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Mutation in *Neurospora crassa* Affecting Some of the Extracellular Enzymes and Several Growth Characteristics

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A mutant of *Neurospora crassa*, strain T9, which shows reduced activity of glucoamylase, was isolated. The mutant glucoamylase was not altered in pH optimum, or thermostability, but altered in elution profile of Bio-Gel filtration. The T9 mutation led to other distinct phenotypic effects: a poor growth response, resistance to the effect of sorbose, a high activity of extracellular acid phosphatase, and an increased sensitivity to high osmolarity of the growth medium. The discussion was based on the idea that the T9 mutation occurred at a gene governing the synthesis of cell wall precursors and resulted in an alteration in various characteristics related to extracellular enzymes and growth.

There are several known enzymes excreted by mycelial cells of *Neurospora crassa*, and several attempts have been made to elucidate the genetic mechanism of enzyme secretion. Gratzner and Sheehan (8, 9) described a mutant exhibiting cell wall alterations due to a single gene mutation associated with the hyperproduction of extracellular α-amylase (α-1,4-glucan 4-glucanohydrolase, EC 3.2.1.1); glucoamylase (α-1,4-glucan glucohydrolase, EC 3.2.1.3), invertase (β-D-fructofuranoside fructohydrolase, EC 3.2.1.26), and trehalase (α,α'-glucoside 1-glucohydrolase, EC 3.2.1.28). Trevithick and Metzenberg (22, 23) have studied the process of excretion of invertase in *Neurospora* and suggested that invertase is secreted through pores in the cell wall. The mechanism of enzyme secretion related to cell wall synthesis has been precisely investigated with yeast protoplasts which produce extracellular invertase containing mannan and glucosamine (7).

The present report describes the studies on a mutant that is altered simultaneously both in activities of some extracellular enzymes and in several growth characteristics.

MATERIALS AND METHODS

Organisms. The following strains of *N. crassa* were used: wild-type strains (76A and 3.1a), *ad-6* (74A-Y234-M200), *ad-7* (74A-Y186-M423), and *os-1* (Em11400). A mutant of *N. crassa*, T9 (76A-T9-M150), which produced a significantly low level of extracellular amylase, has been isolated by spreading mutagenized wild-type conidia on the surface of an agar medium supplemented with 0.1% starch and 1.0% sorbose and observing whether a clear zone was formed around any colony after spreading an iodine solution. Double mutants were prepared from appropriate crosses of the single mutants. Forced heterokaryon cells were obtained by inoculating the mixture of conidia of two strains on a minimal agar medium. The *os-1* mutant was kindly supplied from the Fungal Genetics Stock Center, Arcata, Calif.

Growth measurement. To test growth in various media, a 0.2-ml portion of conidial suspensions (optical density at 620 nm, 0.100) of the strain was inoculated into 15 ml of liquid media in 100-ml Erlenmeyer flasks and incubated at 25 C. Mycelial mats were harvested after designated times, dried, and weighed. Growth was indicated as the milligram dry weight of mycelia thus obtained.

Preparation of culture filtrates and crude extracts. Mycelia were grown at 25 C for the designated times in 1,000-ml Roux bottles containing 100 ml of liquid medium. Mycelial mats were harvested on a Buchner funnel. The filtrates were used as the extracellular enzyme samples. The mycelial mats were washed several times with deionized water and frozen quickly. The frozen mycelia were ground for 5 min with a homogenizer in 0.005 M acetate buffer, pH 5.2, and further disrupted with a French pressure cell at an average pressure of 400 kg per cm^2. The resulting homogenate was centrifuged at 24,000 $\times$ *g* for 60 min, and the supernatant liquid was used as a crude extract.

Chromatography. To obtain samples for chromatography, a 707-g amount of ammonium sulfate was stirred into 1,000 ml of the culture filtrate or crude

extract, the pH was adjusted to 6.0, and the solution was kept at 4 C for 12 h and centrifuged at 12,000 × *g* for 40 min. The precipitate was dissolved in a small volume of 0.005 M acetate buffer, pH 5.2, and dialyzed against 0.001 M acetate buffer, pH 5.2, before loading on the column. The diethylaminoethyl (DEAE)-cellulose column (3.0 by 30 cm) was equilibrated with 0.005 M tris(hydroxymethyl)aminomethane (Tris)-maleate buffer, pH 7.0. A continuous gradient elution was carried out with NaCl from 0 to 0.4 M. A gel filtration was performed through Bio-Gel P-300 (100 to 200 mesh). The gel column (1.0 by 100 cm) was equilibrated and eluted with 0.002 M acetate buffer, pH 5.2.

