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Glutamine Synthetase of *Streptomyces cattleya*: Purification and Regulation of Synthesis

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Glutamine synthetase (GS; EC 6.3.1.2) from *Streptomyces cattleya* was purified using a single affinity-gel chromatography step, and some of its properties were determined. Levels of GS in *S. cattleya* cells varied by a factor of 8 depending upon the source of nitrogen in the growth medium. Of 24 nitrogen sources examined only glutamine or NH_4Cl utilization resulted in very low GS activity. Addition of NH_4Cl to a culture with high GS levels appeared to stop further synthesis and resulted in a progressive decrease in the specific activity of the enzyme. The GS inhibitor methionine sulphoximine (MSX) inhibited GS activity but had no effect on exponentially growing cells. The presence of MSX either lengthened or shortened the period between spore inoculation and initiation of exponential growth, depending on the source of nitrogen. In glutamine minimal medium MSX produced earlier and more efficient spore germination while in glutamate or nitrate minimal medium germination was delayed by its presence.

INTRODUCTION

Streptomyces species produce many medically important antibiotics, but the physiological parameters that regulate their production are poorly understood. We are interested in the regulation of the synthesis of thienamycin, the novel β-lactam produced by *Streptomyces cattleya* (Kahan *et al.*, 1979), and have begun to investigate nitrogen metabolism in this organism. The activity of glutamine synthetase (GS; EC 6.3.1.2), a key enzyme of nitrogen metabolism, is regulated by adenylylation (Streicher & Tyler, 1981) in *S. cattleya* as it is in enteric bacteria such as *Escherichia coli* (Ginsburg & Stadtman, 1973). In this paper we describe a simple single step purification of *S. cattleya* GS and compare some of its properties with the GS from enteric and other bacteria. We also present data on the effect of growth conditions on the levels and adenylylation state of *S. cattleya* GS.

METHODS

Bacterial strains, media and cell growth. Streptomyces cattleya strain MA4297, the original soil isolate (Kahan *et al.*, 1979), was grown in minimal medium containing (per litre): 1·75 g K_2HPO_4, 0·5 g NaCl, 19·5 g MES, 0·1 g $FeSO_4.7H_2O$, 0·1 g $CoCl_2.6H_2O$, and 0·1 g $ZnSO_4.7H_2O$. The pH was adjusted to 6·5 with NaOH. Glucose (1%) was added as a carbon and energy source, and 20 mM-monosodium glutamate as a nitrogen source, except where noted.

E. coli strains EG47 (Goldberg *et al.*, 1974) and ET8002 (ET8000 carrying the *glnA202* allele; MacNeil *et al.*, 1982) were grown in minimal medium containing (per litre): 4·5 g KH_2PO_4, 10·5 g K_2HPO_4, 0·16 g $MgSO_4.7H_2O$, supplemented with 0·4% glucose and 0·2% aspartic acid. For growth on plates, the appropriate medium was solidified by the addition of 1·5% (w/v) agar.

S. cattleya cultures were inoculated with either spores or vegetative cells and were incubated at 37 °C with shaking (220 r.p.m.). Growth was followed by monitoring increases in optical density with a Klett–Summerson

Abbreviations: GS, glutamine synthetase; MSX, methionine sulphoximine.

colorimeter (American Scientific Products, Edison, NJ, USA) with a no. 42 filter. The optical density for this filamentous bacterium was directly proportional to other parameters of cell growth such as cell dry weight, protein content and packed cell volume. Cells were harvested by centrifugation at the indicated times, washed once with 0·85% NaCl, and stored at −80 °C. *E. coli* cells were grown and harvested as previously described (Streicher *et al.*, 1976).

Preparation of crude extracts. Frozen cells were resuspended in 2 vols (w/v) buffer I (20 mM-imidazole.HCl, pH 7·5, 1 mM-$MnCl_2$), and lysed by sonic oscillation (Heat Systems Ultrasonics, 38 East Mall, Plainview, Long Island, NY, USA) for 20 s, unpulsed, at the maximum microtip power in an ice/salt water bath. The lysate was centrifuged for 40 min at 20000 *g* in a Sorvall RC-5B centrifuge with an SS-34 rotor. The supernatant fraction was removed and stored at −80 °C.

Purification of GS. All purification steps were done at 4 °C.

