

## Regulation of secondary metabolism in fungi

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### SUMMARY

Secondary metabolites (idiolites) are structurally diverse and unusual, generally are produced in mixtures with other members of the same chemical family, and usually are formed at low specific growth rates. In batch cultures, processes leading to the production of idiolites are often sequential; the cultures exhibit a distinct growth phase (trophophase) followed by a production phase (idiophase). However, trophophase and idiophase may overlap. Timing depends on the nutritional environment presented to the culture. Specific mechanisms regulating the onset of idiolite synthesis include repression by sources of carbon, nitrogen and phosphate and enzyme induction. Cessation of idiolite biosynthesis occurs via decay of idiolite synthetases as well as feedback inhibition and repression of these enzymes. With regard to control of mycotoxin biosynthesis, most studies have been done on ergot alkaloid, aflatoxin and patulin formation. Ergot alkaloid formation is mainly controlled by carbon source regulation (glucose), phosphate repression, induction (tryptophan), growth rate and feedback inhibition (agroclavine and elymoclavine). Aflatoxin biosynthesis is controlled by nitrogen source repression (nitrate) and induction (glucose); zinc stimulates production in an unknown manner. Patulin production is regulated by nitrogen source repression, induction (6-methylsalicylic acid), growth rate and synthetase decay.

### INTRODUCTION

Secondary metabolites (idiolites) are special metabolites usually possessing bizarre chemical structures and although not essential for the producing organism's growth in pure culture, they have survival functions in nature. Secondary metabolites are produced only by some species of a genus. They possess unusual chemical linkages, such as  $\beta$ -lactam rings, cyclic peptides made of normal and modified amino acids, unsaturated bonds of polyacetylenes and polyenes, and large macrolide rings. Idiolites are produced typically as members of a particular chemical family because of the low specificity of some enzymes involved in secondary metabolism. They include mycotoxins, antibiotics, pigments, and pheromones. An important characteristic of secondary metabolism is that it is usually suppressed by high specific growth rates of the producing cultures. In addition to growth rate control, individual biosynthetic pathways are often affected by regulatory mechanisms such as induction, nutrient repression, synthetase decay, and end-product regulation.

### LATE FORMATION OF IDIOLITES IN BATCH CULTURE

In batch cultures containing nutritionally rich media, high levels of idiolites are usually produced only after most of the cellular growth has already occurred. The growth phase is called the "trophophase" whereas the production phase is termed the "idiophase".

In many idiolite fermentations, typical trophophase-idiophase dynamics occur in complex media capable of supporting rapid growth, but the two phases overlap in defined media supporting slow growth. In a defined medium supporting only slow growth, some nutritional factor may be growth-limiting from the very start of cultivation, thus favoring production while slow growth is occurring.

The timing of product formation should not be used to define a secondary metabolite. Trophophase and idiophase ideally occur at two separate times in batch culture, but they often overlap. A secondary metabolite is secondary only because it is unnecessary for vegetative growth of the producing culture.

Expression of the genes coding for idiolite biosynthesis usually does not occur at high growth rates. The phenomenon suggests that idiolite synthetases are repressed during growth. Indeed, investigators who have monitored synthesis of idiolite synthetases have observed late enzyme formation due to repression during growth. Examples of enzymes whose activity appears only in idiophase are penicillin acyltransferase and the side-chain activating enzyme of penicillin biosynthesis in Penicillium chrysogenum.

The concept of the trophophase-idiophase transition was first put forth during studies on the production of the mycotoxin, patulin, by Bu'Lock *et al.* (1). The two-phase kinetics were clearly defined in Penicillium urticae by GrootWassink and Gaucher (2) under carefully designed conditions, i.e. use of spores as inoculum, silicone-treated flasks and media supporting rapid filamentous growth. As soon as growth rate begins to decline, the first enzyme of the pathway (6-methylsalicylic acid synthetase) appears. When the growth stops (3 hours later), a later enzyme (*m*-hydroxybenzyl alcohol dehydrogenase) appears. The time of appearance of this latter enzyme depends in an inverse manner on the amount of nitrogen source added to the medium, i.e. the higher the nutrient concentration, the later the appearance.

