ISOLATION AND CHARACTERIZATION OF NEUROSPORA MUTANTS AFFECTED IN INVERTASE SYNTHESIS

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ABSTRACT

We have outlined a procedure that allows the large-scale screening of mutagenized *Neurospora crassa* populations for invertaseless mutants. We have isolated and characterized three mutations, *inv(DBL1)*, *inv(DBL9)* and *inv(DBL14)*, which have been mapped at or near the invertase structural gene. One of these, *inv(DBL1)*, is particularly interesting. Our experiments indicate that the reduced level of invertase activity in the *inv(DBL1)*-containing cell can be explained as the result of a reduced number of normal enzyme molecules. We also show that wild-type Neurospora is able to respond rapidly to a change of medium and can dramatically increase its production of invertase within 20 min after a transfer to a carbon-free medium.

SEVERAL extracellular enzymes are synthesized and secreted by *Neurospora crassa* and other fungi in response to carbon deprivation. These include invertases, trehalases, maltases and amylases. These extracellular enzymes are required for growth on sucrose, trehalose, maltose and starch. They are "derepressed" whenever the cell finds itself in a medium having a suboptimal carbon/energy source. The mechanisms that control the production and secretion of these exoenzymes have been of interest to many investigators. Mutations that lead to altered posttranslational modification of these enzymes will often affect their levels (GRATZNER 1972; MURAYAMA and ISHIKAWA 1973). Mutations affecting a single exoenzyme have been used to map the invertase structural gene of *N. crassa* (SARGENT and WOODWARD 1971) and the invertase, α-glucosidase, and maltase structural genes of *Saccharomyces cerevisiae* (MORTIMER and HAWTHORNE 1969).

The only previously isolated Neurospora invertaseless mutant was isolated fortuitously (SARGENT and WOODWARD 1971), and attempts to isolate other invertaseless mutants have failed. We report a screening procedure that can be used for the isolation of *N. crassa* mutants affected in the production of the exoenzyme invertase. The procedure can be used to isolate mutants affected in the production of several of the exoenzymes of Neurospora. Among the mutations characterized is one of particular interest. This mutation allows for the production of a much reduced level of apparently normal enzyme. We report on the characterization of this mutation, *inv(DBL1)*.

MATERIALS AND METHODS

Strains: A temperature-sensitive colonial strain (R501 that carries *cot-1*) obtained from R. L. METZENBERG was used as the parental strain for mutant isolation and initial mutant characterization. In characterizing the invertase produced by the different mutants, we felt it imperative to move the mutations into a wild-type background so as to avoid the problems of other mutations that may be present in the original isolates and to characterize the enzyme from a strain that is free of the *cot-1* mutation. Thus, for most experiments mutants were outcrossed to the wild-type strains, 74-OR23-1A or 74-OR8-1a, and progeny selected that contained the invertaseless mutation in a wild-type background. For these experiments the wild-type strains were used as controls. Mapping was done as described by DAVIS and DESERRES (1970) using strains 3434 and 3661 from the Fungal Genetics Stock Center (containing alcoy *csp-2*) (PERKINS and BJORKMAN 1979), strain R503 (an *al-2 inl* strain obtained from R. L. METZENBERG) and strain R108 (an *al-3 inv* strain obtained from R. L. METZENBERG) which contains the previously isolated invertase structural gene mutation which we designate as *inv(724-2)* (SARGENT and WOODWARD 1971). Vogel's N medium was used throughout with 2% glucose, xylose or sucrose serving as the carbon source (DAVIS and DESERRES 1970).

To test for complementation between different invertaseless mutations "forced" heterokaryons were used. We allowed an *inl inv(724-2)* strain and a second strain containing the invertaseless mutation of interest in the *cot-1* background to grow together and conidiate. Heterokaryons were selected by plating out conidia on an agar medium of inositol-free Vogel's N medium containing glucose as a carbon source and selecting for colonies that grew in a normal mycelial fashion. By maintaining the colonies under these conditions which select against each parent, we can "force" the mycelium to remain heterokaryotic. At the conclusion of the experiment we isolated both of the parental strains from the heterokaryon to verify that the heterokaryon contained nuclei from both of the invertaseless mutants.

To test for the presence of translocations in an isolate we used the method suggested by PERKINS (1974). Translocations affect the fidelity of meiosis in Neurospora and lead to the production of an increased number of abnormal ascospores which can be easily identified because they lack the normal black pigmentation. To test for the presence of a translocation in a strain it was crossed to the normal sequence wild-type strain of the opposite mating type, and the ascospores produced were examined under the microscope.