Enzyme and protein assays. The starch decomposing activity of amylase was measured by the iodine-starch reaction as described by Hagiwara (10). Amylase activity leading to the production of reducing sugars was measured by the method of Bernfeld (2) using 0.005 M acetate buffer, pH 5.2. One unit of amylase activity was defined as the amount of enzyme that produced 1 μmol of glucose per min. The procedure for the invertase and trehalase assays were the same as for the amylase assay except that 1% sucrose or 1% trehalase was used as the substrate in place of 1% starch. One unit of invertase, or trehalase is the amount of the enzyme that produced 2 μmol of glucose-equivalent reducing sugar per min. Acid phosphatase (orthophosphoric monoester phosphohydrolase, EC 3.1.3.2) activity was assayed by measuring the amount of *p*-nitrophenol produced from *p*-nitrophenyl-phosphate (19). One unit of acid phosphatase was defined as the amount of enzyme that produced 1 μmol of *p*-nitrophenol per min.

Protein concentration was determined by the method of Lowry et al. (14).

Chemicals. The following chemicals were obtained commercially: DEAE-cellulose (Brown Co.), Bio-Gel (Bio-Rad Ind.), soluble starch (Merck), sucrose and trehalose (Wako Pure Chem. Co.), and ^{14}C-glucose (Dai-ichi Pure Chem. Co.).

RESULTS

Growth characteristic of mutant T9. At the early stage of growth in a minimal medium containing 1.5% glucose, the growth of strain T9 was slower than that of the wild type (Fig. 1). On glucose minimal medium the growth pattern for the forced heterokaryon cells prepared between strains T9 *ad-7* and *ad-6* was approximately the same as that of the wild type rather than strain T9 (Table 1). This indicates that the altered growth character of strain T9 is recessive. The growth of the wild type was slightly reduced in Fries minimal medium containing 1.5% glucose and 3% NaCl, whereas that of strain T9 was significantly inhibited at the early stage of growth in the same medium (Fig. 1). The growth inhibition by NaCl of strain T9 was similar at early stages of growth to that of one of the osmotic mutants, *os-1*, which is reported as a mutant defective in cell wall structure (4, 13).

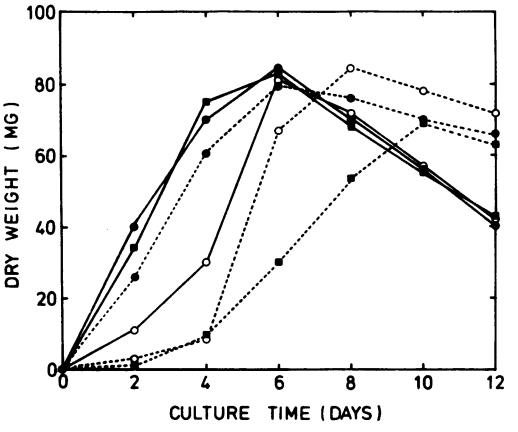

FIG. 1. *Growth of the wild type and strains T9 and os-1 in the minimal medium containing glucose. Growth is indicated as milligrams (dry weight) of mycelia grown in a 100-ml flask containing 15 ml medium with 3% NaCl (dashed line) and without 3% NaCl (solid line) at 25 C. Symbols: ●, wild type; ○, strain T9; ■, strain os-1.*

TABLE 1. *Growth response of forced heterokaryon between strain T9 and the wild type*[a]

Strain	Culture period (days)	Dry weight of mycelia (mg)		
		(A) Grown without 3% NaCl	(B) Grown with 3% NaCl	Ratio (B/A)
T9 *ad-7*	2	19.0	4.2	0.22
	4	54.0	35.5	0.66
	6	67.0	54.1	0.81
T9 *ad-7* + *ad-6*	2	36.4	23.3	0.64
	4	75.8	61.7	0.91
	6	87.4	73.7	0.84
ad-6	2	34.4	28.0	0.81
	4	74.0	70.5	0.95
	6	78.8	79.0	1.00

[a] Strains T9 *ad-7*, *ad-6*, and heterokaryon (T9 *ad-7* + *ad-6*) were grown in 100-ml flasks containing 15 ml of sucrose minimal medium (adenine sulfate, 100 μg/ml, was supplied for strains T9 *ad-7* and *ad-6*) with and without 3% NaCl at 25 C.

For the forced heterokaryon cells prepared between strains T9 *ad-7* and *ad-6*, the sensitivity to high osmolarity was intermediate to the parent strains (Table 1).

