Pleiotropic Deficiency in the Control of Carbon-regulated Catabolic Enzymes in the 'Slime' Variant of *Neurospora crassa*

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A wall-less 'slime' strain of *Neurospora crassa* was shown to be pleiotropically altered in the production and secretion of several enzymes related to carbon catabolism, including invertase, alkaline protease, aryl- β -glucosidase and cellobiase. Invertase and protease were produced constitutively. The two β -glucosidases required induction by cellobiose but were poorly affected by glucose repression. Exoenzymes were released in larger amounts by the 'slime' strain than by the wild-type. Two other carbon-regulated enzymes, isocitrate lyase and phosphoenolpyruvate carboxykinase, were normally regulated in the 'slime' strain. A study of recombinants from a cross of a 'slime'-containing heterokaryon and wild-type suggested that the anomalies observed were inherent in the 'slime' phenotype.

INTRODUCTION

Neurospora crassa, like other filamentous fungi, can produce and secrete many different types of protein, in response to specific environmental stimuli such as nutrient limitation and presence of inducer substrates. The influence of the cell surface on the production of exported proteins has been known for several years. Fungal cells are surrounded by a complex interphase, the periplasmic compartment, delimited by the plasmalemma and the cell wall. Some proteins may be retained in this part of the cell, by covalent bonding to cell wall polymers (Kubicek, 1981; Lampen, 1971), by interaction with the plasma membrane, or by physical restraint due to the cell wall structure, which seems to act as a molecular sieve for proteins (Chang & Trevithick, 1974; Kidby & Davies, 1970; Trevithick & Metzberg, 1966). The pattern of synthesis and secretion of exoenzymes is frequently altered by mutations affecting cell wall structure and composition (Gratzner & Sheenan, 1969; Gratzner, 1972; Murayama & Ishikawa, 1973) as well as by the action of chemicals which cause the same effects (Mishra & Tatum, 1972; Nanda *et al.*, 1986; Sahoo *et al.*, 1986; Silva & Jorge, 1988). A radical condition of cell wall deficiency is present in the 'slime' variant of *N. crassa* (Emerson, 1963). This strain lacks entirely the cell wall structure, and therefore also lacks the periplasmic compartment. 'Slime' cultures develop as rounded, osmotically sensitive spheroplasts, which, unlike spheroplasts produced with lytic enzymes, can actively grow and multiply in solid or liquid medium (Trevithick & Galsworthy, 1977). Because of these properties the 'slime' strain is an interesting system to study the influence of the cell wall and the periplasmic compartment on the control of protein secretion.

In comparison with the wild-type, 'slime' spheroplasts exhibit an abnormally high secretion of alkaline phosphatase (Burton & Metzberg, 1974) and invertase (Bigger *et al.*, 1972). Casanova *et al.* (1987) demonstrated that invertase secretion in the 'slime' variant is resistant to glucose repression. We have now investigated the control of other carbon-regulated enzymes in the 'slime' variant, namely: the extracellular aryl- β -glucosidase (β -D-glucoside glucohydrolase, EC 3.2.1.21), alkaline protease, intracellular cellobiase (EC 3.2.1.21), isocitrate lyase (EC 4.1.3.1) and phosphoenolpyruvate carboxykinase (EC 4.1.1.38). We also studied the production of alkaline phosphatase (EC 3.1.3.1) and invertase (EC 3.2.1.26).