Trophophase-idiophase kinetics are also seen in the aflatoxin fermentation (3) and in the ergot alkaloid process as carried out by certain strains, e.g. Claviceps purpurea, Claviceps fusiformis and Sphaecelia sorghi (for a discussion, see Vining (4)). Enzymes of ergot alkaloid synthesis that only appear after growth are dimethylallyltryptophan synthetase (5) and chanoclavine- I-cyclase (6).

## INTRACELLULAR EFFECTORS THAT CONTROL THE ONSET OF IDIOLITE SYNTHESIS

### 1 Carbon source regulation

Glucose, usually an excellent carbon source for growth, interferes with the biosynthesis of many secondary metabolites. Polysaccharides, oligosaccharides, and oils are often better carbon sources for production than is glucose. In a medium containing a rapidly used carbon source plus a more slowly utilized carbon source, the former usually is used first; idiolite production does not occur in this phase. After the favored carbon source is depleted, the second carbon source is used for idiolite biosynthesis.

Carbon source regulation of secondary metabolism exists in many fermentations. The most well-known case is the repression of penicillin and cephalosporin production by glucose. In penicillin G formation by Penicillium chrysogenum, glucose represses tripeptide formation from L- $\alpha$ -aminoadipic acid, L-valine and L-cysteine (7).

Formation of cephalosporin C by Cephalosporium acremonium is often delayed until the rapidly utilized sugar (glucose) has been consumed and the slowly utilized sugar (sucrose) begins to be used. In comparisons of individual carbon sources, it was found that readily utilized carbon sources (glucose, glycerol, maltose) yield less antibiotic than do slowly utilized sugars (sucrose, galactose). When different concentrations of glucose are compared, the best antibiotic production occurs at the lowest glucose concentration (8). The Cephalosporium fermentation yields two major products, penicillin N and cephalosporin C. Penicillin N is an intermediate in the formation of cephalosporin C but accumulates extracellularly because the enzyme converting it to desacetoxycephalosporin C is a rate-limiting labile enzyme requiring continuous resynthesis throughout the fermentation. Desacetoxycephalosporin C synthetase (expandase) is the enzyme of the pathway most susceptible to carbon source repression. It does not appear in the mycelium until glucose is consumed and growth has ceased. On the other hand, an earlier enzyme, isopenicillin N synthetase (cyclase) appears during trophophase as does the extracellular intermediate, penicillin N (9).

Production of  $\beta$ -carotene by Mortierella ramanniana var. ramanniana is favored by carbon sources which are poor for growth (10).

Carbon source control also occurs in mycotoxin producers. Glucose decreases alkaloid production by Claviceps (11); polyols and organic acids are the preferred carbon and energy sources. When the enniatin fermentation (Fusarium sambucinum) is carried out with glucose (which is rapidly utilized), production occurs only after growth. If the slowly metabolized lactose is the carbon source, production accompanies growth (12).

Penicillium cyclopium does not produce benzodiazapine alkaloids if grown in a medium which contains glucose. If the glucose is replaced by sorbitol and mannitol, alkaloids are produced (13). If alkaloid-producing cells are subjected to glucose addition, there is an immediate inhibition of alkaloid synthesis by over 50%. This suggests that both carbon source repression and inhibition are operative in this organism.

It appears that cyclic adenosine-3',5' phosphate (cAMP) does not mediate carbon catabolite regulation of secondary metabolite synthesis in fungi. cAMP does not reverse glucose

repression of penicillin biosynthesis in *P. chrysogenum* (14), or of benzodiazepene alkaloid biosynthesis in *P. cyclopium* (15). Instead of being involved in synthesis of carbon-repressible enzymes (as it does in bacteria), cAMP activates the cAMP-dependent protein kinase in fungi. This enzyme in turn activates or inactivates fungal enzymes by phosphorylation. Trehalase in yeast is activated by the cAMP-dependent protein kinase (16). On the other hand, yeast NAD-dependent glutamate dehydrogenase is inactivated by cAMP-dependent and cAMP-independent protein kinases and reversibly activated by dephosphorylation (17). A cAMP-dependent protein kinase appears to mediate the effect of cAMP on development in *Dictyostelium discoideum* (18).

