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Conidiogenesis and Secondary Metabolism in *Penicillium urticae*

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Submerged cultures of *Penicillium urticae* (NRRL 2159A) produced the antibiotics patulin and griseofulvin when grown in a glucose-nitrate medium. A high concentration of calcium (i.e., 68 mM) inhibited the production of both antibiotics while stimulating conidiogenesis. Conidial mutants that were defective in an early stage of conidiogenesis produced markedly less patulin, even under growth conditions that favored secondary metabolism. A mutant which lacked the ability to produce the patulin pathway metabolites *m*-cresol, toluquinol, *m*-hydroxybenzyl-alcohol, *m*-hydroxybenzaldehyde, gentisaldehyde, gentisyl alcohol, gentisic acid and patulin, as well as the pathway enzyme *m*-hydroxybenzyl-alcohol dehydrogenase, still produced yields of conidia that were equivalent to or greater than those of the parent strain. Other mutants which were blocked at later steps of the patulin pathway also produced conidia. These results indicate that patulin and the other related secondary metabolites noted above are not a prerequisite to conidiogenesis in *P. urticae*. Environmental and developmental factors such as calcium levels and conidiogenesis do, however, indirectly affect the production of patulin pathway metabolites.

The belief that a producing microbe only benefits from the production of a secondary metabolite and not from the secondary metabolite itself (7) stems largely from the species-specific occurrence of most secondary metabolites. The production of many secondary metabolites is, however, not restricted to a single microbial species (19). Furthermore, the molecular details of regulatory and differentiation mechanisms are often species specific in accord with Alexander's observation (1) that "every organism has an ecological raison d'être" which is based upon specific biochemical traits.

Of the various postulated functions for secondary metabolites (10), their possible role in microbial sporulation is particularly attractive. This is because sporulation is similar to secondary metabolism in a number of respects. The prerequisite conditions for sporulation (16) as well as secondary metabolism (5, 38) are generally of a narrower range than those conditions that permit vegetative growth. In penicillia such as *P. griseofulvum* (3, 26) and *P. urticae* (34, 35) submerged-culture sporulation occurs after the vegetative growth phase, as a response to nutrient (especially nitrogen) limitation. Lastly, the development of spores requires the presence of new enzymes for a limited but specific period of time. This is also true of secondary metabolism (40).

Relatively few secondary metabolites have been implicated in the sporulation process. A structural and/or protective role for phenolic pigments is probable for a number of fungi (6). In some *Penicillium* species, polyphenols, polyphenol oxidase, and spores appear sequentially in that order (18). A conidiogenesis-stimulating agent (i.e., a "morphogen") has been reported to be present in the stationary-growth-phase medium of penicillia and other species (15, 29). The role of a number of fungal sex hormones has been documented (14, 30, 39), and the role of bacterial peptide antibiotics as transcription regulators involved in the transition from the vegetative to sporulation phase has been extensively investigated (20, 31, 32).

P. urticae produces the secondary metabolites patulin (11) and griseofulvin (24) and is capable of submerged-culture sporulation and microcycle conidiation (34, 35). In addition, the patulin biosynthetic pathway and its enzymology have been extensively studied (13, 22, 28, 37). In this communication we report the use of various patulin-minus and sporulation-deficient mutants of *P. urticae* (NRRL 2159A) in considering the question of whether patulin or other patulin pathway metabolites are required by *P. urticae* for the production of viable spores.

MATERIALS AND METHODS

Organism. A white-colony mutant of the common soil fungus *P. urticae* (NRRL 2159A) was used in this

and previous studies of patulin biosynthesis (11). Various mutants used were isolated from this strain as described below and are listed in Table 3.

