An Endogenous Inducer of Sexual Development in *Aspergillus nidulans*

By SEWELL P. CHAMPE,* PREMA RAO AND AMY CHANG

Waksman Institute of Microbiology, Rutgers, The State University of New Jersey, Piscataway,
New Jersey 08854, USA

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During development of the homothallic ascomycete *Aspergillus nidulans*, asexual sporulation is followed by sexual sporulation. We report here the detection of a solvent-extractable activity which inhibits asexual sporulation and stimulates premature sexual sporulation. This activity, called precocious sexual inducer (psi), is overproduced by certain mutants that are blocked in both modes of sporulation. Using partially purified preparations of psi, biological response could be elicited with as little as 50 ng of material. We suggest that psi is a hormone-precursor which is converted to a hormone by normal sporulating strains that respond to psi, but not by the asporogenous mutants that overproduce psi. The stability of psi activity gives promise that the compound can be purified and identified.

INTRODUCTION

The filamentous fungus *Aspergillus nidulans* has been the subject of extensive genetic and biochemical research over the past forty years. It is also an attractive organism for developmental studies because it exhibits a simple and highly regulated programme of cell differentiation. The developmental stages of a confluent agar surface culture originated from dormant spores are shown in Fig. 1. After spore germination and a period of undifferentiated vegetative growth, mitotically-generated asexual spores (conidia) suddenly appear in great numbers and become dark green. As this burst of conidiation ceases, sexual development commences with the formation of cleistothecia. These small spherical shells, ~200 µm in diameter, are the sexual fruiting bodies in which bright-red ascospores are later generated by meiosis. The onset of conidiation is accompanied by the appearance of over 1000 mRNA species not found in vegetative hyphae (Timberlake, 1980).

In previous reports (Butnick *et al.*, 1984*a, b*) we described the properties of three unusual thermosensitive asporogenous mutants which, although non-allelic, exhibited the same biochemical abnormality. At the restrictive temperature (42 °C) each secreted a set of solvent-extractable metabolites into the culture medium at levels much higher than the parental strain or other asporogenous mutants. One of the major components has been identified as diorcinol (3,3'-dihydroxy-5,5'-dimethyldiphenyl ether), the origin of which is probably the polyketide precursor orsellinic acid (Ballantine *et al.*, 1971). Preliminary analyses of several of the other components indicate similar phenolic compounds. Mutants which exhibit this phenotype we call phenolic over producing (POP) mutants and we refer to the overproduced compounds as the POP metabolites. That a single mutation results in the appearance of a variety of compounds suggests that these compounds are accumulated intermediates or shunt products of some metabolic pathway which is blocked by the mutation. Although isolated for their inability to form conidia, and given the gene symbol *aco*, all three POP mutants were found to be blocked in sexual sporulation as well. This report describes the detection of a hormone-like factor, overproduced by POP mutants, that dramatically alters the sporulation schedule of wild-type *A. nidulans*.

Abbreviations: POP, phenolic over producing; psi, precocious sexual inducer.

METHODS

Strains of Aspergillus nidulans. The conidiation-proficient (*aco*⁺) strains used in this study were the original prototrophic Glasgow wild-type FGSC-4 (*ve*⁺), WIM-126 (*yA2 pabaA1, ve*⁺) and WIM-64 (*yA2 pabaA1, veA1*). Strain WIM-145 (*acoC193, yA2 pabaA1, ve*⁺) is a thermosensitive aconidial mutant that overproduces phenolic metabolites (the POP phenotype) and was derived by mutation from WIM-126. The isolation and characterization of this mutant, and of two other non-allelic aconidial mutants having the POP phenotype, have been described previously (Butnick *et al.*, 1984*a, b*).

Growth medium. The medium (YGT) used throughout this study was composed of 0.5% (w/v) yeast extract, 2% (w/v) glucose and trace elements at concentrations given previously (Kurtz & Champe, 1981). For plates, the medium was solidified with 1.5% (w/v) agar.

Preparation of extracts. Liquid YGT medium was inoculated with 2000 conidia ml⁻¹ and shaken at 42 °C. At 96 h the mycelial pellets were removed by filtration and the medium was extracted twice with an equal volume of ethyl acetate. The combined extracts were taken to dryness and the residue was redissolved in a volume of ethyl acetate equal to one-fiftieth the volume of the extracted medium. Such extracts contained about 10 mg of material per 100 ml of extracted medium.