## 2 Nitrogen source repression

A regulatory mechanism that controls the use of nitrogen sources is known in yeasts and molds. Ammonia (or some other readily used nitrogen source) represses enzymes involved in the use of alternate nitrogen sources such as nitrite reductase, nitrate reductase, glutamate dehydrogenase, arginase, extracellular protease and acetamidase.

Idiolite production is negatively affected by  $\text{NH}_4^+$ . For example, production of acetate-derived phenolic idiolites such as trihydroxytoluene by *Aspergillus fumigatus* is under nitrogen source control (19). These polyketides appear in batch culture only when nitrogen is exhausted from the medium. Addition of ammonia completely inhibits their production without affecting growth or pH. Bikaverin formation by *Gibberella fujikuroi* is also under nitrogen regulation (20). Here glycine is used as nitrogen source for the fermentation but bikaverin production begins only upon glycine exhaustion. If glycine is added to a resting mycelial suspension, bikaverin production is decreased.

*C. acremonium* is strongly regulated by nitrogen sources in its formation of  $\beta$ -lactam antibiotics (21). Ammonium concentrations higher than 100mM strongly interfere with  $\beta$ -lactam production whereas L-asparagine and L-arginine are much better nitrogen sources for antibiotic formation. Nitrogen source control is effected by repression of expandase, not cyclase, by  $\text{NH}_4^+$  in a manner similar to carbon source repression. Addition of tribasic magnesium phosphate, which traps the ammonium ion, completely reverses ammonium repression.

Sanchez *et al.* (22) studied nitrogen regulation of penicillin G formation by *P. chrysogenum*. They found a high (85mM)  $\text{NH}_4^+$  level to decrease penicillin production by 2-fold as compared to a control with 8.5 mM  $\text{NH}_4\text{Cl}$ . High  $\text{NH}_4^+$  also decreased glutamine synthetase formation by 80% and the glutamine pool by 33%, but increased the glutamate pool by 2-fold. Resting cells fed with 0.5 mM glutamine in the presence of cycloheximide increased penicillin production to a greater degree than glutamate or  $\text{NH}_4^+$ . The above results indicated that glutamine increases penicillin synthesis and glutamate interferes. However, later work of this group suggests the opposite to be true. Lara *et al.* (23) found glutamate to stimulate penicillin production in fermentations to a much greater degree than did glutamine or  $\text{NH}_4^+$ . Glutamate analogs such as L-glutamic acid  $\gamma$ -monohydroxamate and  $\gamma$ -benzyl-L-glutamate also stimulated production. Glutamate increased by 3.5 fold the dipeptide synthetase activity. The authors considered glutamate to be an inducer of the pathway. In 1984, Mateos *et al.* (24) found that glutamine at higher than 1 mM concentration inhibited resting cell production of penicillin; total inhibition was obtained with 10 mM. Inhibition was not reversed by the three amino acid precursors of penicillin. Glutamine did not inhibit dipeptide synthetase.

With regard to mycotoxin producers, nitrogen source regulation has been studied mainly in the case of aflatoxin formation. Shih and Marth (25) reported that aflatoxin formation by *Aspergillus parasiticus* is inhibited by a high concentration of  $\text{NH}_4^+$ , a level which is best for growth. However, careful examination of their data shows only a minor differential effect by  $\text{NH}_4^+$  on growth and production. A clue to the identity of a nitrogen source repressor more effective than  $\text{NH}_4^+$  was uncovered by the work of Bennett *et al.* (26) on versicolorin production by an *A. parasiticus* mutant. Versicolorin is an intermediate in the aflatoxin pathway. These workers found that whereas ammonium salts support both growth and production, nitrate utilization yields only growth. Kachholz and Demain (27) working with an averufin-(another intermediate) producing mutant showed that nitrate represses averufin production whereas ammonium favors it; repression was not a function of pH changes nor sugar depletion in the medium. They showed that nitrate represses aflatoxin synthesis also in the parent aflatoxin-producing culture. Their data also indicate that nitrogen source regulation is the main nutritional control of aflatoxin biosynthesis. Amino acids reported to be stimulatory to aflatoxin formation include asparagine, aspartic acid, alanine, methionine, proline, and histidine (for review, see ref. 28) but these claims have to be carefully evaluated for effects on growth.