METHODS

Neurospora strains and culture conditions. The following *Neurospora crassa* strains were used: FGSC 424 *a* (wild-type); FGSC 1118 (*fz*; *sg*; *os-1*; 'slime'), FGSC 2713 (*fz*; *sg*; *arg-1*; *cr-1*; *al-1*; *os-1*; *A*) + (*tol*; *pan-1*; *a*) a 'slime'-containing heterokaryon, and BAT 9-5 (*cot-1*; *nic-3*, *a*) an extra-fertile female parent (Cruz & Terenzi, 1981) used for crosses. The first three strains were a gift from the Fungal Genetics Stock Center (Kansas City, Kansas, USA). Mycelial strains were propagated on slants of solid Vogel's (1956) medium with 2% (w/v) sucrose and auxotrophy supplements when required. The 'slime' strain was 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). Liquid cultures were prepared in 250 ml Erlenmeyer flasks containing 50 ml Vogel's minimal medium supplemented with 2% glucose and 0.25 M-sorbitol. Otherwise, medium composition is indicated for each experiment. The inoculum consisted of suspensions of macroconidia (5×10^5 ml⁻¹), or of 'slime' spheroplasts collected by suspending in 0.25 M-sorbitol the growth of a slant culture not more than 3 d old.

Liquid cultures were incubated with agitation (100 r.p.m.) at 30 °C. The 'slime' strain was usually grown for 48 h and mycelial strains for 17 h. Thereafter the cultures were harvested by filtration (mycelial strains) or centrifugation at 159 *g* for 15 min ('slime' strains). Then, the samples were either transferred to fresh medium as indicated for each experiment, or processed for enzyme determination, when both the culture filtrates and supernatants were saved.

Enzyme determinations. 'Slime' spheroplasts, suspended in 10 vols of the appropriate buffer, were disintegrated by vortexing with 1.0 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.

Aryl- β -glucosidase and cellobiase were estimated with *p*-nitrophenyl β -D-glucoside (Eberhart & Beck, 1970). The amount of each enzyme relative to total β -glucosidase activity was estimated by thermal inactivation of cellobiase after incubating one of two paired samples for 1 min at 60 °C. One enzyme unit is defined as 1 nmol *p*-nitrophenol released min⁻¹ at 30 °C. Invertase was determined according to Arnold (1974), using raffinose as a substrate; one enzyme unit is defined as the formation of 1 μ mol glucose (Miller, 1959) min⁻¹ at 37 °C. Alkaline protease was assayed by spectrophotometric measurements of tyrosine released from casein (McDonald & Chen, 1965; Drucker, 1972); one enzyme unit is defined as the colour equivalent of 1 μ g tyrosine min⁻¹ at 37 °C (Hagihara *et al.*, 1958). Isocitrate lyase and phosphoenolpyruvate carboxykinase activities were monitored in a Beckman DU-7 spectrophotometer (Dixon & Kornberg, 1959; Flavell & Fincham, 1968; respectively); one enzyme unit is defined as 1 μ mol product released min⁻¹ at 30 °C. The repressible alkaline phosphatase was assayed with *p*-nitrophenol phosphate (Burton & Metzner, 1974); one enzyme unit is defined as 1 μ mol *p*-nitrophenol released min⁻¹ at 37 °C.

Specific activities were expressed as units per mg protein of crude cell extracts. Protein was determined by the Lowry method, using bovine serum albumin (Sigma) as standard.

Crosses. Crosses were performed according to standard procedures (Davis & de Serres, 1970), using the strain BAT 9-5 as female parent and the heterokaryon FGSC 2713 as male parent, in standard Petri dishes on solid Westergaard & Mitchell's (1947) crossing medium. Recombinants were isolated from plates of minimal Vogel's medium supplemented with 50 μ g nicotinic acid ml⁻¹, 100 μ g arginine ml⁻¹, 1% (w/v) sorbose, 0.1% glucose, 0.1% fructose and 1.0 M-sorbitol. Germinated ascospores were transferred to slants of minimal Vogel's medium supplemented with sucrose, nicotinic acid and arginine. 'Slime-like' recombinants (Emerson, 1963) were submitted to a filtration-enrichment protocol (Nelson *et al.*, 1975) by vegetative propagation in liquid medium of high osmolarity, in attempts to recover stable 'slime' phenotypes. Only in one of several trials was this operation successful (see Results).

Chemicals. *p*-Nitrophenyl β -D-glucoside, *p*-nitrophenyl phosphate, cellobiose, raffinose, sorbitol and sorbose were from Sigma. All other chemicals were analytical grade.