Step 1. The crude extract was dialysed against 500 vols buffer II (buffer I adjusted to pH 6·3) for 6 h with two buffer changes.

Step 2. Affi-gel Blue (Bio-Rad) was washed and equilibrated with buffer II. The dialysed crude extract was added to the gel slurry (10 to 40 GS units per ml of gel) and mixed overnight on a rotating wheel.

Step 3. The equilibrated gel slurry was poured into a column (3 × 18 cm) and washed with one bed volume of buffer II. Non-specifically bound proteins were eluted with buffer II containing 1 M-NaCl until the A_{280} of the eluate returned to the initial baseline reading. The column was next washed with buffer II until all the NaCl was removed, as determined by conductivity measurements. GS was eluted with buffer I containing 5 mM-ADP, and collected in 10 ml fractions.

Step 4. Fractions containing GS activity were pooled and concentrated using an Amicon ultrafiltration cell with a UM-20 membrane filter. Enzyme preparations were stored at 0 °C or at −80 °C. Purified GS was stable for more than three months under both storage conditions. GS from *E. coli* and *Klebsiella aerogenes* was purified as described previously (Streicher & Tyler, 1980).

GS assays. γ-Glutamyl transferase activity of GS was determined as previously described (Bender *et al.*, 1977; Streicher & Tyler, 1981), except that the *S. cattleya* enzyme was assayed at pH 6·9, its pH optimum. The adenylylation state of *S. cattleya* GS was estimated by comparing the transferase activity before and after incubation with snake venom phosphodiesterase (SVPDE; Boehringer-Mannheim), as previously described (Streicher & Tyler, 1981). There is no isoactivity pH for adenylylated and unadenylylated *S. cattleya* GS as is found for other bacterial GS (Bender *et al.*, 1977; Ginsburg & Stadtman, 1973). Whole cell transferase activity was determined as previously described (Pahel *et al.*, 1978; Streicher & Tyler, 1981). One unit of GS produces 1 μmol γ-glutamyl hydroxamate min^{-1} (mg protein)$^{-1}$.

In vitro adenylylation. Adenylylation of GS in crude extracts was done as described previously (Streicher & Tyler, 1981).

Gel electrophoresis. SDS-PAGE was done as previously described (Bender & Streicher, 1979).

Peptide mapping. Peptide mapping by limited proteolysis was done according to the method of Cleveland *et al.* (1977) using trypsin, chymotrypsin and *Staphylococcus aureus* V8 protease (all obtained from Miles Laboratories, Elkhart, Ind., USA).

Protein determination. Protein concentration was determined by either the Lowry method or the Bio-Rad dye-binding protein assay, with bovine serum albumin as the standard. Both assays gave identical results for crude extracts and purified enzyme preparations.

RESULTS

Purification of S. cattleya GS

GS was easily purified using an essentially single step affinity-gel chromatography procedure. SDS-PAGE showed an enzyme preparation 95–98% pure that contained a polypeptide with a molecular weight of 55000 (Table 1, Fig. 1). This procedure also gave high yields of purified enzyme from *E. coli*. Affi-gel Blue (Bio-Rad) had a significantly greater binding capacity for GS, and its use resulted in higher yields and enzymes of greater purity, than did Blue-Sepharose CL-6B (Pharmacia) which utilizes the same affinity ligand. GS binding to the ligand was pH dependent, the greatest binding occurring at pH 6·3. Two other relatively simple purification methods, differential centrifugation and polyethylene glycol precipitation, which were described previously (Streicher & Tyler, 1980), could not be used to purify *S. cattleya* GS.

Since GS could be specifically eluted from the affinity-gel with ADP, the ability of this procedure to purify adenylylated GS, which has an AMP moiety covalently bound to the protein, was investigated. We found that adenylylated GS could not be efficiently purified by our method. When GS in a crude extract was adenylylated, about 73% of the enzyme did not