Bioassay of extracts. Samples of ethyl acetate extracts were dried on 4 mm diameter filter paper discs which were placed on the agar surface at the time of inoculation with 10⁵ conidia of strain FGSC-4 or WIM-126. Inhibition of conidiation around the assay disc was scored after incubating the plates for 40 h at 37 °C. Cleistothecial primordia were visualized and counted by treatment of the plate with a laccase chromogenic substrate (4-amino-2,6-dibromophenol and the coupling agent 3,5-dimethylaniline) which stains the primordia bright green (Hermann *et al.*, 1983).

Chromatography. TLC analysis of ethyl acetate extracts was performed on silica gel plates (Merck Precoated TLC plate, Silica Gel F-254). Precocious sexual inducer (psi) activity (see Results) was located on the plate by bioassay of material eluted from the adsorbent scraped from defined areas. Psi activity migrated with an *R_F* of 0.1–0.2 with a solvent (CEE) composed of chloroform/ethyl acetate/diethyl ether (10:4:2, by vol.) and with an *R_F* of 0.5–0.6 with a solvent (CM) composed of chloroform/methanol (8:2, v/v).

Preparative separations were performed on silica gel columns. A 35 × 2 cm column of 70–230 mesh Merck Kieselgel 60 eluted with CEE was used for the initial fractionation of up to 1 g of crude material. Fractions with psi activity were pooled and rechromatographed on a higher-resolution (230–400 mesh) adsorbant. Repeated rechromatography using chloroform/methanol in various proportions progressively eliminated many non-active components.

RESULTS AND DISCUSSION

A possible cause of the failure of POP mutants to sporulate is that one or more of the accumulated metabolites acts to block sporulation. To determine the effect of the POP metabolites on sporulation, an ethyl acetate extract of a 96 h culture of the POP mutant *acoC193* was applied, on small filter paper discs, to confluent lawns of the parental strain at the time of spore inoculation. Conidiation was strongly inhibited in the vicinity of the assay disc containing the mutant extract (Fig. 2). This inhibition was strong if the mutant extract was applied to the plate within the first 12 h after spore inoculation, but rapidly weakened with later times of application.

Our standard cultivation medium contains yeast extract, a component of which could conceivably be converted to the inhibitor by a simple transformation. However, the activity is also produced in a glucose/glutamate minimal medium, and so must be formed *de novo* by the fungus. It is to be noted that inhibition is obtained with as little as 2 µl of extract, which corresponds to 0.1 ml of culture medium. The inhibitory activity is produced by agar surface cultures as well as by liquid shake cultures.

An activity that inhibits conidiation is also produced by the *aco*⁺ parental strain but at a much lower level. From the relative responses to varying amounts of extract used in Fig. 2 it can be seen that, at the harvest-time of 96 h, the mutant had about 25-fold more inhibitory activity than its parent. Similar assays of extracts of younger cultures indicated that the activity first appeared in parental and mutant cultures at about 40 h. For the mutant cultures activity steadily increased with culture age, whereas for the parental cultures activity declined after a period of increase. Two other non-allelic POP mutants (Butnick *et al.*, 1984*b*) exhibited activity comparable to *acoC193*.

Fig. 1. The developmental stages of *A. nidulans*. At $t = 0$, 10^5 conidia of strain WIM-64 were spread on the surface of an agar plate which was incubated at 37°C . Plugs of fixed area were excised from the plate at various times and assayed for conidia, cleistothecia and ascospores by methods described previously (Hermann *et al.*, 1983; Serlupi-Crescenzi *et al.*, 1983; Yager *et al.*, 1982).

Fig. 2. Bioassay of ethyl acetate extracts. Extracts of WIM-126 (*aco*⁺) and WIM-145 (*acoC193*) were prepared and bioassayed as described in Methods using the green conidial strain FGSC-4 as indicator. The dark areas on the plates are the green conidia, whereas the light (yellowish) areas around the assay discs consist mainly of cleistothecial primordia. Identical extracts of sterile or freshly inoculated medium were without effect. The observed response to the extract is stronger for strains, such as FGSC-4, that carry the *ve*⁺ allele of the *velvet* gene which was described by Kafer (1965). Extract volume (μl): (a) 50, (b) 10, (c) 2.