RESULTS

 β -Glucosidase activities in the wild-type and in 'slime' strain FGSC 1118

Neurospora crassa produces two β -glucosidases: aryl- β -glucosidase, which is constitutive during conidiogenesis, and cellobiase. Neither enzyme is synthesized by exponentially growing vegetative mycelium, unless induced, cellobiose being the most effective inducer. Aryl- β -glucosidase is extracellular and accumulates preferentially on the surface of conidia. In contrast, cellobiase is intracellular (Eberhart & Beck, 1970, 1973).

The two β -glucosidase activities were induced by cellobiose, in both wild-type and 'slime' cultures (Table 1). The basal (zero time) activities were somewhat higher in the 'slime' strain

Table 1. β -Glucosidase specific activities in the wild-type and in the 'slime' strain FGSC 1118

The 'slime' strain was grown for 48 h at 30 °C, with agitation, in Vogel's medium supplemented with 2% glucose and 0.25 M-sorbitol. Wild-type cells were grown under the same conditions but only for 17 h. At that time (zero time) the cultures were harvested by centrifugation ('slime') or filtration (wild-type) and resuspended in fresh Vogel's medium plus 0.25 M-sorbitol, with 2% glucose (repressed), 0.2% cellobiose (induced) or a mixture of both sugars at the same concentrations. After 8 h incubation at 30 °C the cultures were harvested and β -glucosidase activities were determined in the culture medium and cell extracts, as indicated in Methods. Specific activities (intracellular and extracellular) are expressed as units per mg of crude extracted protein.

Conditions	Wild-type				'Slime'			
	Aryl- β -gluc.		Cellobiase		Aryl- β -gluc.		Cellobiase	
	Intra	Extra	Intra	Extra	Intra	Extra	Intra	Extra
Zero time	1.73	0.41	ND	ND	2.83	ND	2.70	ND
Glucose	1.10	0.19	1.10	ND	2.84	ND	3.17	ND
Cellobiose	52.30	11.40	18.50	0.32	8.00	35.54	19.30	2.75
Cellobiose plus glucose	2.40	ND	1.33	ND	5.60	3.34	15.50	2.43
No carbon source added	4.01	1.50	0.69	ND	2.83	ND	2.08	ND

ND, Not detected.

than in the wild-type, possibly because of the longer period of incubation required to grow the 'slime' strain. Nevertheless, the specific activities of induced 'slime' and wild-type cultures were similar. Induction by cellobiose was specific in both strains: other carbon sources tested such as galactose, maltose, trehalose and arabinose were ineffective or much less effective than cellobiose (not shown).

About 82% of the aryl- β -glucosidase produced by induced 'slime' cultures was secreted into the culture medium. In contrast, the wild-type secreted only 18%. However, cellobiase activity was predominantly intracellular in both 'slime' and wild-type cultures. Some cellobiase activity was detected in the culture medium of the 'slime' strain, probably released from cells damaged during manipulation.

Glucose repression strongly affected the production of aryl- β -glucosidase and cellobiase in wild-type cultures but it was much less effective in the 'slime' strain. For instance, intracellular and extracellular aryl- β -glucosidase and (intracellular) cellobiase activities of 'slime' cultures incubated with glucose plus cellobiose were 70%, 9.4% and 80.3% of the respective induced activity (plus cellobiose only). This result was strikingly different from that obtained with the wild-type, in which these values were 5% for intracellular aryl- β -glucosidase, negligible for the extracellular activity and 7.2% for cellobiase.

Invertase and alkaline protease activities in the wild-type and in 'slime' strain FGSC 1118

Invertase and alkaline protease are carbon-controlled exoenzymes. Invertase synthesis can be induced by galactose and is repressed when glucose is present (Metzenberg, 1962) as shown for the wild-type in Table 2, where it can also be observed that about 72% of the induced invertase activity remained cell bound. These results are in agreement with those of other authors (Bigger *et al.*, 1972; Metzenberg, 1963). In contrast, invertase appeared to be constitutive in the 'slime' strain, which in the absence of inducer produced four times more invertase per mg protein than did wild-type cultures. Addition of galactose did not increase the enzyme activity further. On the average, about 97% of the invertase activity produced by the 'slime' strain was extracellular.