Culture conditions. Surface cultures of both the parent and mutant strains were grown on Czapek-Dox agar (49 g of Czapek solution agar [Difco] plus 5 g of Bacto agar [Difco] in 1 liter of double-deionized water) slants in 8-dram (29.6-ml) vials for 7 days at 28°C. For the inoculation of submerged cultures, a spore suspension was prepared by adding 10 ml of a detergent solution (450 μg of Aerosol OT [Fisher Scientific Co.] per ml of water) to one agar slant and shaking vigorously. A portion of this spore suspension was added to each 500-ml Erlenmeyer flask containing 50 ml of seed culture medium (D-glucose, 50 g; $NaNO_3$, 2.5 g; yeast extract [Difco], 1.0 g; and distilled water to 1 liter, pH 7.4) to yield a final concentration of $\sim 8 \times 10^6$ spores/ml. For conidiation-deficient mutants, a cell suspension was prepared by adding 10 ml of the above detergent solution to an agar slant and rubbing the surface of the culture with a wire loop. Flasks (500 ml) containing 50 ml of seed culture medium were inoculated with 2.5 ml of this cell suspension. Five-day-old shake cultures were homogenized (1 min at maximum speed in a Sorvall Omnimixer), and 1 ml of the homogenate was used to inoculate a further series of flasks containing 50 ml of seed culture medium. After shaking for 1 (conidial strains) or 2 (aconidial strains) days, 50-ml portions of these cultures were filtered (Whatman no. 1), washed with sterile double-deionized water, suspended in 10 ml of sterile double-deionized water, and homogenized as described above. A portion of this cell suspension (1 ml; ~15 mg [dry weight] of cells) was used to inoculate each of a series of 500-ml Erlenmeyer flasks containing 50 ml of either of two culture media. These were: glucose-nitrate medium (D-glucose, 50.0 g, 278 mM; $NaNO_3$, 2.5 g, 29 mM; KH_2PO_4, 1.0 g, 7.3 mM; $MgSO_4 \cdot 7H_2O$, 0.5 g, 2.0 mM; KCl, 0.5 g, 6.7 mM; $FeSO_4 \cdot 7H_2O$, 1.9 mg, 6.8 μM; $ZnSO_4 \cdot 7H_2O$, 4.5 mg, 15.7 μM; $MnSO_4 \cdot H_2O$, 0.23 mg, 1.4 μM; $CuSO_4 \cdot 5H_2O$, 0.15 mg, 0.60 μM; and double-deionized water to 1 liter, pH 6.5) and glucose-yeast extract medium (the same as the glucose-nitrate medium except for the substitution of 5.0 g of yeast extract [Difco] for the $NaNO_3$). These media were supplemented with $CaCl_2 \cdot 2H_2O$ as required. All shake cultures were incubated at 28°C on a rotary shaker (NBS model G-10 gyratory shaker, 250 rpm, 1-inch [ca. 2.54-cm] stroke). To minimize wall growth, all flasks were coated with a water-repellent silicone film (Dri-Film SC-87, Pierce Chemical Co.).

Isolation of conidiation-deficient mutants. A conidial suspension ($\sim 10^8$/ml) in a 450-μg/ml Aerosol OT solution was prepared from an agar slant of strain NRRL 2159A. A 0.2-ml portion of this spore suspension was added to 1.8 ml of seed culture medium in a test tube (16-mm diameter) and shaken (NBS model R82 reciprocal shaker, 120 displacements/min, 4-cm stroke) for 7 h at 28°C to initiate germination. A 0.2-ml portion of ethyl methane sulfonate (EMS) (Eastman Organic Chemicals) was then added, and the suspension of germinating conidia was shaken for another 1.5 h. The suspension was then homogenized by repeated (~100) Pasteur pipettings, and diluted 100-fold with Aerosol OT solution (450 μg/ml), and 0.1-ml portions of the diluted suspension were plated on petri dishes containing 15 ml of a modified Czapek-Dox agar (as described above but supplemented with 10 g of yeast extract and 0.13 g of $CaCl_2 \cdot 2H_2O$ per liter). After the plates stood for 3 days at 28°C, the conidiation of individual colonies was assessed visually with a Nikon stereoscopic microscope (×40). A 6% survival ratio was obtained and the aconidial nature of visually selected mutants was verified by examining the conidiation of submerged cultures grown in calcium-supplemented media.