It is well known that L-sorbose added to the growth medium causes wild-type mycelia of *Neurospora* to grow as very restricted colonies on an agar plate (21). Figure 2 shows the typical growth patterns of the wild type and strain T9

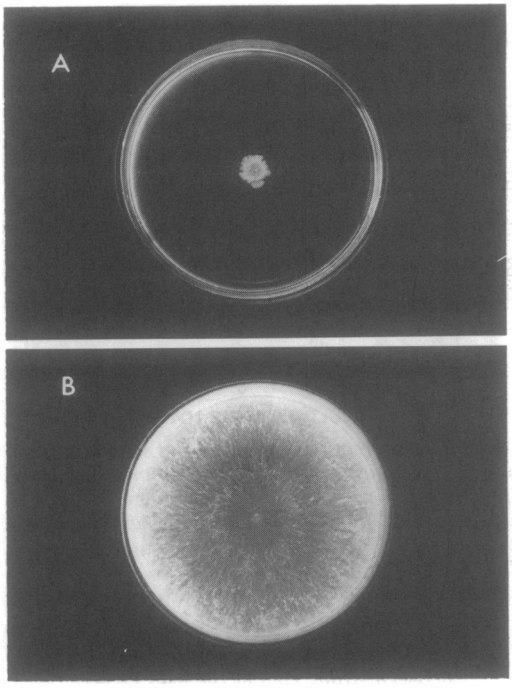

FIG. 2. *Effect of sorbose on growth of the wild type and strain T9. The wild type (A) and strain T9 (B) were grown on minimal agar medium containing 1.0% sorbose and 0.2% sucrose at 25 C for 5 days.*

on a minimal agar medium which contains 1.0% sorbose and 0.2% sucrose as the carbon sources. The T9 strain grown on sorbose showed normal growth behavior in contrast to the restricted colonial growth of the wild type. This result indicates that the strain T9 is resistant to the sorbose effect. Forced heterokaryon cells prepared between strains T9 *ad-7* and *ad-6* were not sorbose resistant. That is, the sorbose resistance in the strain T9 is recessive.

Amylase activity of the wild type and T9. Wild-type and T9 mycelia were grown on sucrose, and the amylase activities of the culture media and crude mycelial extracts were assayed. Throughout the culture period examined significant differences in the activities of extracellular amylase were observed between the wild type and strain T9 (Fig. 3A). After 12 days of cultivation, the extracellular amylase activity of strain T9 was about one-sixth of that of the wild type. The cell-bound amylase activity in both strains reached a plateau after 6 days of cultivation, and the activity of strain T9 at that time was approximately one-half that of the wild type (Fig. 3B).

The extracellular amylase activity of forced heterokaryon cells prepared between strains T9 *ad-7* and *ad-6* was measured over a 12-day period. As indicated in Fig. 3A, the amylase activity of the heterokaryon was always an intermediate level between the wild type and strain T9, although the growth of such a heterokaryon was the same as that of the wild type (Table 1).

Two kinds of amylase, α-amylase and glucoamylase, are known to exist in the mycelial extract of *Neurospora* (24). The amylase was fractionated to identify and characterize the amylase species responsible for the reduced amylase activity observed in strain T9. Culture filtrates of the wild type and strain T9 grown on sucrose for 14 days were fractionated by DEAE-cellulose column chromatography (Fig. 4A and C). The amylase activity of each strain was recovered as a single component which was eluted without adsorption to DEAE-cellulose. Crude mycelial extracts of the wild type and of strain T9 were also fractionated by DEAE-cellulose column chromatography, and two major components were found in the amylase assay (Fig. 4B and D). The first component to be eluted corresponds to that found in the medium; the second component was eluted with 0.2 M NaCl. Comparing the chromatographic patterns of the culture filtrates and crude extracts of the wild type and strain T9, the first

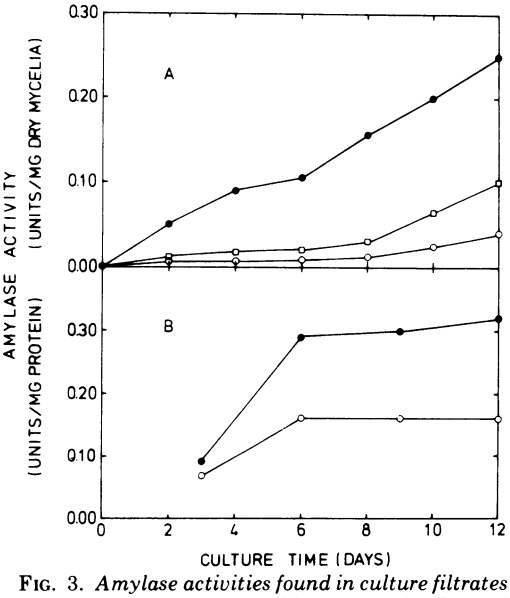

FIG. 3. *Amylase activities found in culture filtrates and crude extracts of the wild type, strain T9, and heterokaryon. Culture filtrates (A) and crude extracts (B) were prepared from cultures grown in 100-ml flasks containing 15 ml of minimal medium with 1.0% sucrose for various periods. Amylase activity was assayed by measuring production of glucose. Symbols: ●, wild type; ○, strain T9; □, heterokaryon (T9 ad-7 + ad-6).*