Proteolytic activity is induced by proteins in combination with carbon, sulphur or nitrogen limitation (Drucker, 1972; Hanson & Marzluf, 1975). The main component of the proteolytic system of *N. crassa* is an inducible, extracellular alkaline protease activity (Abbot & Marzluf, 1984; Drucker, 1975). Table 3 shows the effect of a protein, gelatin, in cultures of the wild type and 'slime' strains. Alkaline protease activity of wild-type cultures incubated with gelatin was 13-fold and 25-fold higher in cells and external medium, respectively, than that of cultures incubated with glucose. Glucose strongly counteracted the inducing effect of gelatin. On the other hand, glucose-grown 'slime' cultures produced seven times more alkaline protease than

Table 2. *Invertase specific activities in the wild-type and in 'slime' strain FGSC 1118*

Culture conditions were as described in the legend for Table 1. Induced cultures were incubated for 15 h with 1.35% galactose. Invertase activity was determined as indicated in Methods. Specific activities (intracellular and extracellular) are expressed as units per mg of crude extracted protein.

Conditions	Wild-type		'Slime'	
	Intra	Extra	Intra	Extra
Zero time	1.72	0.15	0.37	7.07
Glucose	3.63	0.02	0.33	13.91
Galactose	16.11	6.38	0.55	18.48
Galactose plus glucose	4.36	0.44	0.40	20.85

Table 3. *Alkaline protease specific activities in the wild-type and in the 'slime' strain FGSC 1118*

Culture conditions were as described in the legend to Table 1. Induced cultures were incubated for 7 h with 0.25% gelatin. Alkaline protease activity was determined as described in Methods. Specific activities (intracellular and extracellular) are expressed as units per mg of crude extracted protein.

Conditions	Wild-type		'Slime'	
	Intra	Extra	Intra	Extra
Zero time	1.13	2.82	2.63	17.41
Glucose	0.81	1.51	3.37	13.88
Gelatin	10.89	38.50	1.72	21.31
Gelatin plus glucose	2.38	9.39	3.73	17.82

glucose-grown wild-type cultures, and the presence of gelatin did not alter this result. About 86% of the alkaline protease was extracellular in 'slime' cultures at zero time, as compared to 78% in induced wild-type cultures (Table 3).

Phosphoenolpyruvate carboxykinase and isocitrate lyase activities in the wild-type and in 'slime' strain FGSC 1118

To determine whether the 'slime' defects described above extended to other carbon-regulated enzymes, we examined phosphoenolpyruvate carboxykinase and isocitrate lyase activities. These are intracellular enzymes related to intermediary metabolism, which appear in low concentration in glucose-grown cells and become induced in the presence of gluconeogenic substrates such as acetate (Flavell & Woodward, 1970). Phosphoenolpyruvate carboxykinase occurs in the cytosol, while isocitrate lyase is associated with glyoxysomes. As shown in Table 4, phosphoenolpyruvate carboxykinase and isocitrate lyase increased 4.5- and 22-fold respectively in wild-type cells induced by acetate, and this effect was counteracted by glucose. For the 'slime' strain, the induced levels of both enzymes were lower than those of the wild-type, but the two enzymes were evidently regulated in the 'slime' strain in the same way as in the wild-type.

