Effects of cell wall deficiency on the synthesis of polysaccharide-degrading exoenzymes: a study on mycelial and wall-less phenotypes of the fz; sg; os-1 ('slime') triple mutant of Neurospora crassa

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The present investigation extends previous reports from our laboratory (Pietro et al., 1989, 1990) of the pleiotropic effects of cell wall deficiency on the control of synthesis and secretion of catabolic exoenzymes in the fz (fuzzy); sg (spontaneous germination); os-1 (osmotic) multiple mutant of Neurospora crassa (Emerson, 1963). Strains with these mutations ('slime'-like) are morphologically abnormal and tend to lose irreversibly the ability to synthesize cell wall when repeatedly subcultured at high osmotic pressure, giving rise to isolates which grow as rounded, osmotically-sensitive spheroplasts (stable 'slime') (Emerson, 1963; Nelson et al., 1975; Pietro et al., 1989, 1990). The use of stable mycelium-forming (mycelial intermediate) as well as spheroplast-forming (stable 'slime') derivatives from a single fz; sg; os-1 ('slime'-like) segregant has enabled us to study the effects of cell wall deficiency on different phenotypes with identical genetic backgrounds (Pietro et al., 1990), and we have demonstrated that characteristic defects of stable 'slimes', such as hypersecretion and deregulation of catabolic exoenzymes, are only expressed in the spheroplast-forming stable 'slime' and not in mycelium-forming intermediates. The latter, although altered in morphology, exhibit wild-type regulation and secretion of carbon-controlled catabolic exoenzymes (Pietro et al., 1990). The present study aimed to investigate the production of exoenzymes which hydrolyse large carbohydrate polymers.

Wild-type N. crassa produces exoenzymes which attack carbohydrate polymers such as cellulose (Eberhart et al., 1964; Rao et al., 1983), pectin (Roboz et al., 1952), xylan (Mishra et al., 1984) or starch (Gratzner & Sheehan, 1969; Sigmund et al., 1985). These enzymes are not normally synthesized and secreted in large amounts during the growth phase, unless appropriate substrates are present in the surrounding medium. In addition, active production of these enzymes is repressed by carbon catabolites. The production of exoenzymes which attack polysaccharides and other macromolecules is therefore frequently regarded as a substrate-induced

Abbreviation: CMC, carboxymethylcellulose.
process. Many authors have questioned how large, insoluble molecules, which by their very nature do not directly contact the cytoplasm, can be recognized by the cell. A widely accepted view is that the true inducers are low-molecular-mass products of partial polymer hydrolysis, mediated by enzymes somehow trapped on, or linked to, the cell wall. Uninduced cells are thought to express genes that participate, either through enzymic or through steric mechanisms, in the recognition of complex macromolecules which are substrates for extracellular enzymes.

We therefore considered it interesting to investigate the production of enzymes which attack microcrystalline cellulose, carboxymethylcellulose (CMC), xylan, and polygalacturonic acid, in mycelium-forming intermediates and a wall-less stable 'slime' obtained by vegetative selection from a single segregant of a 'slime' × wild-type cross (Pietro et al., 1990). The unrelated stable 'slime' strain FGSC 1118 was also included in this study.

**Methods**

*Neurospora crassa strains and culture conditions.* N. crassa strains FGSC 1118 (fs; sg; os-I ('slime') and RCP-3 (fs; sg; os-I; al-1; a) were used. The former strain was a gift from the Fungal Genetics Stock Center (Kansas City, Kansas, USA). Strain RCP-3 was obtained from a cross of a 'slime'-containing heterokaryon and a wild-type strain (Pietro et al., 1990). The three phenotypes of segregant RCP-3, namely the mycelial intermediate, the spheroplast-hyalphal intermediate and the stable 'slime', were isolated by submitting the ascospore germling of segregant RCP-3 to a filtration-enrichment protocol of vegetative selection (Nelson et al., 1975). A detailed description of the three phenotypes of strain RCP-3 was given by Pietro et al. (1990).