As an alternative to EMS treatment, 5 ml of a conidial suspension in Aerosol OT (see above) was placed in an open petri dish (100 by 10 mm) about 20 cm below an ultraviolet (UV) lamp (254 nm) and irradiated for several minutes to kill $\sim 10^3$ conidia/ml. UV-treated conidia were then plated onto modified Czapek-Dox agar and examined as before.

Isolation of patulin-negative mutants. Germinated conidia were treated with EMS as described above, and portions of a diluted, homogenized suspension were plated on petri dishes containing 15 ml of another modified Czapek-Dox agar (as described above but supplemented with 1 g of yeast extract and 0.8 g of sodium deoxycholate per liter). The added deoxycholate promotes the formation of small dense colonies (23). After the plates stood for 2 days at 28°C, the small colonies were cut out with a cork borer (4-mm diameter), and the agar plugs were transferred with tweezers to an empty petri dish (17). After a further 2 days at 28°C, the colony-bearing agar plugs were transferred to an agar slab containing *Bacillus subtilis*. Each agar slab was prepared as described below and had a capacity of ~70 agar plugs. Agar plugs bearing the parent strain had accumulated sufficient patulin to yield a clear inhibitory zone after standing for 24 h at 28°C on the bioassay plate. Colonies that did not inhibit the growth of *B. subtilis* were tested for secondary metabolite production by cultivation under submerged-culture conditions followed by thin-layer chromatographic (TLC) examination of medium extracts. A single-colony isolate was then obtained from each mutant of interest.

Dry weight determinations. Culture samples were suction filtered through Whatman no. 1 filter paper and washed twice with 0.04 M citrate-phosphate buffer (pH 5.0) and twice with double-deionized water. The washed cells were frozen, lyophilized or dried overnight in an oven at 110°C, and cooled to room temperature before weighing.

TLC detection of secondary metabolites. Samples of filtered culture medium (2 ml) were acidified to pH 2.0 with 4 N HCl and extracted with 2 equal volumes of ethyl acetate. These extracts were concentrated to 1 ml by evaporation with a stream of air, and 20-μl portions were spotted onto 250-μm thick fluorescent indicator-containing silica gel plates (Woelm Silica Gel GF_{254}) that had been activated for 2 h at 110°C and equilibrated at room temperature without desiccation. Pure standards (5 μg of each except for 20 μg of *m*-cresol) were rou-

tinely spotted on each plate (see legend to Fig. 2). The eluants used were either chloroform-glacial acetic acid (9:1, vol/vol) or chloroform-glacial acetic acid-methanol-butanol (12:4:1:1, by volume).

Chromatographed compounds were detected by two methods. Under UV light nonfluorescent spots were readily detected if they contained as little as 0.5 μg of *m*-cresol, *m*-hydroxybenzaldehyde, *m*-hydroxybenzyl-alcohol, *m*-hydroxybenzoic acid, gentisaldehyde, gentisic acid, or griseofulvin and less than 0.5 μg of 6-methylsalicylic acid (6-MSA) or patulin. Larger amounts or aging of the TLC plates for a few days was necessary for adequate detection of toluquinol or gentisyl alcohol. After UV examination, the plates were sprayed with a solution (100 mg/20 ml of deionized water) of 3-methyl-2-benzothiazolinone-hydrazone hydrochloride monohydrate (MBTH; Aldrich Chemical Co.) and developed for 15 min at 130°C (33). The detection limits for patulin, which appeared immediately as a yellow spot on a white background, were 0.05 μg (3.2×10^{-4} μmol) per spot under visible light and 0.01 μg/spot under long-wavelength UV light. The detection limit for 6-MSA (purple), toluquinol (red-orange), gentisyl alcohol (red), *m*-hydroxybenzyl-alcohol (purple), and gentisic acid (brown) was 0.5 to 2.0 μg/spot, whereas for *m*-hydroxybenzaldehyde (violet, creme center) and gentisaldehyde (light brown) it was 1.0 to 5.0 μg/spot. *m*-Hydroxybenzoic acid (gray) and *m*-cresol were less readily detected and griseofulvin was not detected with MBTH. The detection limits of some of the compounds improved upon 1 to 2 days of aging while covered with a glass plate.