Invertase assay: Invertase was assayed using the procedure of GASCON and LAMPEN (1968). The assay employs glucose oxidase, horseradish peroxidase and *o*-dianisidine in measuring the glucose released by the action of invertase on sucrose.

To characterize the residual invertase activity present in the invertaseless mutant the thermostability of the activity was tested. Aliquots of cellular extracts were heated to 60° for various lengths of time, and the remaining invertase activity was assayed. K_m values were determined by assaying the activity with differing concentrations of sucrose. The results were plotted as described by LINEWEAVER and BURKE (1934).

Mutant isolation: A procedure using glucose oxidase and horseradish peroxidase to assay colonies *in situ* was employed in screening large numbers of mutagenized conidia for invertaseless mutants. *cot-1* macroconidia were mutagenized by a 15-min exposure to UV radiation. Typically, we get 1% survival of the mutagenized conidia. Mutagenized conidia were plated out (40 viable conidia/Petri dish) on Vogel's N medium with xylose as the carbon source and placed at 33° to allow the germinating conidia to grow in a colonial mode. We used about 100 dishes for each screening. After 4 to 5 days of growth the colonies were ready for screening. We added 2 ml of a sterile solution containing 0.1 g of sucrose and 0.02 g of *o*-tolidine and gently tapped each colony with a sterile glass rod to ensure that the sucrose and *o*-tolidine penetrated each colony. After allowing the sucrose and *o*-tolidine to be absorbed by the agar we added an additional 1 ml containing glucose oxidase (10 units) and horseradish peroxidase (10 units) in 250 mM Tris, pH 8.0. The colonies containing invertase activity developed a dark blue halo of oxidized *o*-tolidine within 45 min. Mutants lacking invertase remained without a halo. Control experiments showed that sucrose, *o*-tolidine, peroxidase and glucose oxidase were all necessary for the staining reaction.

Colonies showing no halo were picked and retested using the screening procedure to ensure

that they were invertaseless. Following the second screening the mutants were further characterized individually.

Antibody production: Neurospora invertase was purified as described by MEACHUM, COLVIN and BRAYMER (1971). Pure invertase (500 mg) in complete Freund's adjuvant was injected subcutaneously and intramuscularly into each of two New Zealand white male rabbits. A booster of 500 mg of pure invertase in complete Freund's adjuvant was injected subcutaneously 6 weeks later. The rabbits were bled 10 days after the booster and every 8 to 10 days thereafter until the titer began to decrease. The antiserum obtained was able to immunoprecipitate only a small fraction of the invertase in an extract. However, we were able to precipitate all of the invertase if we used goat antirabbit antibody to form an indirect immunoprecipitate. The immunoprecipitation of invertase was done as described earlier (FREE and METZENBERG 1982) except that polyethylene glycol was present at a concentration of 10% (w/v, molecular weight 8000). The 10% polyethylene glycol precipitates the rabbit IgG and gave results similar to those we have obtained using goat antirabbit antisera or protein A to precipitate the rabbit IgG.

Time course studies: To study the production of invertase following transfer to a carbon-free medium we inoculated Erlenmeyer flasks (500 ml flasks containing 100 ml of medium) with 1 to 2×10^6 conidia/ml of Vogel's glucose medium and incubated with vigorous shaking (150 rpm) at 30° for 18 to 24 h. The cells were collected on a Buchner funnel and washed with 4 volumes of water. They were then placed in Vogel's medium devoid of a carbon source and further incubated with vigorous shaking at 30°. Aliquots were taken at various times, harvested and frozen at −20°. Extracts were prepared by grinding with levigated alumina in a mechanical Dounce homogenizer.

Materials: *o*-Tolidine was purchased from Eastman Kodak Company. All other reagents were obtained from the Sigma Chemical Company.

RESULTS

Isolation of mutants: The procedure used in isolating invertaseless mutants is based on the *in situ* assay of colonies derived from mutagenized spores and is described in MATERIALS AND METHODS. Using the screening procedure we have isolated several mutants impaired in the ability to grow on sucrose as a sole energy/carbon source. We report on the characterization of three of these invertaseless mutants, DBL-1, DBL-9 and DBL-14. The DBL-1 mutant is of particular interest to us. The specific activity of invertase in DBL-1 was found to be 0.053 as compared to a value of 3.54 in a wild-type cell under the same conditions (DBL-1 has 1.5% of the wild-type level of invertase activity). We have not been able to detect invertase activity in DBL-9 and DBL-14. These low levels of invertase are inadequate to allow these mutants to grow on sucrose as a carbon/energy source.