It is interesting that although most fungi cannot carry out nitrification (i.e. nitrate production from  $\text{NH}_4^+$  or amino acids), *Aspergillus flavus* and *A. parasiticus* can (29-31). White and Johnson (32) established a correlation between the presence of nitrification and that of aflatoxin production in *A. flavus* strains. These facts suggest that nitrification

might have some role in turning off aflatoxin formation in the producing organism. It would be of interest to determine whether heavy application of nitrate might prevent aflatoxin formation in the field and in storage.

It should also be noted that the onset of patulin-forming enzymes by *P. urticae* can be delayed for hours by provision of too great a concentration of nitrogen source (2).

The above discussion has dealt with the general nitrogen repression phenomenon. However, there exists another type of nitrogen control in which specific amino acids repress and/or inhibit production of secondary metabolites because they are derived from the same branched pathway and exert feedback control of that branched pathway.

Such pathways have an early common part which then branches to the synthesis of a primary metabolite, on the one hand, and to a secondary metabolite, on the other. In some cases, the primary end-product feedback inhibits the common part of the pathway and thus inhibits idiolite biosynthesis. For example, lysine interferes with penicillin and cephalosporin production by fungi. It acts by inhibiting and repressing homocitrate synthase, the initial enzyme of the branched pathway leading to lysine and  $\beta$ -lactam antibiotics (for review, see ref. 7).

### 3 Phosphate regulation

Phosphate is the crucial growth-limiting nutrient in many secondary metabolite fermentations. Phosphate in the range of 0.3-300 mM generally supports extensive cell growth, but concentrations of 10 mM and above suppress the biosynthesis of many secondary metabolites.

In *C. acremonium*, cephalosporin C production is negatively affected by phosphate but the effect is not a strong one. The work of Kuenzi (33) suggests that the phosphate effect is indirect, acting by increasing the consumption of the carbon source repressor, glucose.

Bikaverin production is also depressed by levels of inorganic phosphate which have no inhibitory effect on growth (34).

Inorganic phosphate, which at high levels totally inhibits alkaloid formation (35-36), has been shown to repress the first enzyme unique to alkaloid biosynthesis (dimethylallyl-tryptophan synthetase) (5) as well as chanoclavine-I-cyclase in *Claviceps* SD58 (6).

An important question is whether intracellular orthophosphate is the ultimate effector or whether it merely regulates the level of some other intracellular effector that controls expression of idiolite biosynthesis, e.g. ATP. A mutant of *Fusidium coccineum* producing 10 times more fusidic acid than a low producing strain, contains less intracellular ATP during production and less polyphosphate throughout the fermentation than the low producer (37).

It is also possible that phosphate control is exercised on enzyme activity by phosphorylation and dephosphorylation via protein kinases and phosphoprotein phosphatases (38). Yeasts contain at least two types of protein kinases, cAMP-dependent and cAMP-independent.

### 4 Enzyme induction

Many groups have reported on the stimulation of ergot alkaloid synthesis in *Claviceps* by the precursor tryptophan (39-42). Induction of a key enzyme by tryptophan was postulated by Floss and Mothes (40) and Bu'Lock and Barr (42). This concept was supported by data showing stimulation by tryptophan analogs which were not alkaloid precursors (40,43-44) and by timing studies on tryptophan addition (40,45). Finally, it was shown by Krupinski et al. (5) that tryptophan induces dimethylallyltryptophan synthetase, the first enzyme of the pathway. An even more effective inducer of alkaloid biosynthesis is the tryptophan analog, DL- $\beta$ -2-naphthylamine (46).