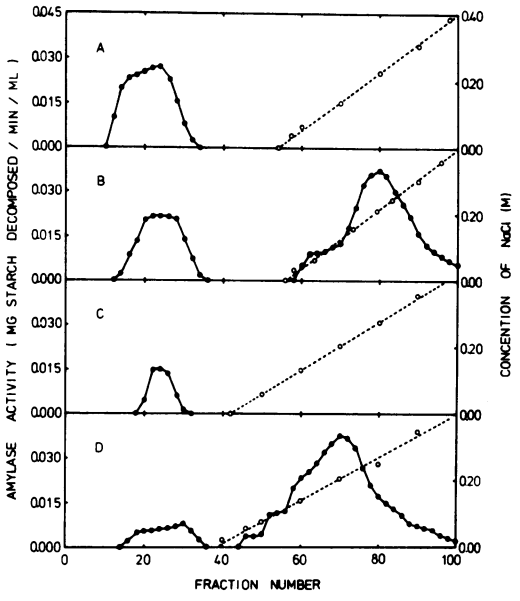

FIG. 4. *DEAE-cellulose chromatography of amylase from wild type and strain T9. Crude extracts and culture filtrates were obtained from a 14-day-old culture. A 40-ml amount of crude extract (80 mg of protein) or a 50-ml amount of culture filtrate (7.5 mg of protein) was put onto a column and eluted with 0.005 M Tris-maleate buffer, pH 6.0, containing NaCl (0 to 0.4 M) into 4-ml fractions. Each fraction was assayed for amylase activity by starch-iodine reaction (solid line) and titrated for concentration of NaCl by Mohr's method (dashed line). A, Culture filtrate of the wild type; B, crude extract of the wild type; C, culture filtrate of strain T9; D, crude extract of strain T9.*

component of amylase activity was always smaller in strain T9 but the second component showed approximately the same mobility and amount as the wild type.

Two methods have been employed to assay amylase activity in both the culture filtrates and the fractions corresponding to the two components found in DEAE-cellulose column chromatography of a mycelial extract of the wild type (Table 2). The culture filtrate and the first component from the mycelial extract showed significantly higher ability to produce reducing sugar than did the second component. The products obtained after the decomposition of starch with the culture filtrate and the first component from the mycelial extract of the wild type were individually identified by paper chromatography using a *n*-butanol-acetic acid-water system (4:1:5, vol/vol/vol) as single components which correspond to glucose. No detectable components corresponding to maltose were found in these experiments. These results indicate that the amylase found in either the culture filtrate or the early eluted component on DEAE-cellulose column chromatogram of the mycelial extract is glucoamylase which hydrolyzes the nonreducing terminals of the starch molecules to generate glucose.

TABLE 2. *Starch-decomposing activity and reducing sugar-producing activity of amylase samples obtained from culture filtrate and mycelial extract of the wild type*

Enzyme sample	(A) Starch decomposed[a]	(B) Reducing sugar produced[b]	Ratio (B/A)
Filtrate[c]	3.50	3.82	1.09
First peak of DEAE-cellulose chromatogram[d]	0.87	0.79	0.91
Second peak of DEAE-cellulose chromatogram[d]	1.43	0.06	0.04

[a] Milligrams of starch decomposed by 1 ml of enzyme solution in 1 h.

[b] Milligrams of glucose produced by 1 ml of enzyme solution in 1 h.

[c] The culture filtrate of the wild type grown in a 100-ml flask containing 15 ml of sucrose minimal medium at 25 C for 14 days.

[d] The first and second components found on DEAE-cellulose chromatogram (Fig. 4) of crude extract of the wild type grown in Roux bottles containing 100 ml of sucrose minimal medium at 25 C for 14 days.

These results suggest that *Neurospora* mycelia contain two kinds of amylase, α-amylase and glucoamylase, that the mycelia excrete the latter into the culture medium, and that the T9 mutation results in a reduced activity of glucoamylase.

Comparison of glucoamylase from the wild type and strain T9. The molecular sizes of extracellular glucoamylase and invertase from the wild type and strain T9 were compared by Bio-Gel filtration. Invertase of *Neurospora* is eluted as two components. The molecular weight of the first is 210,000 and it is dissociated into the second under alkaline conditions (17, 18). Glucoamylase obtained from culture filtrate or crude extract of the wild type was eluted as a single component behind the second fraction of invertase (Fig. 5A). In contrast to the single large component found for the wild-type enzyme, glucoamylase obtained from culture filtrate of strain T9 was eluted as one, two, or three small components depending on the experiment. In one experiment the mutant glucoamylase was eluted as three components (Fig. 5B and 6A); the first peak (enzyme I) was eluted