Mapping the mutation in DBL-1 responsible for the reduced level of invertase: In a preliminary mapping experiment in which we crossed DBL-1 with an *al-2 inl* strain, the DBL-1 mutation responsible for the inability to utilize sucrose was located on the right arm of linkage group V at a distance of 13 cM from the inositol locus. This is very close to the site of the previously isolated invertase structural gene mutation (SARGENT and WOODWARD 1971). Thus, we performed a cross between an *al-3 inv(724-2)* strain and DBL-1. The only progeny from this cross able to grow using sucrose as a carbon source would be either those having a recombinational event between *inv(724-2)* and the DBL-1 mutation so as to restore a wild-type sequence or revertants from the two parents. We have been unable to obtain revertants from DBL-1 even after selecting for revertants from 10^7 mutagenized conidia. From 6.5×10^4 ascospores germinated and selected

for the ability to grow on sucrose-containing medium, 20 colonies were obtained. Of these, 17 had the wild-type allele of the *al-3* gene (from the DBL-1 parent). Since we have been unable to revert the DBL-1 mutation these progeny were analyzed as the products of recombinational events. If it is assumed that the three *al-3*-containing colonies also were derived from recombinational events, this gives a map distance of 0.06 cM between the two mutations. This suggests that the two mutations are in the same gene. Thus, we designate the new mutation *inv(DBL1)*. In as much as most of the invertase-containing colonies had the wild-type allele of the *al-3* gene we can conclude that the order of the loci is *al3*, *inv(724-2)*, *inv(DBL1)*. *al-3* is located 15 map units centromeric of *inv*.

Further evidence that the mutation in DBL-1 is an allele of the invertase structural gene comes from the analysis of heterokaryons. We found that both the previously isolated *inv* mutation and *inv(DBL1)* were recessive in forced heterokaryons. Heterokaryons containing nuclei from either of these *inv* strains and from a strain having the wild-type allele of *inv* were able to utilize sucrose as a sole carbon/energy source. A forced heterokaryon was made between an *inv(DBL1)* strain and a strain carrying the previously isolated *inv(724-2)* mutation. *inv(DBL1)* was unable to complement the *inv* mutation. This complementation analysis demonstrates the *inv(DBL1)* is an allele of the invertase structural gene.

Other invertaseless mutants: In addition to DBL-1 two other invertaseless mutants, DBL-9 and DBL-14, contained mutations that mapped to the *inv* locus. In mapping the mutation present in DBL-9 we found that this strain has a translocation, as demonstrated by the presence of a large number of abnormal ascospores. In looking at the invertaseless progeny from a mating of DBL-9 with the wild-type cell, we found that 100% of the invertaseless progeny had a translocation. We conclude that DBL-9 has a translocation in or very near the invertase structural gene.

We have been unable to demonstrate any invertase activity in DBL-14. The mutation in DBL-14 maps at the same locus as the other *inv* mutations. In a cross between DBL-14 and the *al-3 inv(724-2)* strain we found the two mutations to be within 0.1 cM of each other. This suggests that the invertaseless mutation present in DBL-14, *inv(DBL14)*, lies within the invertase structural gene.

Characterizing mutant enzyme activities: Since the strains were isolated following a heavy mutagenesis we crossed them to the wild-type and isolated progeny having the mutations of interest in a wild-type background. We used these strains having the invertaseless mutations in a wild-type background in characterizing the effects of the different mutations upon invertase. As part of the characterization of the mutants we tested the residual invertase activity in our mutant strains for thermolability. The results of such an experiment are shown in Figure 1. We have compared the thermolability of the enzyme produced by DBL-1-203, a strain having the *inv(DBL1)* mutation in a wild-type background, with the wild-type enzyme and have been unable to distinguish between the enzymes from the two cell types. We have repeated these experiments at a number of temperatures and with six different DBL-1-203 and wild-type extracts. We also found a small amount of residual activity in the previously isolated *inv(724-2)* mutant. This activity is thermolabile, having a half-life of 2 min at 60°, consistent with the *inv* locus being the structural gene for invertase (Figure 1).

