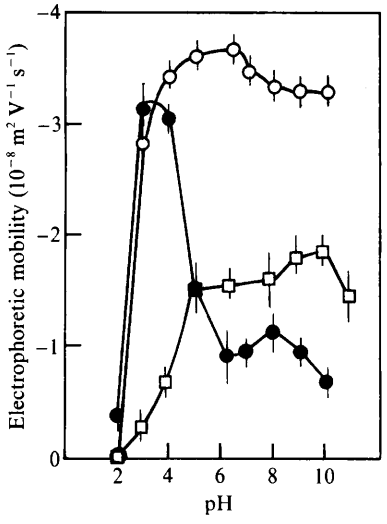

Fig. 1

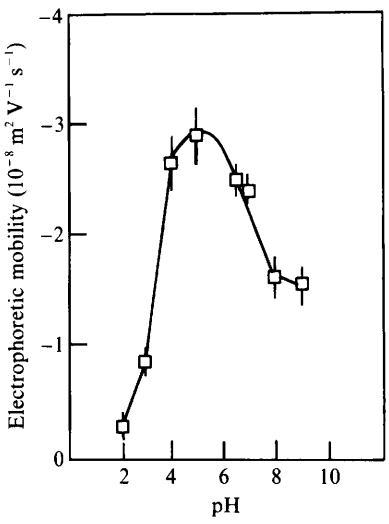

Fig. 2

Fig. 1. pH–mobility curves of freshly harvested spores of *A. niger* (□), *P. chrysosporium* (○) and *G. candidum* (●). Vertical lines indicate the standard deviations from the mean.

Fig. 2. pH–mobility curve of swollen spores (incubated for 7·4 h at 25 °C in Vogel's medium, pH 6·5) of *A. niger*. Vertical lines indicate standard deviations from the mean.

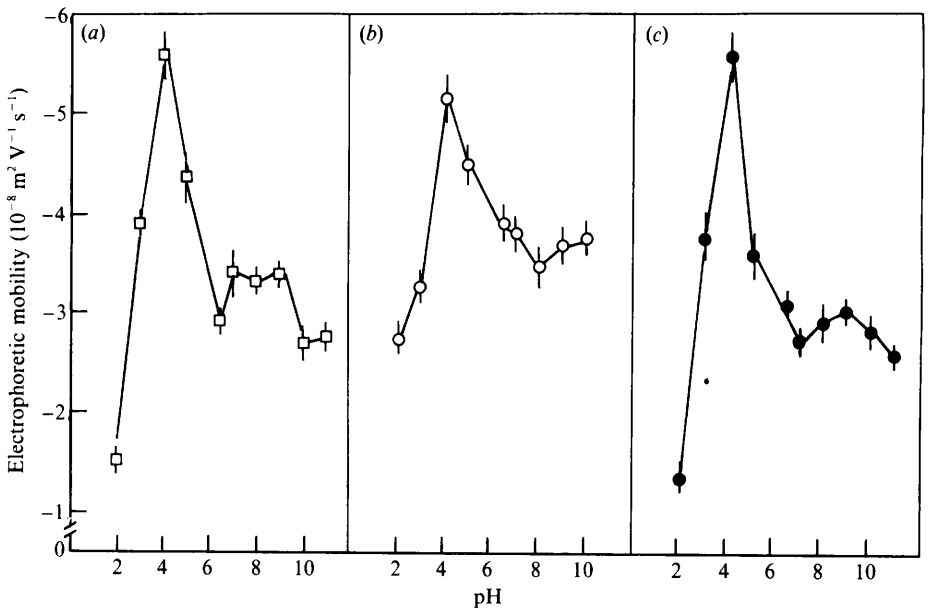

Fig. 3. pH–mobility curve of spores of (*a*) *A. niger* (□), (*b*) *P. chrysosporium* (○) and (*c*) *G. candidum* (●) after treatment with 0·2% (w/v) Junlon-110 in Vogel's medium at pH 6·5 for 1 h. Vertical lines indicate standard deviations from the mean.

($-5{\cdot}58 \times 10^{-8}$ m^2 V^{-1} s^{-1} for *A. niger*, $-5{\cdot}12 \times 10^{-8}$ m^2 V^{-1} s^{-1} for *P. chrysosporium* and $-5{\cdot}65 \times 10^{-8}$ m^2 V^{-1} s^{-1} for *G. candidum*).