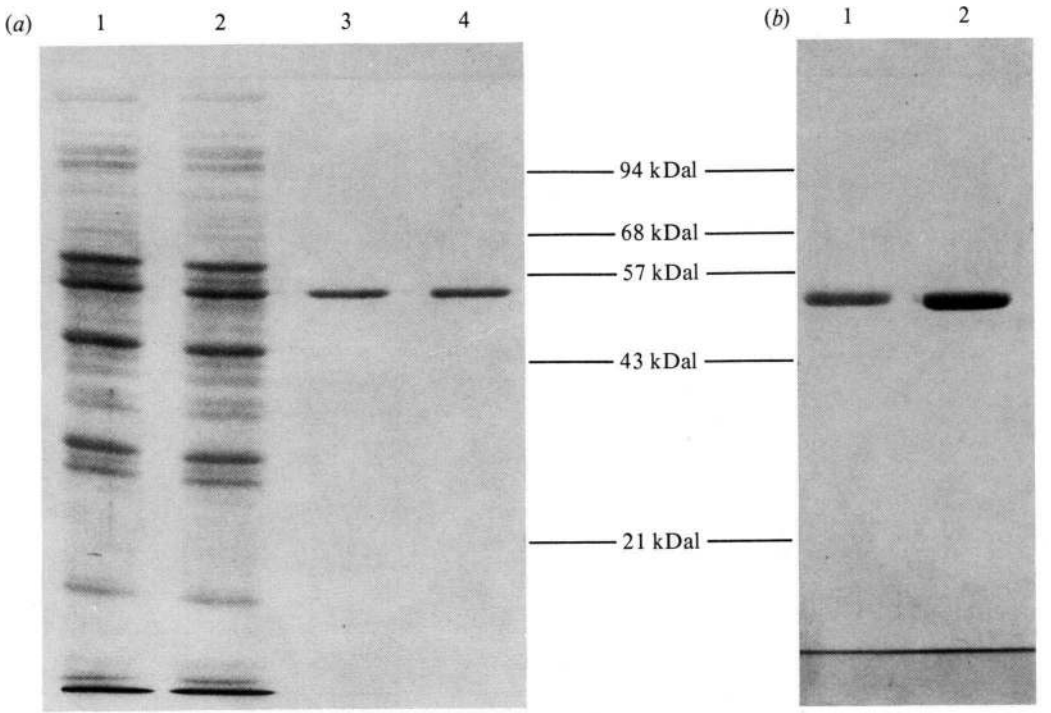

Fig. 1. (*a*) 10% SDS-PAGE of *S. cattleya* GS at each step of purification. Lane 1, crude extract; lane 2, dialysed crude extract; lane 3, Affi-gel Blue ADP eluate; lane 4, Amicon concentrate. All lanes contained 0·45 units GS activity. Molecular weight markers were phosphorylase B, 94 kDal; bovine serum albumin, 68 kDal; pyruvate kinase, 57 kDal; ovalbumin, 43 kDal; and soybean trypsin inhibitor, 21 kDal. (*b*) Comparison of purified GS from *S. cattleya* and *E. coli*. Lane 1, 0·23 unit *E. coli* GS; lane 2, 1·0 unit *S. cattleya* GS.

bind to the affinity-gel and was detected in the column run-through and washes. This is in contrast to the unadenylylated control extract where less than 5% of the GS activity eluted non-specifically.

Some properties of S. cattleya GS

S. cattleya GS required Mn^{2+} for stability in crude extracts and purified enzyme preparations. Extracts prepared without $MnCl_2$ lost more than 60% of their GS activity within 24 h. $MgCl_2$ could not substitute for $MnCl_2$ to prevent loss of GS activity.

Electron microscopy of purified *S. cattleya* GS showed a dodecameric structure (data not shown) similar to that of the enzyme from *E. coli* and *Bacillus subtilis* and *B. stearothermophilus* (Valentine *et al.*, 1968; Deuel *et al.*, 1970; Wedler & Hoffman, 1974). Amino acid analysis of *S. cattleya* GS revealed a composition similar to the enzyme from these other organisms (data not shown). Peptide maps generated by partial digestion using the proteolytic enzymes trypsin, chymotrypsin or *S. aureus* V8 protease revealed distinct differences between the GS from *S. cattleya* and that from two Gram-negative bacteria, *E. coli* and *Klebsiella aerogenes* (Fig. 2).

GS from both *S. cattleya* and *E. coli* could be adenylylated *in vivo* and *in vitro* under the same conditions, but the adenylylation system of one organism was incapable of modifying the GS of the other. Crude extracts were prepared from a *glnA E. coli* strain and *S. cattleya* cells grown in glutamine-supplemented minimal medium to ensure that each contained little or no endogenous GS. Each extract catalysed the adenylylation of purified homologous GS, but not that from the other strain, when incubated under a variety of conditions.