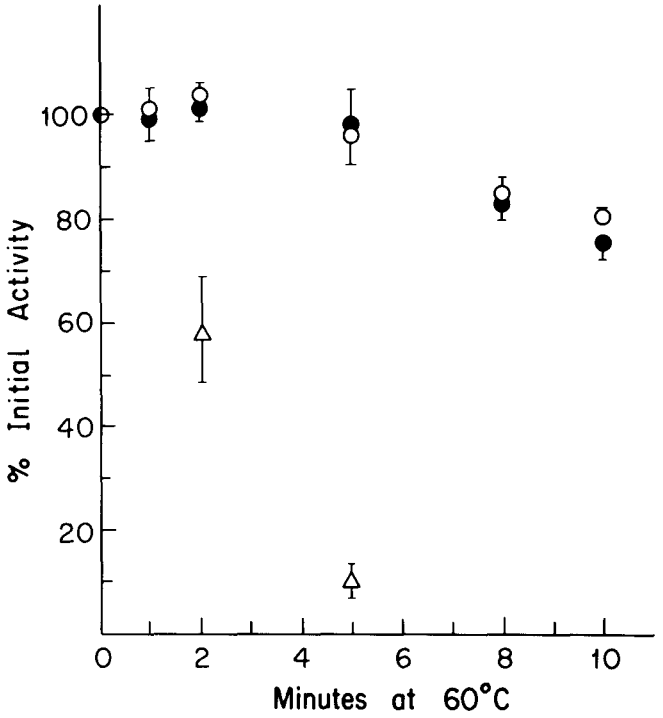

FIGURE 1.—Testing DBL-1-203 invertase for temperature sensitivity. Crude extracts from wild type and DBL-1-203 were incubated at 60° for various lengths of time before being assayed. Wild-type enzyme (○); DBL-1-203 enzyme (●); enzyme from previously isolated *inv* mutant (△).

The DBL-1-203 activity was further characterized with regard to its K_m value. The DBL-1-203 invertase and wild-type enzyme both have a K_m of 12 mM (Figure 2).

We have tested the DBL-1-203 cellular extract for the presence of an invertase inhibitor by mixing DBL-1-203 extracts with wild-type extracts and looking for the loss of invertase activity from the wild-type extract. We have been unable to demonstrate the presence of an inhibitor in DBL-2-203 extracts. In a much as *inv(DBL1)* maps at or near the structural gene and is recessive in heterokaryons, we would not expect an invertase inhibitor to be the cause of the low level of invertase activity in DBL-1-203.

We have also compared the antigenicity of the DBL-1-203 invertase with the wild-type enzyme. Using invertase purified as described by MEACHUM, COLVIN and BRAYMER (1971), we raised rabbit antibody directed against Neurospora invertase. The antiserum obtained was of low titer, and, thus, we used polyethylene glycol to form an immunoprecipitate. We have also used goat antirabbit antisera to form indirect immunoprecipitates and got the same results as when using polyethylene glycol. Figure 3 shows the results of an experiment in which invertase from DBL-1-203 and from the wild-type strain were titered by adding increasing amounts of rabbit antisera to an equal amount of invertase activity from the two sources and adding polyethylene glycol to precipitate the rabbit antibody. We assayed the immunoprecipitates for activity in determining the