Fig. 4 shows that the negative electrophoretic mobility of spores of *P. chrysosporium* pre-treated for 1 h with different concentrations of Junlon-110 in Vogel's medium prior to making mobility measurements increased linearly with the concentration of Junlon-110 used in the pre-

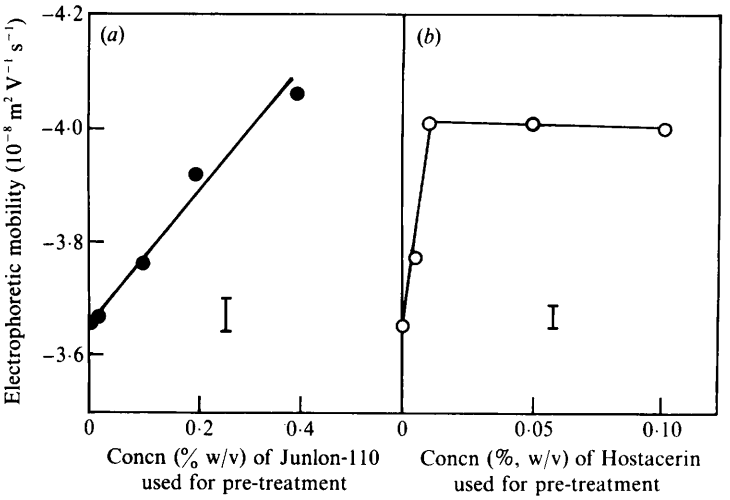

Fig. 4. Electrophoretic mobility at pH 6·5 of spores of *P. chrysosporium* before (zero reading) and after treatment with different concentrations of Junlon-110 (●) or Hostacerin (○) in Vogel's medium at pH 6·5 for 1 h. The vertical lines give the average standard deviation from the mean.

treatment. However, the negative electrophoretic mobility of spores pre-treated with Hostacerin only increased up to a Hostacerin pre-treatment concentration of 0·01% (w/v), and thereafter remained constant. These observations suggest that Hostacerin is more effective (lower concentration required) than Junlon at increasing the negative charge on spores and that the spore surface became saturated with Hostacerin. No saturation occurred with Junlon 110; mobility increased even up to 1% (w/v) concentration, which gave an electrophoretic mobility of $-5{\cdot}52 \times 10^{-8}$ m^2 V^{-1} s^{-1} even at pH 6·5.

The present and previous results (Trinci, 1983) suggest that Junlon-110, like Carbopol-934 (Elmayergi *et al.*, 1973; Seviour & Read, 1985), is adsorbed to the surface of fungal spores and hyphae. The similarity of the pH–mobility curves of Junlon-110-treated spores of the three species (Fig. 3) certainly suggests that their negative mobility is largely determined by surface-bound polyacrylic acid. Thus, as suggested by Elmayergi *et al.* (1973), Junlon-110 may prevent aggregation because of the electrostatic repulsion between the polymer-coated spores.

A key to understanding this mechanism is an adequate explanation for the cause of the aggregation in the absence of these polymers. Electrostatic attraction is an obvious possibility, and, certainly, freshly harvested (Fig. 1) and swollen (Fig. 2) spores of *A. niger* lack a net charge at pH 2·0 and aggregation and pellet formation does not occur in cultures grown at this pH (Galbraith & Smith, 1969; Seviour & Read, 1985). Seviour & Read (1985) discussed the paradox of accounting for aggregation by electrostatic attraction in a population of particles showing a uniformly net negative charge. One possible mechanism is for divalent cations to form 'salt bridges' between negatively charged cells (Mill, 1964). The possibility that anionic polymers reduce aggregation of fungal spores and hyphae by sequestering divalent cations from the medium and thereby preventing 'salt-bridge' formation (G. S. Byrne & S. Ward, personal communication) can be discounted since, in our experiments, aggregation occurred in Junlon-supplemented medium when the polymer was contained within a dialysis bag (results not shown).

It is feasible for fungal spore aggregation to be due to electrical attraction because, although the spores have a net negative charge, this does not mean that they are devoid of positively charged sites. Further, a population of germinating spores will be heterogeneous in particle size, in surface electrochemistry (compare Figs 1 and 2), and in surface topography. Thus, differences in net charge between different particles, and in charge distribution over the surface of particles, can readily account for initial aggregations which then, by amplifying these differences, act as

foci for further aggregation. However, the mechanism of aggregation is not necessarily the inverse of, nor even related in nature to, the mechanism of action of the polymers. By coating the spore with a spatially extensive and evenly distributed layer of polymer which is uniformly charged, Junlon and Hostacerin produce a population of particles in which the tendency to aggregate is abolished.

G. candidum, unlike *A. niger* and *P. chrysosporium*, does not aggregate to form pellets when grown in submerged culture (Robinson & Smith, 1976). In seeking explanations both for aggregation and its avoidance, it may therefore be significant that freshly harvested spores of the 'non-aggregating' fungus *G. candidum* (Fig. 1) and Junlon-110 treated spores of *A. niger* and *P. chrysosporium* (Fig. 3) had similar pH–mobility profiles.

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