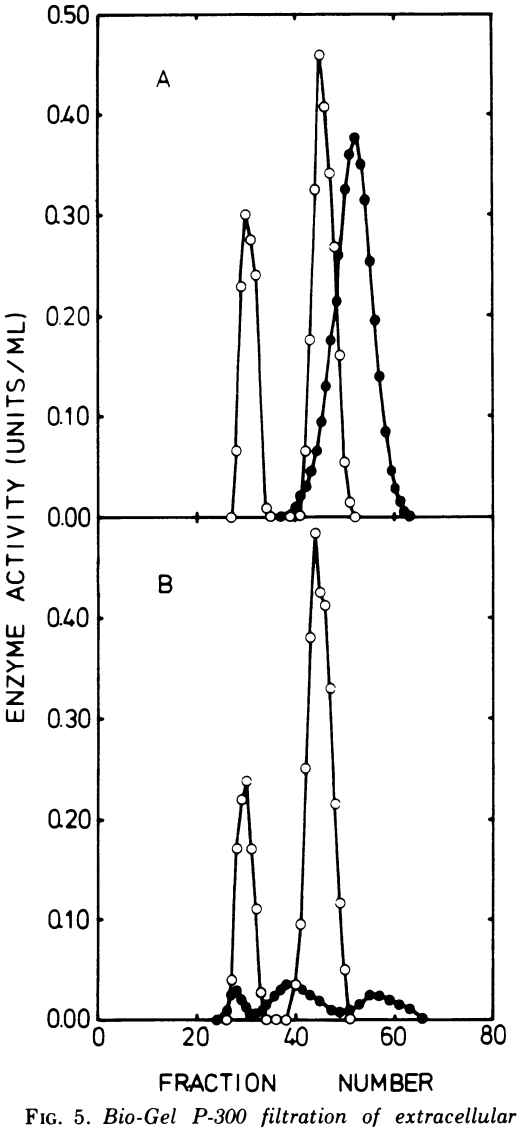

FIG. 5. *Bio-Gel P-300 filtration of extracellular amylase and invertase. Enzyme samples were prepared from the culture filtrates of the wild type (A) and strain T9 (B) grown in 1,000-ml Roux bottles containing 100 ml of sucrose minimal medium at 25 C for 14 days. A 2-ml amount of the sample (6 mg of protein) was put onto a column and eluted with 0.002 M acetate buffer, pH 5.2, into 2-ml fractions. Each fraction was assayed for amylase activity and invertase activity by measuring production of glucose. Symbols: ●, amylase; ○, invertase.*

ahead of the first component of invertase, the second (enzyme II) was eluted in between the two invertase fractions, and the third (enzyme III) was eluted at the same position as that of the wild type. In five independent experiments the glucoamylase activity of strain T9 was distributed between enzymes II and III (Fig. 6B), in one experiment it was eluted as two fractions at the positions of enzymes I and III (Fig. 6C), and in one experiment it was eluted as a single component at the position of enzyme I (Fig. 6D). No such heterogeneity of molecular sizes has been encountered in five independent experiments fractionating glucoamylase from the wild type.

No significant difference in the pH optimum was observed among glucoamylases obtained from the wild type and enzymes II and III of strain T9. Also, no significant difference in the stability at 60 C was observed among these enzyme samples. The heat inactivation of these enzyme samples followed first-order kinetics with a half-life of about 10 min.

Comparison of other extracellular enzymes from the wild type and strain T9. Three extracellular enzymes of *Neurosora*, concerned with carbohydrate metabolism, amylase, invertase, and trehalase, have been reported to be coordinately derepressed (8, 9). In connection with this, the effect of the T9 mutation on the activities of these enzymes was investigated. As

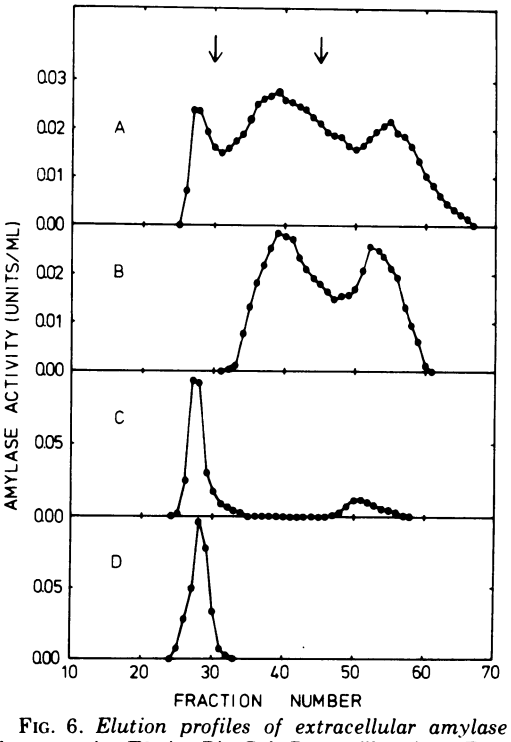

FIG. 6. *Elution profiles of extracellular amylase from strain T9 in Bio-Gel P-300 filtration. Four enzyme samples (A to D) were prepared independently and chromatographed by the same methods as those used in Fig. 5. Arrows indicate the positions where invertase was eluted.*