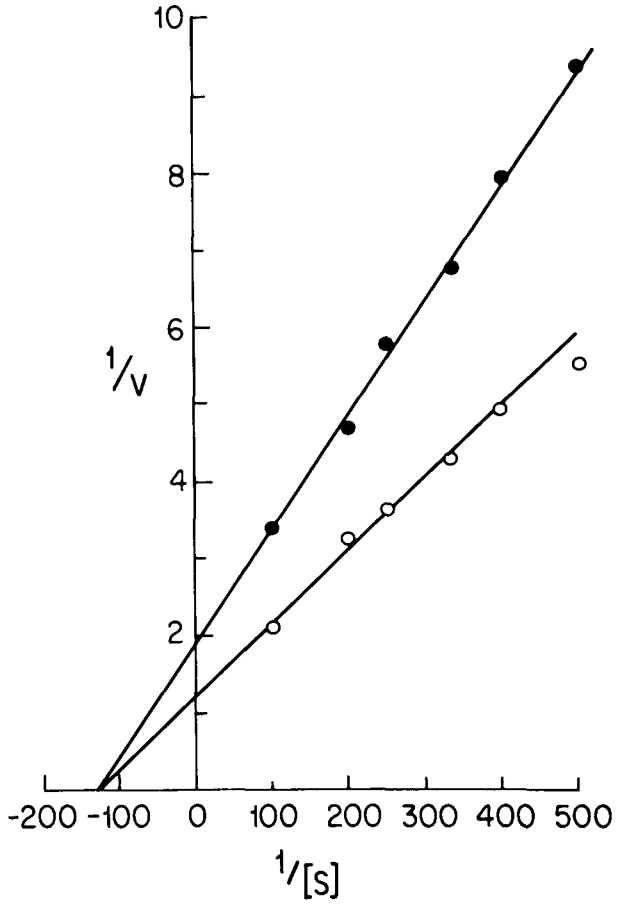

FIGURE 2.—Determination of the Michaelis constant (K_m) values for invertases. Crude extracts were incubated under standard assay conditions with various amounts of sucrose, and the rate of sucrose hydrolysis was determined. The data are plotted as suggested by LINEWEAVER and BURK (1934). Wild-type enzyme (O); DBL-1-203 enzyme (●).

amount of enzyme precipitated. Control experiments showed that the antibody does not affect the enzyme activity and that invertase does not precipitate under the experimental conditions with preimmune antisera. Figure 3 shows that it requires the same amount of rabbit sera to precipitate an equal amount of activity from the two sources. A given amount of our antibody recognizes the same amount of enzymatic activity from DBL-1-203 as from the wild-type cell. These experiments suggest that the mutation of interest affects the number of enzyme molecules produced without affecting the invertase molecule itself.

Time course of invertase production as cells are transferred into a glucose-free medium: We have characterized the production of invertase as wild-type cells enter carbon deficiency. Mycelium from conidia that have been germinated and allowed to grow for 20 hr in Vogel's glucose medium were harvested, extensively washed and resuspended in Vogel's medium devoid of a carbon/energy source. At various times thereafter cells were harvested, and the level of invertase was

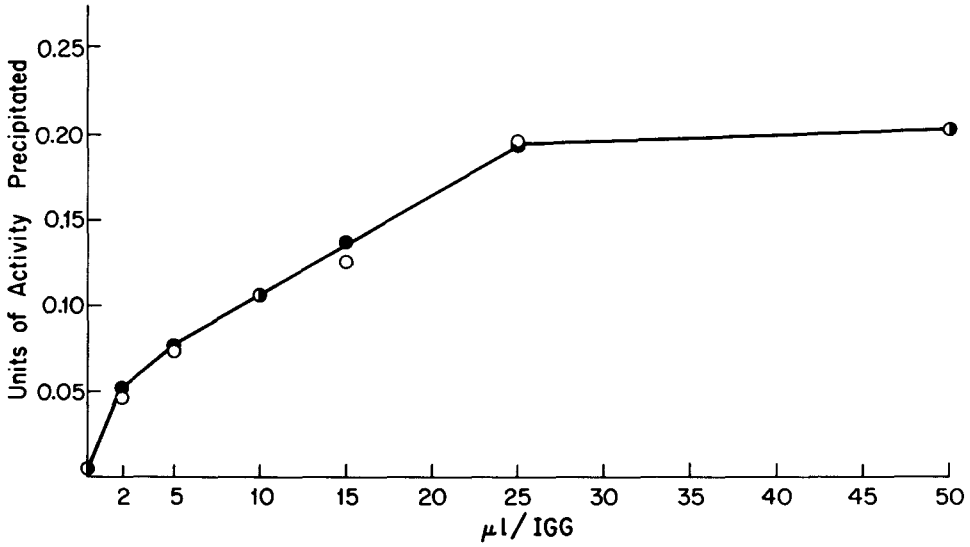

FIGURE 3.—Titering of invertases. Equal amounts of enzymatic activity from wild-type and DBL-1-203 cells were incubated at 37° for 1 hr with increasing amounts of rabbit antisera. Following the incubation an equal volume of 20% polyethylene glycol was added to precipitate the rabbit IgG. The precipitate was collected by centrifugation for 15 min at 10,000 × *g* and assayed for invertase activity. Wild-type enzyme (●); DBL-1-203 enzyme (○).

determined. Starting 20 min after the transfer, we found a dramatic increase in invertase activity (Figure 4). We found the specific activity of invertase to increase more than 50-fold during the first 2 hr of carbon starvation. The level of invertase in DBL-1 cells that have not been derepressed is less than the level of detection of our assay. Thus, we have not been able to quantify accurately the derepression of invertase in DBL-1. However, the fact that we can measure invertase levels in DBL-1 after placing the cells in the carbon-free medium but not before suggests that invertase is able to be derepressed in DBL-1. The derepressed level of invertase in DBL-1 is lower that the "basal" invertase level in the wild-type cell.