Table 1. *Purification of glutamine synthetase from S. cattleya and E. coli*

	S. cattleya					*E. coli*				
Step	Total protein (mg)	Total activity (units)	Sp. act. [units (mg protein)$^{-1}$]	Percentage yield	Purification (fold)	Total protein (mg)	Total activity (units)	Sp. act. [units (mg protein)$^{-1}$]	Percentage yield	Purification (fold)
1. Crude extract*	79·8	882·6	11·1	100	1·0	27·2	29·3	1·1	100·0	1·0
2. Dialysed extract	75·0	1003·6	13·4	114	1·2	27·2	18·4	0·7	62·8	0·6
3. Affi-gel Blue eluate	1·9	345·0	185·5	39	16·7	—	18·7	—	63·8	—
4. Amicon concentrate	1·2	286·1	235·3	32	21·2	0·3	16·8	51·4	57·3	46·7

—, Not determined.

* Crude extracts were prepared from 3·0 g *S. cattleya* cells and 1·5 g *E. coli* cells.

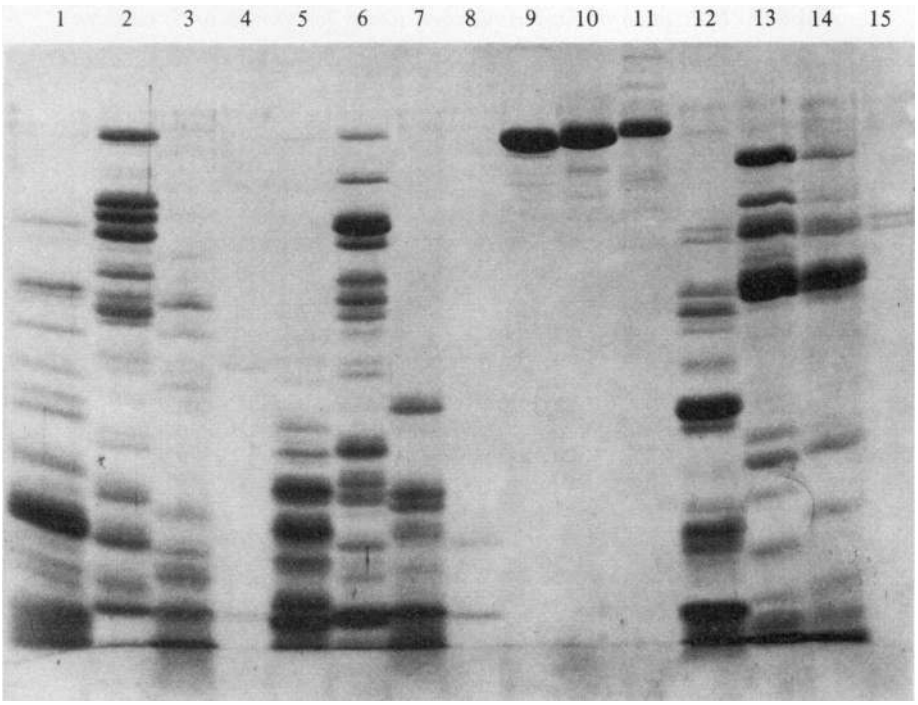

Fig. 2. Peptide map of GS from *S. cattleya*, *E. coli* and *Klebsiella aerogenes*. Purified enzyme (50 μg) from each organism was partially digested with trypsin, chymotrypsin or *S. aureus* V8 protease (2·5 μg each) and electrophoresed in a 15% SDS-polyacrylamide gel. Lanes 1–3, trypsin digestion of GS from *S. cattleya* (S.c.), *E. coli* (E.c.) and *K. aerogenes* (K.a.). Lane 4, trypsin (2·5 μg). Lanes 5–7, chymotrypsin digestion of GS from S.c., E.c. and K.a. Lane 8, chymotrypsin (2·5 μg). Lanes 9–11, 15 μg each of undigested GS from S.c., E.c. and K.a. Lanes 12–14, *S. aureus* V8 protease digestion of GS from S.c., E.c. and K.a. Lane 15, *S. aureus* V8 protease (2·5μg).