shown in Fig. 7, the activities of invertase and trehalase of strain T9 were lower than those of the wild type. This may be due to longer lag period of growth of strain T9 than that of the wild type (Fig. 1). Contrarily, the activity of acid phosphatase in the culture filtrate of strain T9 was higher than that of the wild type (Fig. 7). The molecular sizes of trehalase, invertase, and acid phosphatase obtained from culture filtrates were compared between the wild type and strain T9. Trehalase was eluted as a single component from a Bio-Gel P-300 filtration, and acid phosphatase was eluted as a single component between the first fraction of invertase and trehalase (Fig. 8A). The elution positions of these enzymes from strain T9 are identical with those from the wild type (Fig. 8).

Incorporation of glucose. The T9 mutant showed a lower growth response than the wild type in a minimal medium containing glucose (Fig. 1). To test the possibility that sugar transport is damaged in strain T9, the ability to incorporate ^{14}C-glucose was compared between conidia of the wild type and strain T9 by the method of Marzluf and Metzenberg (16). As shown in Fig. 9, no significant difference in the ability to incorporate ^{14}C-glucose was observed between the wild type and strain T9.

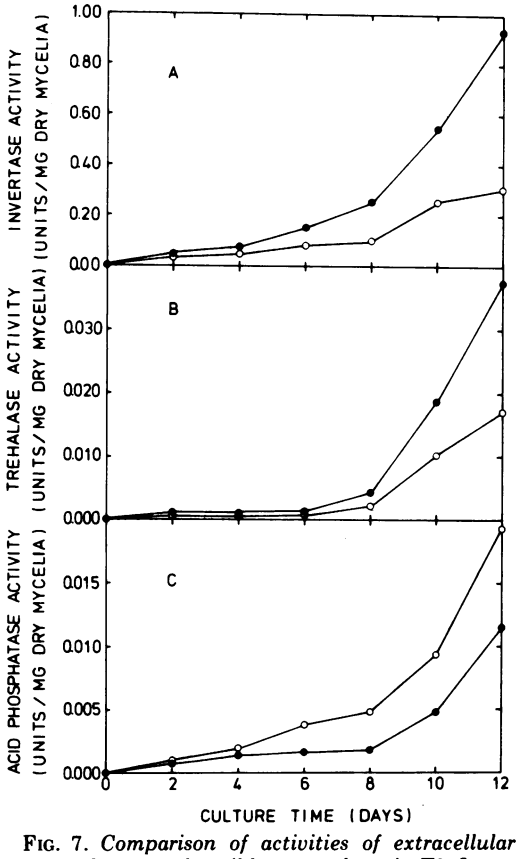

FIG. 7. *Comparison of activities of extracellular enzymes between the wild type and strain T9. Invertase (A), trehalase (B), and acid phosphatase (C) activities in the culture filtrates from the wild type and strain T9 grown in 100-ml flasks containing 15 ml of sucrose minimal medium at 25 C for various periods. Enzyme activities are indicated as units per milligram of mycelia (dry weight) in the culture. Symbols: ●, wild type; ○, strain T9.*

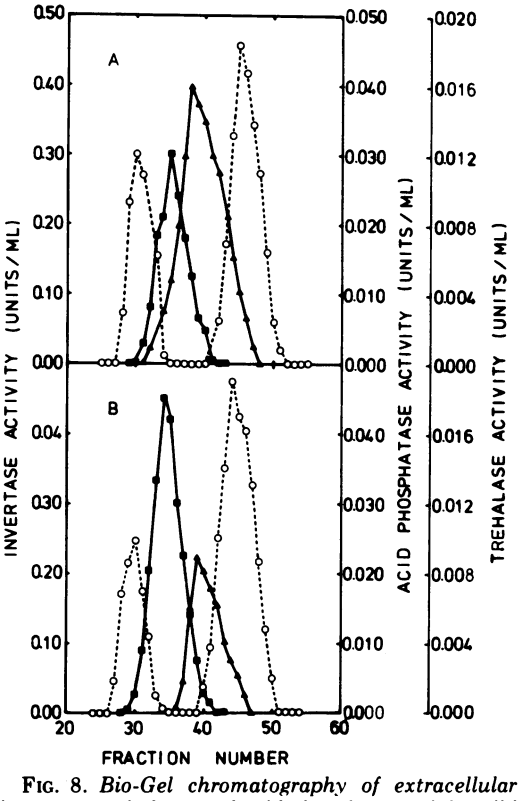

FIG. 8. *Bio-Gel chromatography of extracellular invertase, trehalase, and acid phosphatase of the wild type and strain T9. Enzyme samples and methods were same as those used in Fig. 5. A, Wild type; B, strain T9. Symbols: ○, invertase; ▲, trehalase; ■, acid phosphatase.*