Mycelial strains (mycelial and spheroplast-hyalphal intermediates of strain RCP-3) were propagated on slants of solid Vogel (1964) medium supplemented with 2% (w/v) glucose. Stable 'slime' strains were maintained by weekly transfers on slants of solid Vogel's medium supplemented with 2% glucose, 0.75% Bacto-peptone (Difco) and 0.75% yeast extract (Difco) (complete medium). Liquid cultures were prepared in 250 ml Erlenmeyer flasks containing 50 ml Vogel's complete medium supplemented with 2% glucose. Otherwise, medium composition is indicated for each experiment. The inoculum consisted of mycelial fragments (mycelial intermediate and spheroplast hyphal intermediate of strain RCP-3) or of 'slime' spheroplasts, collected by suspending in 0.25 M-sorbitol the growth of a slant not more than 3 d old.

Cultures were incubated with agitation (100 r.p.m.) at 30°C for 24 h, then harvested by filtration (mycelial intermediate of strain RCP-3) or centrifugation at 150 g for 15 min (spheroplast-hyalphal intermediate of strain RCP-3 and 'slime' spheroplasts). The resulting samples were either washed with 0.3 M-sorbitol to remove the original nutrients and transferred to fresh medium as indicated for each experiment, or processed for enzyme assay, in which case both the culture filtrates and supernatants were saved.

**Enzyme determination.** 'Slime' spheroplasts, suspended in 10 vols of the appropriate buffer, were disintegrated by vortexing with 1 g of glass beads for two 90 s rounds, separated by an interval of cooling in ice. Mycelia were ground in a porcelain mortar with glass beads and extracted with 10 vols of the appropriate buffer. The supernatants of a low-speed centrifugation (500 g) and samples of culture media were dialysed overnight and used directly for enzyme determinations.

Invertase activity was assayed as previously described (Pietro et al., 1989). Polysaccharide-degrading activities were measured at 37°C in 37-5 mM-sodium acetate buffer (pH 5.5) with the appropriate substrate: 10 mg ml⁻¹ CMC (sodium salt, Sigma); 10 mg ml⁻¹ polygalacturonic acid (sodium salt, Sigma); 10 mg ml⁻¹ microcrystalline cellulose (Avicel, Merck) or 5.4 mg ml⁻¹ xylan (larchwood, Sigma) and enzyme conveniently diluted, in a final volume of 5.0 ml. Substrate concentrations are final concentrations. Reaction rates were calculated from duplicate points taken after 15 min, 30 min and 60 min of incubation. Enzyme activity units (U) were defined as 1 μmol of reducing-sugar equivalent (Miller, 1959) expressed as glucose (or monogalacturonic acid for polygalacturonic-acid-degrading activity) min⁻¹. Specific activities are expressed as units per mg protein of crude cell extracts. Total activities were calculated by adding the enzyme units of crude cell extracts (considering the whole cell mass of the culture) to the enzyme units of the culture medium (considering the respective culture volume). Protein was determined by the Lowry method using bovine serum albumin (Sigma) as standard.

**Chemicals.** Monosaccharides and polysaccharides were from Merck or Sigma. All other chemicals were of the best quality available.

**Results**

Micro-organisms degrade extracellular glycosidic polymers by the action of extracellular enzyme complexes, composed of different proteins endowed with specific catalytic activities (e.g. exo- and endoglycosidase). It is common for a single activity (as defined in terms of catalytic specificity) to be represented by more than one protein; such isoforms may differ in terms of primary structure or may be the same protein with different degrees of glycosylation. Therefore, all the enzyme activities described in the present study, except that of invertase, are most likely a consequence of the action of multimolecular entities. For that reason, they will be referred to generically as 'Avicel-degrading’ activity, ‘CMC-degrading’ activity, ‘xylan-degrading’ activity or ‘polygalacturonic-acid-degrading’ activity.

**Polysaccharide-degrading activities in mycelial forms of segregant RCP-3**

Cellulosic substrates stimulate production and secretion of enzymes of the cellulolytic complex in many microbial systems (Mandels et al., 1962; Breuil & Kushner, 1976; Kubicek, 1987). As shown in Fig. 1, incubation in microcrystalline cellulose (Avicel) or CMC elicited the synthesis of a cellulose-degrading activity in the RCP-3 mycelial intermediate strain. This activity degraded CMC more efficiently than Avicel (not shown). Analogous induction occurred for the RCP-3 spheroplast-hyalphal intermediate strain, although in this case the
specific enzyme activity was less than half that of the corresponding mycelial intermediate cultures. Fig. 1 also shows that for both strains most of the cellulose-degrading activity was extracellular.