Nitrogen source utilization and GS regulation

S. cattleya is able to utilize a variety of compounds as its sole source of nitrogen (Table 2). All but four of the common amino acids supported growth on solid medium when carbon and energy were supplied by either glucose or glycerol. Eight amino acids served additionally as sources of carbon and energy. Identical growth responses were obtained with either spore or vegetative cell inocula. GS activity assayed in crude extracts of cells grown in media containing different nitrogen sources varied greatly but did not correlate with the growth rate. This was strikingly demonstrated in liquid medium where, despite similar growth rates (generation times of 120 min and 100 min respectively), cells grown on glutamate had six to eightfold higher GS activity (8·6 units per mg protein) than cells grown on NH_4Cl or glutamine (1·5 units per mg protein and 1·0 units per mg protein respectively). Conversely, cells grown on either glutamate or nitrate had comparable GS activity (8·6 and 7·7 units per mg protein respectively) but markedly different growth rates (generation times of 120 min and 300 min respectively). The differences in GS activity were due to changes in the amounts of GS protein present in the various cells, and not to the relative degree of adenylylation. The highest level of adenylylation found in growing cells was only 16% (NH_4Cl + glutamate as nitrogen source). The phase of cell growth, from early to late exponential phase did not affect the specific activity of GS, or the ability of cell extracts to catalyse adenylylation.

We have previously shown that the addition of NH_4Cl to cultures containing high GS levels caused a rapid loss of activity due to adenylylation (Streicher & Tyler, 1981; Wax *et al.*, 1982). The specific activity of GS, after *in vitro* deadenylylation by SVPDE treatment, in such a shocked culture progressively decreased: 6·5 h after NH_4Cl addition it was approximately equal to that found in cultures grown continuously in NH_4Cl-containing medium. The total amount of

Table 2. *Utilization of various nitrogen soures for growth of S. cattleya*

Minimal medium plates, with or without glucose (0·8%), and supplemented with the indicated nitrogen source, were inoculated with either spores or washed vegetative cells, and incubated for 3 d at 37 °C.

Nitrogen source	Concn (mM)	Growth + glucose*	GS activity†	Growth − glucose
Alanine	20	+++	++++	++
Arginine	20	+++	++	++
Asparagine	20	+++	+++	+
Aspartate	20	+++	+++	+
Cysteine	20	0	ND	0
Glutamine	20	++++	+	+++
Glutamate	20	++++	++++	+++
Glycine	20	+++	+++++	0
Histidine	20	+++	+++	++
Isoleucine	20	+	+++	0
Leucine	20	+	+++	0
Lysine	20	+++	+++++	0
Methionine	20	++	+++++	0
Phenylalanine	4	+	++++	0
Proline	20	+++	++++	++
Serine	20	0	ND	0
Threonine	20	0	ND	0
Tryptophan	4	+	++	0
Tyrosine	4	0	ND	0
Valine	20	+++	+++++	0
γ-Aminobutyrate	20	+++	+++++	+
Adenosine	4	+++	+++++	++
NH_4Cl	20	+++	+	ND
$NaNO_3$	20	++	ND	ND

ND, Not determined.

* Growth was determined qualitatively comparing the diameters of isolated colonies and scored on a scale of ++++ (very good) to 0 (none).

† GS activities were determined qualitatively by the whole cell transferase assay. Colonies were picked from plates and incubated in assay mix in the wells of a microtitre dish for 30 to 60 min at 37 °C. Activity was evaluated on a scale of +++++ (high levels) to 0 (none detected), based upon the intensity of the brown colour produced by the assay.

GS protein in the ammonia-shocked culture appeared to remain constant, however, and the change in specific activity was probably due to cell growth with a concomitant increase in total cellular protein. Degradation of pre-formed GS did not appear to be occurring at any significant rate since incubation of crude extracts, even for long periods (24 h or longer) at 37 °C, did not result in loss of GS activity or changes in cellular proteins as analysed by 2-dimensional gel electrophoresis (data not shown).