Production of carbon-controlled exoenzymes in recombinants from a cross between the wild-type and a 'slime'-containing heterokaryon

Casanova *et al.* (1987) suggested that lack of glucose repression of invertase in a 'slime' variant is not related to the phenotypic absence of the cell wall. According to these authors, the invertase defect could be the result of an additional mutation, harboured by the 'slime' strain, different from those which determine the 'slime' wall-less phenotype. We investigated this possibility, using a genetic approach. A heterokaryon (FGSC 2713) containing 'slime' nuclei was used to fertilize wild-type 'female receptor' mycelium. Only the 'slime' component of the heterokaryon participated in the cross (Nelson *et al.*, 1975). The heterokaryon itself exhibited normal production of exoenzymes. For instance, the two β -glucosidases, invertase and alkaline protease were inducible, and fully repressible by glucose. Furthermore, not more than 36% and 6.8% of

Table 4. *Phosphoenolpyruvate carboxykinase (PEPCK) and isocitrate lyase (ICL) specific activities in the wild-type and in the 'slime' strain FGSC 1118*

Culture conditions were as described in the legend to Table 1. Induced cultures were incubated for 6 h with 0.3% sodium acetate. Enzyme activities were determined in crude cell extracts as indicated in Methods. Specific activities are expressed as units per mg of crude extracted protein.

Conditions	Wild-type		'Slime'	
	PEPCK	ICL	PEPCK	ICL
Zero time	39.00	8.90	11.56	2.61
Glucose	39.60	8.90	19.92	7.10
Acetate	178.40	194.80	38.68	46.32
Acetate plus glucose	40.00	10.00	20.39	6.80

aryl- β -glucosidase and invertase, respectively, were extracellular. Another enzyme which is secreted in excess by 'slime' strains, the repressible alkaline phosphatase (Burton & Metzenberg, 1974), was 98% cell-bound in the heterokaryon. Thus, it seemed clear that the 'slime' phenotype behaved as recessive. Crosses produced abundant perithecia and ascospores. Germination was good (about 60–70%), giving rise to non-osmotic and various classes of osmotic phenotypes, classified on the basis of morphology and sensitivity to high osmotic pressure (Perkins, 1959). Other phenotypes (i.e. abortive germination), as described by Emerson (1963), were also recognized. One percent or less of ascospores produced a plasmodioid outgrowth resulting in a very small colony of 'slime-like' cells. None of them maintained this characteristic, but developed mycelium after transfer to medium without sorbitol, or even in sorbitol solid medium after long periods of incubation. In an attempt to recover a stable 'slime' phenotype (i.e. producing spheroplasts regardless of osmotic concentration), ten such germinating ascospores were isolated, incubated in liquid medium of high osmolarity, and submitted to a filtration-enrichment procedure (Nelson *et al.*, 1975). Only one stable 'slime' strain, designated S-4, was recovered. No attempt was made to characterize further the genotype of the recombinants with 'osmotic' phenotype obtained from the cross. Thus, the sample studied might contain other markers in addition to *os-1*, which confers the osmotic-sensitive phenotype. The same might be true for the non-osmotic class selected.

Alkaline phosphatase secretion was not higher than 1–2% in any mycelial strain (non-osmotic and osmotic phenotypes), but was over 50% in the 'slime' recombinant S-4. In this respect S-4 behaved like the 'slime' strain studied by Burton & Metzenberg (1974). Furthermore, β -glucosidase activities were normally regulated in all osmotic and non-osmotic strains, but not in the S-4 'slime' recombinant, in which the production of β -glucosidase activities was practically identical to that shown in Table 1 for the 'slime' strain FGSC 1118.

Intermediate phenotypes were observed for invertase and alkaline protease production (Table 5). Production and secretion of these exoenzymes exhibited a wild-type pattern in non-osmotic recombinants (compare with the data in Tables 2 and 3). The 'slime' strain S-4, like FGSC 1118, produced the two enzymes constitutively and secreted large amounts of them into the culture medium. All osmotic recombinants exhibited an intermediate phenotype, characterized by partially constitutive levels of invertase and alkaline protease, incomplete glucose repression, and higher levels of secretion than the wild-type. This phenotype was not affected by culturing these strains in the presence or absence of sorbitol (1.0 M) (not shown).