Incubation in the presence of pectin or galactose elicited synthesis and secretion of polygalacturonic-acid-degrading activity in both mycelial forms of RCP-3 (Fig. 2). The inductive effect of pectin was more evident for the spheroplast-hyphal intermediate while galactose was more effective for the mycelial intermediate. Overall, more than half of the polygalacturonic-acid-degrading activity was extracellular.

Xylan, CMC and to some extent Avicel (not shown) elicited synthesis and secretion of xylan-degrading activity in the mycelial forms of segregant RCP-3 (Fig. 3). Induction by carbohydrate polymers other than xylan indicated that the inducer requirement was nonspecific, as reported by Mishra et al. (1984) for a wild-type N. crassa strain.

All the activities mentioned above were regulated in an apparently normal fashion in the mycelial forms of segregant RCP-3. They were very sensitive to glucose repression, and no activities were detected in cultures incubated in the absence of a carbon source, indicating a requirement for induction.

**Polysaccharide-degrading activities in the stable 'slime' derived from segregant RCP-3**

The stable 'slime' derived from segregant RCP-3 did not produce any significant Avicel-degrading activity (not shown); neither did it respond to pectin or galactose by synthesizing polygalacturonic-acid-degrading activity (Fig. 2). Nevertheless, these cultures produced and secreted invertase constitutively (Fig. 2), demonstrating that under the conditions studied synthesis and secretion of enzymes was not generally impaired. On the other hand, the stable 'slime' RCP-3 produced significant levels of CMC-degrading (Fig. 1) and xylan-degrading (Fig. 3) activities. These enzymes were produced constitutively by the stable 'slime' (i.e. in the absence of inducer substrates), but remained fully sensitive to glucose repression.

**Polysaccharide-degrading activities in the stable 'slime' strain FGSC 1118**

The stable 'slime' strain FGSC 1118 produced and secreted cellulose-degrading activity (Fig. 1), polygalacturonic-acid-degrading activity (Fig. 2) and xylan-degrading activity (Fig. 3) at specific levels even higher.
Polygalacturonic-acid-degrading specific activities in mycelial and stable 'slime' derivatives of the 'slime'-like segregant RCP-3 and in the unrelated stable 'slime' strain FGSC 1118. Culture conditions were as described in the legend to Fig. 1. Carbon source concentrations were: 1% pectin (pec); 1.35% galactose (Gal); 2% glucose. Polygalacturonic-acid-degrading activity was determined as indicated in Methods. Figures at the top of the bars indicate the percentage of extracellular activity (± SD among cultures with enzyme activity) relative to total culture activity. Triangles represent invertase specific activity. The results of a representative experiment are shown. R, repressed (glucose); D, derepressed (no carbon source added); I, induced (pectin or galactose, as indicated); I/R, induced and repressed (inducer plus 2% glucose); M, mycelial intermediate; S/H, spheroplast-hyphal intermediate; S, stable 'slime'.

Fig. 3. Xylan-degrading specific activities in mycelial and stable 'slime' derivatives of the 'slime'-like segregant RCP-3 and in the unrelated stable 'slime' strain FGSC 1118. Culture conditions were as described in the legend to Fig. 1. Carbon source concentrations were: 2% glucose; 1% CMC; 1% xylan (xyl). Xylan-degrading activity was determined as described in Methods. Figures at the top of the bars indicate the percentage of extracellular activity (± SD among cultures with enzyme activity) relative to total culture activity. The results of a representative experiment are shown. R, repressed (2% glucose); D, derepressed (no carbon source added); I, induced (xylan or CMC, as indicated); I/R, induced and repressed (inducer plus 2% glucose); M, mycelial intermediate; S/H, spheroplast-hyphal intermediate; S, stable 'slime'.