Effect of glutamine analogues on growth and GS activity

Four compounds that would be expected to interfere with nitrogen metabolism were examined for their effect on *S. cattleya*. Three of the four (γ-glutamyl hydroxamate, D-glutamine and β-2-thienylalanine) inhibited growth. Methionine sulphoximine (MSX), however, did not inhibit growth at any level tested. This is in contrast to its effect as a growth inhibitor in Gram-negative bacteria (Arp & Zumft, 1983; Miller & Brenchley, 1981; Pahel *et al.*, 1978). The presence of MSX (10 mM) in glutamate-supplemented liquid medium delayed the initiation of exponential growth for 7 h when the culture was inoculated with spores. This delay also occurred when the source of nitrogen was NH_4Cl or $NaNO_3$. However, when glutamine was the source of nitrogen, exponential growth began significantly earlier than in the control cultures. The same effects upon the initiation of exponential growth were observed when MSX was added to cultures at any time up to 8 h post inoculation. The growth rate, once exponential growth started,

was unchanged by the presence of MSX. These results suggest that MSX is affecting some stage of spore germination. The presence of MSX in glutamine medium resulted, after 8 h, in a 100-fold increase in the number of spores with germ tubes, compared to the untreated controls. At 12 h post-inoculation, about 90% of the spores in the MSX-treated culture had germinated, with evident mycelial branching, while in the untreated control only about 5% of the spores had germinated. These results are in contrast to those from glutamate-grown cultures where, after 12 h, the treated one had about 5% spore germination, and the untreated control had about 50% spore germination with little mycelial branching.

The addition of MSX to exponentially growing cells caused a significant decrease in GS activity which, unlike the decrease caused by addition of NH_4Cl or glutamine, could not be recovered by incubation of crude extracts with SVPDE. The low GS activity was not due to inhibition of the transferase assay by free unreacted MSX in the crude extracts. When a crude extract of MSX-treated cells was mixed with a crude extract from untreated cells, the specific activity of GS was the average of the two extracts.

DISCUSSION

We purified GS using a modification of an affinity-gel chromatography procedure described by Lepo *et al.* (1979). In our experience neither the second affinity-gel column nor the ion exchange column they used was required to obtain nearly homogeneous enzyme preparations from *S. cattleya* or *E. coli*. Adenylylated GS from neither *S. cattleya* nor *E. coli* bound to the affinity matrix under our conditions and therefore could not be purified.

Purified *S. cattleya* GS has a native dodecameric structure similar to that of all bacterial GS (Ginsburg & Stadtman, 1973). Its subunit molecular weight is 55000, the same as *E. coli* GS. The peptide mapping experiment revealed distinct differences between *S. cattleya* GS and that from the enterics *E. coli* and *K. aerogenes*. It was somewhat surprising that the two enteric enzymes appear by this analysis to be so different since they can substitute for one another for *in vivo* and *in vitro* adenylylation/deadenylylation, and that normal growth and regulation occur in interspecies hybrid strains (Streicher *et al.*, 1976; Tyler, 1978). *S. cattleya* GS is, however, different enough that, *in vitro* at least, no heterologous adenylylation activity was observed.

Under balanced growth conditions the levels of GS activity in cells grown on different nitrogen sources can vary by a factor of 8. These differences appear to reflect changes in the levels of enzyme synthesis and not adenylylation. Adenylylation levels did not exceed 16% unless an ammonia or glutamine shock occurred. The long term result of ammonia shock on a glutamate culture was a progressive decrease of GS specific activity to the low level found in ammonia + glutamate grown cells, probably due to the maintenance of the pre-shock GS level as cells continued to grow and increase their protein content. Although continued GS synthesis coupled with an increased rate of GS degradation could have caused the specific activity to decrease, this was probably not occurring since GS activity in crude extracts was stable in the presence of Mn^{2+} and proteolytic activity was not observed. It is most probable that long term regulation of GS levels occurs by altering expression of the structural gene (*glnA*).

Methionine sulphoximine (MSX) had a profound effect on spore germination, either accelerating or delaying it, depending on the source of nitrogen. After germination MSX did not have any effect on growth, even at high concentrations. The addition of MSX to exponentially growing cells caused GS activity to drop 10- to 100-fold. MSX may be inactivating other enzymes in addition to GS or may be causing the derepression of some that have roles in early spore germination. In other bacteria, MSX addition, besides inactivating GS, resulted in elevated levels of other enzymes (Garber *et al.*, 1980; Smith *et al.*, 1981). In some of the initial studies of the effect of MSX on growth in solid media we noticed that the formation of aerial mycelia was enhanced and that sporulation occurred earlier. This suggests that some stages of differentiation leading to spore formation are also perturbed by MSX. It is not clear whether the effects on germination and sporulation are related.

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