To determine the presence of intracellular metabolites, washed cells from 96-h cultures grown in glucose-nitrate or glucose-yeast extract media were lyophilized, and cell-free extracts were prepared as described below. Extracts from 30 mg of dry cells were extracted with ethyl acetate and spotted on TLC plates as described above.

6-MSA calorimetric assay. An assay previously described by Forrester and Gaucher (11) and based upon the formation of a colored 6-MSA-$FeCl_3$ complex was used in this study despite the fact that colored culture filtrates and other compounds can interfere with this assay. The detection limit was 0.25 μmol (38 μg) of 6-MSA per ml of culture filtrate.

Patulin bioassay. Culture medium filtrates were assayed for patulin by an agar plate diffusion assay using *B. subtilis* as the antibiotic-sensitive organism (J. W. D. Groot Wassink, unpublished procedure). Recrystallized patulin was used to construct a standard plot of log patulin concentration versus inhibition zone diameter. The sensitivity of the assay was 0.2 μmol or 31 μg of patulin per ml. Since 0.1-ml samples were used, <3 μg of patulin could be confidently detected.

Preparation of cell-free extracts and assay of *m*-hydroxybenzyl-alcohol dehydrogenase. To determine the presence of intracellular secondary metabolites as well as *m*-hydroxybenzyl-alcohol dehydrogenase (EC 1.1.1.97), cell-free extracts were prepared by ballistic disruption of lyophilized cells in a Braun shaker as previously described (13). The dehydrogenase assay used has been fully described (13) and can detect as little as 6 to 10 mU (i.e., 6 to 10 nmol of reduced nicotinamide adenine dinucleotide phosphate oxidized per min) of enzyme per mg of dry cells.

Conidia counting. Culture samples were stored frozen and then thawed, and 5-ml portions were diluted with 5 ml of deionized water and homogenized for 1 min in an Omnimixer (Sorvall) at maximum speed. A 0.2-ml portion of a Macerozyme (Kinki Yakult Mfg. Co. Ltd.) solution (100 mg/ml of 0.2 M phosphate buffer, pH 6.0) was added to 2 ml of this homogenate and incubated for 15 min at 30°C. A 1-ml portion of this enzyme-treated homogenate was added to 0.3 ml of Aerosol OT (450 μg/ml) and 2.0 ml of double-deionized water, and the suspension was sonically treated for 1 min at 80% of the maximal power of a Biosonik III (Bronwill Scientific Inc.) equipped with a 2-cm-diameter probe. This treatment did not destroy any conidia, but did free conidia from attachment to hyphal cells as well as extensively rupturing these latter cells. Duplicate samples of each conidial suspension were placed on a hemacytometer (Resistance, Fisher Scientific Co.; 0.0025-mm rulings, 0.1-mm depth), and the total number of conidia in 10^{-4} ml (i.e., within an area of 1 mm^2) of each sample was counted under a microscope (×400). The counting reproducibility was ±7%, and a conidial concentration of $<2.0 \times 10^5$ conidia/ml of original culture was below the detection limit of the assay. Assay results were routinely reported as the number of conidia per milligram of dry cells.

Determination of conidia viability. Conidia from agar slants of NRRL 2159A and mutant S15 were germinated in seed culture medium exactly as described above. Germination began at about 7 h and was essentially complete by 12 h. The low calcium content of the seed culture medium prevented any submerged-culture conidiation. Samples (5 ml) obtained at 0 and 12 h were treated as described above except for the omission of the Macerozyme step. Since the sonic treatment step eliminated most cell aggregation while causing some disruption of viable cells, the number of nonviable conidia (i.e., those that had not undergone spherical growth or germination [34]) was readily counted. The percent viability of conidia was obtained by subtracting the number of conidia per milliliter of a 12-h culture from that of a 0-h culture.