Discussion

Cell-wall-forming phenotypes (mycelial intermediate and spheroplast-hyphal intermediate) of the f2; sg; os-1 multiple mutant strain RCP-3 produced exoenzymes
which attacked cellulose, xylan and polygalacturonic acid in an apparently normal fashion. These enzyme activities were only produced in the presence of polysaccharidic substrates and in the absence of glucose. In contrast, the stable 'slime' derived from this strain, as well as the stable 'slime' FGSC 1118, synthesized these enzymes constitutively, except stable 'slimes' RCP-3 which, even in the presence of the substrate, did not produce polygalacturonic-acid-degrading activity.

Comparing the production of polysaccharide-degrading activities by the stable 'slimes' RCP-3 and FGSC 1118, FGSC 1118 is markedly resistant to glucose repression while RCP-3 is fully sensitive. In fact, every carbon-related catabolic enzyme of strain FGSC 1118 so far studied appears to be produced constitutively even in the presence of glucose. On the other hand, the exoenzymes invertase and \( \beta \)-glucosidase are not fully repressed by glucose in the stable 'slime' RCP-3 (Pietro et al., 1990).

The different sensitivity to glucose repression for stable 'slimes' RCP-3 and FGSC 1118 might have a genetic origin. The two strains have different genetic backgrounds because they were obtained from crosses of different 'slime' parents with unrelated wild-type strains (Emerson, 1963; Pietro et al., 1990). The genetic analysis of \( fz; sg; os-l \) mutant strains is complicated by the fact that even the mycelial forms of these mutants are female-sterile, a condition attributed to the \( sg \) mutation (Emerson, 1963; unpublished observations). This precudes the study of the segregation of enzyme phenotypes by the use of reciprocal crosses. However, the two strains also differ in terms of vegetative lifetime. Our stock of FGSC 1118 has been subcultured weekly for about fifteen years, while the \( fz; sg; os-l \) segregant RCP-3 gave rise to a stable 'slime' clone just one year ago. During continuous propagation, 'slime' strains lose some of the characteristics of wall-forming strains, such as the ability to form heterokaryons and the ability to function as the fertilizing parent in crosses (Selitrennikoff et al., 1979). The stable 'slimes' RCP-3 and FGSC 1118 also differed in these respects; RCP-3 is still effective as a fertilizing parent (Pietro et al., 1990) but all recent attempts to cross our FGSC 1118 stock have been unsuccessful. The possibility of existing epigenetic differences between the stable 'slimes' RCP-3 and FGSC 1118 cannot therefore be eliminated.

Notwithstanding the uncertainty of linking the glucose-resistant synthesis of polysaccharide-degrading enzymes of strain FGSC 1118 with the loss of cell wall, it seems likely that this defect caused abnormal enzyme synthesis in the absence of inducer substrates for both stable 'slimes'. Uninduced synthesis of a lytic enzyme, amylglucosidase has been observed for the \( exo-l \) mutant of \( N. \) crassa (Gratzner, 1972). This strain produces high amylolytic activity under conditions of carbon starvation which fail to produce the same effect in the wild-type. The \( exo-l \) mutant exhibits alterations in cell-wall composition which, according to Gratzner (1972), may explain the defective regulation of exoenzymes. This author and others (Lampen, 1965; Yabuki & Fukui, 1970), have suggested a regulatory model for explaining the influence of the microbial cell surface on the synthesis of exoenzymes, which proposes the existence of specific binding sites for a particular enzyme. These sites, when occupied, would repress further synthesis of that enzyme. The model also proposes the existence of 'masking factors', which would only be active in the presence of inducers (Yabuki & Fukui, 1970) and which, by competing with an enzyme for its binding site, would promote the release of the enzyme to the medium, thus stimulating continuous synthesis. This regulatory model implies that components of the cell wall (or the periplasmic compartment) would restrain enzyme synthesis in the absence of inducers. If so, we suggest that alteration to, or loss of, the critical elements could result in constitutive synthesis and excessive protein release. The results obtained in the present study are consistent with this model.

Unfortunately, this hypothesis is not yet substantiated with molecular information, due to the paucity of knowledge about the highly complex and organized structure of the fungal cell wall and periplasm, and the no less complex physiological functions which must take place in this part of the cell.

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References


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