Another precursor which functions as an inducer of alkaloids is phenylalanine in the production of benzodiazapene alkaloids in *Penicillium cyclopium* (15). It is interesting that compartmentation of phenylalanine exists in this organism so that exogenous phenylalanine mainly goes to protein whereas endogenous phenylalanine is incorporated predominantly into alkaloids (47).

The concept of induction of secondary metabolic enzymes prior to idiophase metabolism in the patulin fermentation was first suggested by Bu'Lock et al. (48). Grootwassnik and Gaucher (2) showed that the initial enzyme is formed at the end of trophophase as the result of nitrogen source exhaustion. Some four hours later, the remaining enzymes appear (actually only the 2nd, 4th and 7th enzymes were measured) as a result of coordinate induction by 6-methylsalicylic acid, the product of the first enzyme. Thus it appears that all the enzymes are idiophasic, the first being nitrogen (or growth-rate?)

repressible and the remainder inducible (49). It is interesting that once formed, the first three metabolites of the pathway (6-methylsalicylic acid, m-cresol and m-hydroxy-benzylalcohol) function as inducers for the rest of the pathway.

Studies on aflatoxin formation indicate that a product of glucose metabolism induces the aflatoxin pathway. Production of the mycotoxin is stimulated by increasing concentrations of glucose and the onset of the idiophase is accompanied by an increased rate of glucose consumption (3). In contrast to glucose, peptone as carbon source allows good growth but no aflatoxin formation (50). When peptone-grown cells are added to a replacement culture containing glucose, aflatoxin synthesis occurs. That this is not due to nitrogen source derepression was shown by adding peptone to the glucose replacement culture and observing even better production than in glucose alone. The effect was not specific for glucose; also active were ribose, fructose, sorbose, mannose, maltose, sucrose, raffinose and glycerol (51). Lactose, lactate, pyruvate, oleate, citrate, and acetate were inactive. At present neither the mechanism of this induction nor the intracellular metabolite responsible for it is known.

Methionine stimulation of cephalosporin production acts via enzyme induction. Although the prototrophic *C. acremonium* culture is merely stimulated in cephalosporin production by methionine (since it can make its own methionine but apparently not enough for optimal idiolite synthesis), methionine auxotrophs show an absolute requirement for methionine over and above the amount required for optimal growth. This excess methionine can be replaced by norleucine which contains no sulfur - thus methionine does not stimulate cephalosporin synthesis by supplying sulfur. In support of the regulatory hypothesis, it has been shown that growth in methionine or norleucine induces both the cyclase and expandase enzymes 3- to 7-fold (52).

Production of carotene in *Phycomyces blakesleeanus* is induced by light and non-precursors such as vitamin A and  $\beta$ -ionone (53). The effect is eliminated by cycloheximide and thus appears to involve enzyme induction.

Induction in fungi appears to occur by positive control (54), i.e. the product of the regulator gene is required for transcription of inducible enzymes.

## 5 Growth rate

The mechanism(s) by which idiolite production is frequently delayed until the end of the trophophase involves repression of the enzymes of secondary metabolism during growth (as described above) by sources of carbon, nitrogen and phosphorus. However, it also appears to involve growth rate.

In batch cultures of *G. fujikuroi*, growth occurs first (maximum rate at 18h), then bikaverin is synthesized (at 40h) and finally gibberellin is made (70h). In the chemostat, bikaverin is formed best at a dilution rate of  $0.05\text{h}^{-1}$  whereas gibberellin is made at  $0.01\text{h}^{-1}$ . At this latter dilution rate, bikaverin is not produced at all (20).

In patulin biosynthesis by *P. urticae*, the trophophase-idiophase shift is the result of growth cessation at 20-21 h and the appearance of the first enzyme of the pathway (6-methylsalicylic acid synthetase) at that same time. About 4 h later, the 2nd, 4th and 7th enzymes appear. The appearance of the 1st and 4th enzymes have been shown to be due to *de novo* biosynthesis of RNA and protein. The onset of secondary metabolism is brought on by growth cessation caused by nitrogen depletion. Since other means of stopping growth also bring on secondary metabolism, the key event appears to be cessation of growth. A secondary factor is induction (49).