If this conidiation-inhibiting activity is responsible for the aconidial phenotype of POP mutants, it might be expected that an extract would block sexual development as well, because the mutants are also sexually defective. Contrary to this expectation, microscopic examination revealed that the fungal tissue present in the yellowish zone around the assay disc was not undifferentiated hyphae, but rather consisted of masses of cleistothecial primordia. In areas

Fig. 3. Conidial and cleistothecial abundance as a function of distance from the psi assay disc. Two radial strips (4 mm wide) were excised from a 40 h psi assay plate similar to those of Fig. 2 and cut into 2.5 mm serial segments. The segments of one strip were homogenized and assayed for conidia as colony forming units, while those of the second strip were stained and assayed for cleistothecial primordia as described in Methods. To enhance the visibility of the stained primordia, WIM-126, which produces yellow conidia, was used as the indicator strain.

distant from the assay disc primordia did not appear until many hours later. These visual observations were confirmed by direct measurements of conidial and primordial densities as a function of distance from the assay disc (Fig. 3). The primordia that formed in response to the extract matured into normal-appearing cleistothecia upon further incubation. Twenty mature cleistothecia were picked at random sites in the conidia-less area around the assay disc and assayed individually for ascospore content as colony forming units. The average content was 7.3×10^4 and the lowest was 3.0×10^4 . The mutant extract thus produced two effects: inhibition of asexual sporulation and induction of precocious sexual sporulation. From this latter property we term the responsible factor(s) psi. The dual effects of the extract could, of course, be due to the presence of two distinct factors, but in the course of extensive fractionations, no separation of the two effects has been detected.

Analysis of crude ethyl acetate extracts by TLC showed that psi activity migrated as an apparently single compact zone which included a number of the POP metabolites. Fractionation of extracts by silica gel column chromatography has yielded preparations of psi factor with greatly increased specific activity and has shown, moreover, that the activity does not reside with any of the major UV-absorbing POP metabolites seen on thin-layer chromatograms of crude extracts. We estimate that the active component must constitute less than 1% of the mass of a crude extract. With some purified preparations, the biological response of Fig. 2 could be elicited with as little as 50 ng of material. Further fractionation by high-resolution HPLC is being used to prepare material for structural analysis. Psi activity appears to be sufficiently stable to withstand the rigours of fractionation; activity survived repeated air drying and heating to 100 °C, but was destroyed by heating in the dry state at 200 ° for 1 h.

Can the asporogenous phenotype of POP mutants be explained in terms of the dual effects of psi factor? The failure of both asexual and sexual sporulation in POP mutants could be due to the presence of inhibiting factors, the absence of stimulating factors, or both. However, the observation that psi activity is transient in the parent strain but accumulates in the mutant suggests that psi factor is an intermediate which can be further metabolized by the parent but not by the mutant. If it is assumed that the metabolic products of psi factor include inducers of both asexual and sexual sporulation, POP mutants will lack these inducers and consequently be defective in both modes of sporulation. When psi factor is applied to the parental strain at the time of spore inoculation, the sexual inducer may be generated prematurely, thus causing

precocious sexual development. Inhibition of conidiation by psi factor could be an indirect effect resulting from competing early sexual development. Certain mutants that exhibit early sexual development are, in fact, aconidial (Serlupi-Crescenzi *et al.*, 1983), suggesting that when the two modes of sporulation are in competition, the sexual mode prevails. Conversely, it is possible that psi factor directly inhibits conidiation and that, as an indirect consequence, sexual development commences prematurely. If so, the mere failure to form conidia cannot be sufficient to allow early sexual development because aconidial mutants do not, in general, have this characteristic. Such a mechanism is also difficult to reconcile with our observation that conidiation was inhibited by psi factor only if the extract was applied during the first 12 h after spore inoculation.

The relationship between psi factor and the POP metabolites is unclear. It is possible that both are intermediates of the same metabolic pathway, specific steps of which are blocked in the mutants, but it is equally conceivable that psi factor and the POP metabolites are metabolically unrelated. The mere presence of the POP metabolites, for example, could influence the metabolism of psi factor. It is notable, however, that psi factor and the POP metabolites are produced in submerged liquid culture, a growth condition that suppresses sporulation. Indeed, the vast majority of the sporulation-specific mRNAs which are formed by conidiating surface cultures fail to appear during submerged growth (Timberlake, 1980). The genes responsible for psi factor and the POP metabolites thus appear to be expressed independently of those whose expression requires surface growth.

Very few hormones of the filamentous fungi have been characterized in detail (for a recent review see Gooday, 1983), and the three whose structures are known are terpenoids but are otherwise dissimilar. One of these, produced by the *Mucorales* group of fungi, is trisporic acid, the effect of which is reminiscent of psi factor in that it causes a total switch from asexual reproduction to sexual sporulation. Trisporic acid is not species-specific, inducing sexual development in many members of the *Mucorales* group. By contrast, psi factor appears to be species-specific; no response was observed when we applied highly active extracts to several other sexual or imperfect *Aspergillus* species including *A. ruber*, *A. ornatus* and *A. niger*.

A. nidulans is becoming an increasingly useful organism for the study of gene organization and the control of gene expression during development. The existence of a chemical signal that regulates sporulation, and the availability of mutants that modulate the level of the signal, add a new dimension to such investigations.

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