DISCUSSION

We have reported a procedure that allows the large-scale screening of mutagenized Neurospora populations for mutants affected in the production of the extracellular enzyme invertase. The procedure can be easily adapted to screen for mutants affected in the ability to produce and secrete a number of other extracellular enzymes that release glucose as a hydrolysis product. The procedure is easy and rapid. SARGENT and BRAYMER (1969) suggested the use of a similar screening procedure but were unable to isolate mutants using their suggested screening. We are at a loss to explain why we were successful when they were not. We found it helpful to use xylose medium and the *cot-1* strain because this allowed us to screen the colonies under conditions in which high levels of invertase are present.

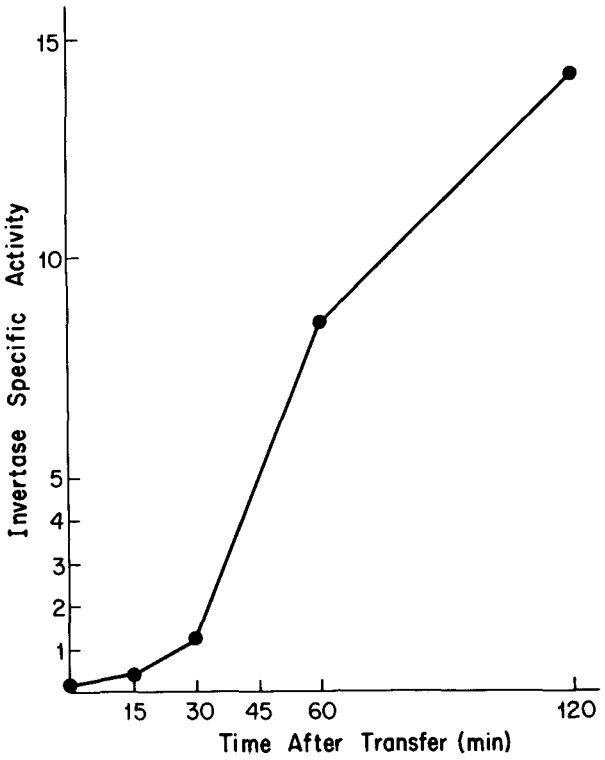

FIGURE 4.—Derepression of invertase. Wild-type cells were transferred from a Vogel's glucose medium to a medium containing Vogel's salts but no carbon source. Aliquots of cells were removed at various times following the transfer and assayed for invertase activity.

Using our screening procedure we have isolated mutants affected in the ability to produce invertase. We have studied one of these mutations, *inv(DBL1)*, in some detail. The *inv(DBL1)*-containing cell produces approximately 1.5% of the wild-type level of invertase. We have mapped the *inv(DBL1)* mutation and shown it to be at or very close to the invertase structural gene. Complementation analysis indicated that the two invertaseless mutations are both recessive and are unable to complement each other further, substantiating the conclusion that the two are in the same gene.

We have been unable to distinguish between the wild-type enzyme and the enzyme produced by *inv(DBL1)*-containing strains such as DBL-1-203 using such criteria as K_m values and thermostability.

We have also used antibody directed against wild-type invertase to question whether we could distinguish between the invertase present in DBL-1-203 extracts and wild-type extracts. If DBL-1-203 produced inactive invertase molecules or had an invertase that was antigenically distinguishable from the wild-type enzyme, we might expect that it would require more antibody to precipitate the same amount of invertase activity from a DBL-1-203 extract as compared to a wild-type extract. We used 10% polyethylene glycol to precipitate all of the added rabbit antibody (in effect doing an indirect immunoprecipitation) so that

we could detect small amounts of cross-reacting material or small differences in antigenicity. We have been unable to demonstrate any immunological differences in the enzyme molecules produced by DBL-1-203 and wild-type cells. The most likely conclusion from our stidies is that *inv(DBL1)* affects the number of enzyme molecules produced but has no effect on the molecule itself. We suggest that the *inv(DBL1)* mutation might be in a region of the invertase structural gene that affects the ability of the cell to synthesis invertase.

We have also characterized the response of wild-type cells as they are transferred from a glucose-containing medium to a medium devoid of any usable carbon/energy source. In the experiments we used 20-hr germinating conidia to avoid areas of localized differentiation which could be expected to be present in older hyphae. We found that within 20 min after transfer to a carbon-free medium the cells produce invertase.

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