Enzymes of ergot alkaloid biosynthesis are synthesized in the idiophase after phosphate is depleted in certain ergot alkaloid producing strains, i.e. *C. purpurea*, *C. fusiformis* and *S. sorghi* (4) in the presence of the inducer tryptophan. It is an open question whether these idiophase events are brought about strictly by nutrient exhaustion or primarily by the low growth rate which results upon exhaustion. That the growth rate may play a key role is indicated by the work of Brar *et al.* (35) with *C. paspali*. This fungus usually produces ergot alkaloids during growth, unlike the other fungi which normally display a distinct trophophase and idiophase. However, even *C. paspali* can be forced into two-phase dynamics when it is grown on a complex medium supporting a high growth rate, i.e. on a defined medium supporting only a slow growth rate, trophophase and idiophase overlap but with rapid growth on a complex medium, trophophase and idiophase occur at different times.

## CESSATION OF BIOSYNTHESIS

There are two known reasons for the cessation of idiolite biosynthesis: 1) irreversible decay of one or more enzymes of the idiolite-synthesizing pathway; 2) a feedback effect of the accumulated product.

### 1 Synthetase decay

The main cause of cessation of patulin production in *P. urticae* is the decay of the first enzyme, 6-methylsalicylic acid synthetase which has an in vivo half-maximal lifetime of seven hours (56). Later enzymes, *m*-hydroxybenzyl alcohol dehydrogenase (the 4th) and isoeopxydon dehydrogenase (the 7th) have longer half-lives (17 and 19 hours, respectively).

### 2 Feedback regulation

The role of feedback regulation in controlling secondary metabolism is well-known. Many secondary metabolites inhibit or repress their own biosynthesis. For example, myco-phenolic acid (57) limits its own synthesis by inhibiting the final enzyme, an *O*-methyl-transferase, in *Penicillium stoloniferum*.

Feedback inhibition of ergot alkaloid biosynthesis by *Claviceps* occurs at the first step, dimethylallyltryptophan synthetase, which is inhibited by agroclavine and elymoclavine (58,59). Feedback repression does not appear to be important. Elymoclavine also inhibits a later enzyme, chanoclavine-I-cyclase (6).

A second type of feedback inhibition in the ergot alkaloid pathway involves inhibition of tryptophan synthesis at the first enzyme of the tryptophan biosynthetic branch, anthranilate synthetase. This affects alkaloid production since tryptophan is both a precursor and inducer of alkaloid biosynthesis. This important enzyme is inhibited by elymoclavine and chanoclavine (60,61).

$\beta$ -carotene regulates its own synthesis by repression in *P. blakesleeana* (53). This is indicated by the enormous accumulation of precursors when formation of  $\beta$ -carotene is inhibited genetically or chemically. The gene car5 is thought to code for a repressor protein since mutations in this gene lead to overproduction of carotene.

## OTHER FACTORS

### 1 Metals

In the aflatoxin fermentation, zinc is needed at a concentration greater than that required for growth in order to get high production of the mycotoxin (62). Other investigators have reported stimulatory effects of cadmium, manganese, cobalt, boron, molybdenum, copper, iron and calcium (for review, see ref. 28) but these claims have to be carefully evaluated for effects on growth. Excess manganese is necessary for patulin production (63). The site of manganese action appears to be after the first step since 6-methylsalicylic acid is accumulated at sub-optimal levels of the metal.

### 2 Oxygen

High levels of oxygen that provide good growth are inhibitory to the aflatoxin fermentation (64). Aflatoxin degradation may play a role in this phenomenon (28).

### 3 Light

Considerable controversy exists concerning the possible negative effect that light has on the formation of aflatoxins (65). On the other hand, light stimulates the formation of enniatins by *F. sambucinum* (66).

### 4 Temperature

Temperatures lower than that required for optimum growth are required for maximum production of enniatins (66) and aflatoxins (25). In the production of aflatoxins, temperature shifts upward during the fermentation appear to markedly increase production on a solid substrate (rice) (69).

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