Effect of the T9 mutation on cell wall material. Cell wall material was prepared from lyophilized mycelia of the wild type and strain T9 as described by Mahadevan and Tatum (15). As shown in Table 3, no significant difference in the yield of cell wall material was found between the wild type and strain T9. The cell wall materials thus obtained were fractionated into four fractions (F-I to F-IV) by the method of Mahadevan and Tatum (15). Although considerable variations in the amount of each fraction appears to be inevitable in this experiment, as was also recognized by Mahadevan and Tatum

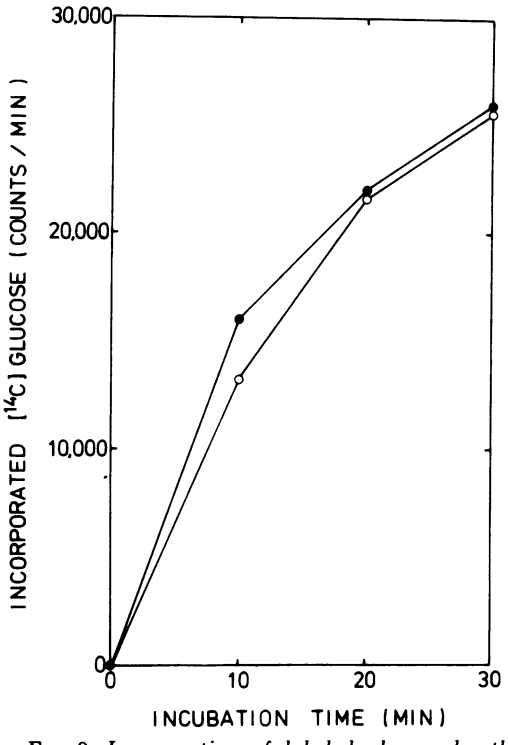

FIG. 9. *Incorporation of labeled glucose by the wild-type and strain T9 conidia. The mixture containing 0.5 ml of 0.05 M sodium succinate buffer, pH 5.0, 0.2 ml of glucose-1-^{14}C solution (135,450 counts per min per ml), and 0.3 ml of conidial suspension (optical density at 420 nm, 3.33) was incubated at 37 C with shaking. At various time intervals (0 to 30 min), a 5-ml amount of water was added to the mixture, and after rapid mixing conidia were collected on a prewetted membrane filter (Millipore Corp.) and washed six times with 5-ml portions of water. The radioactivity of conidia dissolved in the scintillation fluid was counted. Glucose incorporation is indicated as total counts of glucose incorporated into 0.3 ml of conidial suspension under the condition described above. Symbols: ●, wild type; ○, strain T9.*

(15), no significant difference in percentage of each fraction was found between the wild type and strain T9 (Table 3).

Genetic analysis of the T9 locus. The phenotypes exhibited by isolates from a cross between the wild type and strain T9 were examined in special reference to poor growth in hypertonic medium, sorbose resistance, and low amylase activity. Of 101 random spores tested, 59 isolates showed the T9 phenotype and 42 isolates showed the wild-type phenotype. No segregation of these T9 characteristics was observed. From these data, it appears that the T9 characteristics are the result of a single mutation at one locus.

Genetic analysis was performed to determine the linkage relationship between the T9 mutation and known markers. It was found that the T9 mutation was located 5 units from the centromere, 6 units proximal to the mating type locus on the left arm of linkage group I.

DISCUSSION

The T9 mutant was obtained as a mutant defective in an extracellular amylase. The wild-type mycelia produce two kinds of amylase, α-amylase and glucoamylase; α-amylase was detected in mycelial extract and glucoamylase was detected in mycelial extract and culture medium. Glucoamylase of *Neurospora* was first found by Tsujisaka and Fukumoto (24). This enzyme exolytically hydrolyzes both α-1,4 and α-1,6 linkages from the nonreducing end of starch molecules producing glucose. This is in contrast to α-amylase which endolytically hydrolyzes α-1,4 linkages in starch molecules finally to produce maltose. The T9 mutant showed significantly reduced activity of glucoamylase in both the mycelia and the culture medium.

The molecular size of the glucoamylase obtained from the wild type was smaller than the second peak of invertase described by Meachun et al. (17), whereas glucoamylase obtained from strain T9 showed heterogeneity in the Bio-Gel elution profile. At most three molecular species of T9 glucoamylase were separated on its Bio-Gel elution profile. One or two species of T9 enzyme disappeared depending on the experiment. Heterogeneity in the molecular form of extracellular glucoamylase of strain T9 may result from differences in the degree of autolysis. However, it is difficult to explain by this mechanism why only one component of glucoamylase of smaller molecular weight was found in crude mycelial extracts prepared from 7-day-old, wild-type cultures.