DISCUSSION

The 'slime' strain of *N. crassa* was pleiotropically defective for the regulation of several carbon-catabolic enzymes, including invertase, alkaline protease, aryl- β -glucosidase and cellobiase. Invertase and alkaline protease were produced constitutively, and aryl- β -glucosidase and cellobiase, although inducible by cellobiose, were less sensitive than wild-type to carbon catabolite repression. In fact cellobiase synthesis in the 'slime' strain was almost fully resistant to

Table 5. *Invertase and alkaline protease activities in recombinants from a cross between the wild-type and a 'slime'-containing heterokaryon*

Recombinants were obtained and scored as indicated in Methods. Culture conditions and other procedures were as indicated in the legends to Tables 2 and 3.

Phenotype	Percentage specific activities*							
	Invertase				Alkaline protease			
	A	B	C	D	A	B	C	D
Non-osmotic	4	5	3	14	56	79	4	23
Osmotic	27	20	16	28	44	73	33	30
'Slime'	80	79	127	112	85	88	80	133

* Figures represent relative percentage activities of the pooled results of seven independent isolates of non-osmotic and seven osmotic phenotypes, and one true 'slime' isolate. A, Extracellular activity in glucose-incubated cultures relative to total (extracellular plus intracellular) activity of the same cultures; B, extracellular activity in induced cultures relative to total activity of the same cultures; C, total activity in glucose-incubated cultures relative to total activity of induced cultures; D, total activity in cultures incubated with glucose plus the inducer relative to total activity of cultures incubated with the inducer only.

glucose repression. Exoenzymes were released by the 'slime' cells in larger amounts than for the wild type. This was not an artifact due to the fragility of 'slime' cells because cellobiase, which is not an exportable protein, was normally retained in the cells. At least one other exoenzyme, alkaline phosphatase, is also secreted in large amounts by the 'slime' strains FGSC 1118 and S-4 studied here, and by at least one other (Burton & Metzberg, 1974).

The cause of these 'slime' defects is not clear. The observations performed using recombinants from a 'slime' $\times$ wild-type cross did not reveal with certainty whether a mutation different from *os-1*, *sg* and *fz*, which are essential for the wall-less phenotype, was the cause of the abnormal regulation of catabolic enzymes. However, if the hypothetical mutation existed, it is clear that it could be only expressed in a stable, self-propagating, 'slime' phenotype.

The mechanism by which a stable 'slime' phenotype arises is rather puzzling. The very few ascospores from crosses with the 'slime' strain which germinated into plasmodioid outgrowth ('slime-like'; Emerson, 1963) invariably developed mycelium in media of normal osmolarity. In order to recover, rarely, a stable 'slime' phenotype, several cycles of vegetative propagation and enrichment in liquid and solid medium of high osmolarity are necessary (Nelson *et al.*, 1975). Vegetative selection permits, occasionally, the derivation of clones which actively proliferate as pure spheroplasts regardless of osmolyte concentration (Emerson, 1963). Such phenotypic change involves drastic alterations in morphology, growth habit, cell-wall-organizing ability and several other characteristics. The mechanisms underlying this phenomenon are obscure and deserve more investigation. One possible explanation is that vegetative propagation selects secondary mutations influencing the conversion of 'slime-like' cells into stable 'slime' ones. However, this seems unlikely because Emerson (1963) demonstrated by genetic analysis that all essential mutations required for the expression of a stable 'slime' phenotype exist in 'slime-like' isolates. Furthermore, if other mutations different from *fz*, *sg* and *os-1* determine the expression of 'slime', the inheritance of this phenotype in subsequent crosses should improve, but this has not been demonstrated (Emerson, 1963; authors' unpublished observations). No doubt the genetic basis of 'slime' would benefit from more investigation, for example of the *fz* and *sg* loci, which have not been mapped, or of the biochemical effects of *os-1*.

The reason why phenotypic expression of 'slime' always requires a prolonged vegetative selection is not clear, but it might represent a gradual change occurring at the cell surface level, for instance, occasional loss of regulatory elements which participate in the expression of a number of cell functions such as organization of the cell wall, recognition of environmental signals, etc., which in 'slime' strains appear to be radically altered.

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