RESULTS

Effect of metal ions on conidiogenesis. The effect of various metal ions on the growth and conidiation of *P. urticae* (NRRL 2159A) shake cultures was examined. Detectable submerged-culture conidiation did not occur unless the glucose-nitrate medium was supplemented with relatively high concentrations (i.e., ≥34 mM) of calcium chloride (Table 1). Chloride itself (i.e., NaCl or KCl) had no such effect, and the prolific conidiation of *P. urticae* surface cultures did not require the addition of calcium to the agar medium. Of the other "alkaline earth met-

TABLE 1. *Effect of various cations on P. urticae (NRRL 2159A) submerged-culture conidiation* [a]

Divalent cation[b]	Concn (mM)	Cell dry wt (mg/ml)	Conidia (no./mg of dry cells)
$CaCl_2 \cdot 2H_2O$	68 (10 g/liter)	7.35	1.6×10^7
	34 (5 g/liter)	7.05	1.5×10^7
	0.34 (0.05 g/liter)	8.69	—[c]
$SrCl_2 \cdot 6H_2O$	40	7.67	1.3×10^7
	1	7.02	—
$BaCl_2 \cdot 2H_2O$	40	2.56	—
	1	7.07	—

[a] Shake cultures were grown for 48 h in glucose-nitrate medium supplemented with various divalent cations.

[b] The following cations were also tested in glucose-nitrate medium (the initial medium concentration and the cell dry weight at 72 h are given in parentheses): LiCl (40 mM, 5.92 mg/ml; 1 mM, 8.35 mg/ml), NaCl (100 mM, 7.42 mg/ml), KCl (40 mM, 6.36 mg/ml), $MgSO_4 \cdot 7H_2O$ (40 mM, 7.65 mg/ml), $BaCl_2 \cdot 2H_2O$ (40 mM, 4.85 mg/ml), $MnSO_4 \cdot H_2O$ (40 mM, no growth; 1 mM, 7.88 mg/ml), $FeSO_4 \cdot 7H_2O$ (40 mM, no growth; 1 mM, 9.25 mg/ml), $NiCl_2 \cdot 6H_2O$ (40 mM, no growth; 1 mM, 3.77 mg/ml), $CuSO_4 \cdot 5H_2O$ (40 mM, no growth; 1 mM, 0.20 mg/ml), $ZnSO_4 \cdot H_2O$ (40 mM, no growth; 1 mM, 7.88 mg/ml), and $HgCl_2$ (40 mM, no growth; 1 mM, 0.22 mg/ml). In every case 72-h samples exhibited no measurable number of conidia.

[c] Below the limit of accurate detection.

als" (i.e., Mg^{2+}, Sr^{2+}, Ba^{2+}) only strontium exhibited a lesser but positive stimulation of conidiation at these high concentrations. Of the other divalent metal ions tested, some (Mn^{2+}, Fe^{2+}, Zn^{2+}) prevented growth at high concentrations (i.e., 40 mM), whereas others (Ni^{2+}, Cu^{2+}, Hg^{2+}) were also very inhibitory at the lower concentration of 1 mM.

Effect of calcium on conidiogenesis and secondary metabolism. In calcium-supplemented (i.e., 1.36 mM) medium, cultures attained a maximum growth yield and conidia yield at about 48 h, whereas patulin production lagged behind and did not reach a maximum yield until about 72 h (Fig. 1). Increasing concentrations of calcium had little, if any, effect on growth, but a rather dramatic effect on secondary metabolism and conidiation (Table 2). The rate of patulin production and the ultimate yield of patulin were severely decreased as the medium concentration of calcium chloride was increased. Calcium had a similarly negative effect on the production of 6-MSA, the first metabolite of the patulin pathway, and of *m*-hydroxybenzyl-alcohol dehydrogenase, the fourth enzyme of the pathway. In contrast, conidiation was dependent upon the presence of calcium and was maximal (i.e., ~1.6×10^7 conidia/mg of dry cells) at a calcium concentration of about 14 mM. At a high calcium concentration of 68 mM, little or no adverse effect on growth and conidiation was observed, but patulin biosynthesis was severely inhibited. Thus, patulin production appeared to follow rather than precede conidiation, and calcium had markedly opposite effects on these two nongrowth-associated phenomena.