Besides the altered characteristics of glucoamylase activity, the T9 mutation led to the following distinct characteristics. (i) The T9 mutation resulted in a reduced growth ability on minimal medium. The poor growth of strain T9 may not be due to the inability to incorporate the carbon source into cells, because the ability to incorporate glucose into T9 cells was not different from that of the wild-type cells. (ii) The T9 mutant was resistant to the effect of sorbose which is known to alter the colonial morphology in the wild type. (iii) The growth of strain T9 was inhibited by high osmotic pressure from the culture medium. (iv) The T9 mutation resulted in higher activity of extracellular acid phosphatase than the wild type. This

TABLE 3. *The yield of cell wall material from lyophilized mycelia of the wild type, 76A, and strain T9, and amount of cell wall fractions*[a]

Strain	Carbon sources	Yield of cell wall[b]	F-I[c]	F-II[d]	F-III[e]	F-IV[f]
76A	Sucrose (1.5%)	22.1	13.8	24.4	21.8	19.7
	Glucose (1.0%) + Fructose (0.5%)	20.8	16.4	26.5	23.2	16.0
	Glucose (0.3%) + Fructose (1.2%)	23.9	14.7	17.6	21.9	17.4
T9	Sucrose (1.5%)	20.4	14.6	20.4	23.3	13.8
	Glucose (1.0%) + Fructose (0.5%)	20.5	15.0	24.6	27.6	15.8
	Glucose (0.3%) + Fructose (1.2%)	19.0	18.3	20.4	22.7	18.3

[a] Cell wall material was prepared from lyophilized mycelial powder by treatment with 1% sodium dodecyl sulfate. The cell wall material thus obtained was fractionated into four fractions (F-I to F-IV) as described by Mahadevan and Tatum (15).

[b] Percentage of lyophilized mycelia.

[c] Lyophilized soluble fraction obtained after the first treatment with 2 N NaOH for 16 h. Percentage of cell wall material.

[d] Glucose in soluble fraction obtained after 1 N H_2SO_4 treatment at 90 C of the portion resistant to the first NaOH treatment. Percentage of cell wall material.

[e] Lyophilized soluble fraction obtained after the second 2 N NaOH treatment for 30 min of the portion resistant to the 1 N H_2SO_4 treatment. Percentage of cell wall material.

[f] Lyophilized fraction resistant to the second 2 N NaOH treatment of the fraction resistant to the 1 N H_2SO_4 treatment. Percentage of cell wall material.

evidence suggests that the T9 mutation may not occur at the structural gene of glucoamylase. In relation to the characteristics of strain T9 described above, it has been shown that the sorbose-induced colonial morphology and the resistance to the sorbose effect in *Neurospora* are accompanied by changes in chemical composition of the cell wall (3, 15). Furthermore, a characteristic of the *os-1* mutant, sensitivity to a high osmolarity of the culture medium, results from an altered composition of the cell wall (13). From these studies, it is suggested that the T9 mutation causes primarily an alteration of the cell wall structure and, as a secondary pleiotropic effect, this results in the altered growth characteristics such as the slow growth, the resistance to sorbose, and the sensitivity to high osmolarity.

The assumption that the cell wall structure of strain T9 is altered may further suggest that the activity of extracellular enzymes such as glucoamylase and acid phosphatase may be modified in strain T9 in relation to the alteration of cell wall structure. Several studies on extracellular enzymes showed that the mechanism of enzyme secretion is related to the cell wall structure (8, 22, 23). In addition, Eylar (5) suggested that the extracellular proteins are glycoproteins, and Andrews (1) showed that the apparent molecular weights of some glycoproteins estimated by gel filtration are significantly larger than those estimated by other methods, depending on the carbohydrate content of the glycoproteins. Therefore, it may be postulated that glucoamylases I and II of the mutant may be differently glycosylated forms of the wild-type enzyme as a result of an abnormality in the regulation of cell wall biosynthesis. It is known that restriction of the synthesis of a particular carbohydrate moiety of the glycoprotein exerts a control on the synthesis of the active enzyme by inhibition of the formation of the protein moiety (6, 11, 12, 20). Part of the evidence against this idea may be the fact that no significant difference in cell wall composition has been detected between the wild type and strain T9 by following the fractionation methods of Mahadevan and Tatum (15).

An alternative assumption is that the T9 mutation causes primarily an alteration of cell membrane and, as a secondary effect, this results in alterations in sorbose uptake, the sensitivity to high osmolarity, and the production of extracellular enzymes.

Further work on this mutant is obviously necessary to elucidate the mechanism for the alteration of the cell wall metabolism and the molecular structure of T9 glucoamylase.

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