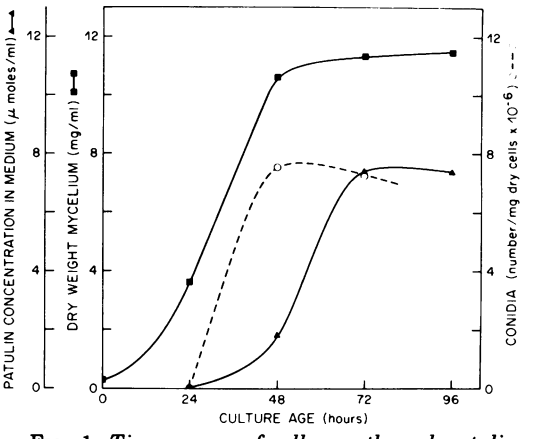

FIG. 1. *Time course of cell growth and patulin and conidia production in shake cultures of P. urticae (NRRL 2159A) grown in glucose-nitrate medium supplemented with 0.2 g of $CaCl_2 \cdot 2H_2O$ per liter.*

Characterization of patulin-negative mutants. Patulin-deficient mutants (Table 3) were isolated from *P. urticae* NRRL 2159A. Mutants J1, J2, S11, and S15 and the parent strain NRRL 2159A were cultivated in glucose-nitrate and glucose-yeast extract medium, and 48-h samples were extracted and examined by TLC (Fig. 2).

Mutant J1 produced gentisyl alcohol, *m*-hydroxybenzyl-alcohol, a little toluquinol and gentisic acid, and three unknown compounds, but no patulin (Fig. 2). Mutant J2 produced gentisyl alcohol, *m*-hydroxybenzyl-alcohol, considerable toluquinol, and perhaps a little gentisic acid, but none of the above-mentioned unknowns and no patulin. The glucose-yeast extract medium generally tended to favor secondary metabolite production in both mutants, and older (i.e., 96-h) cultures of both mutants accumulated larger amounts of the dead-end metabolite gentisic acid. Both surface and shake cultures (2 g of $CaCl_2 \cdot 2H_2O$ added per liter) of mutants J1 and J2 produced normal numbers of conidia.

Mutant S11 produced very small amounts of gentisyl alcohol, and *m*-hydroxybenzyl-alcohol

TABLE 2. *Effect of calcium on the production of secondary metabolites, m-hydroxybenzyl-alcohol dehydrogenase, and conidia by submerged cultures of P. urticae (NRRL 2159A)*

$CaCl_2 \cdot 2H_2O$[a] (g/liter, mM)	Culture time (h)	Cell dry wt (mg/ml)	Medium pH	Patulin		6-MSA (μmol/ml)	Dehydrogenase[c] (U/mg of dry cells)	Conidia (no./mg of dry cells)
				μmol/ml	%[b]			
0, 0	24	4.80	6.3	–[d]	–	0.74	73	–[d]
	48	8.45	4.8	9.6	74	0.69	157	–
	72	9.55	4.7	13.5	104	1.02	58	–
	96	9.13	5.9	13.0	100	1.43	27	
0.2, 1.36	24	3.65	6.9	–	–	0.34	–	2.0×10^4
	48	10.6	4.9	1.9	26	0.93	38	7.5×10^6
	72	11.3	4.7	7.3	100	0.72	24	7.2×10^6
	96	11.4	4.9	7.3	100	1.19	15	
2, 13.6	24	4.65	6.2	–	–	–	–	5.0×10^6
	48	8.18	4.0	0.84	14	0.28	49	1.7×10^7
	72	9.85	4.0	5.6	90	0.43	41	1.5×10^7
	96	10.1	4.0	6.2	100	0.40	22	
10, 68.0	24	4.32	6.6	–	–	–	–	3.9×10^6
	48	8.17	4.0	0.31	9.4	0.43	–	1.6×10^7
	72	9.75	4.2	2.2	67	0.58	20	1.8×10^7
	96	11.0	4.1	3.3	100	0.62	14	

[a] Shake cultures were grown in glucose-nitrate medium that was supplemented with various concentrations of $CaCl_2 \cdot 2H_2O$.

[b] Relative percent yield of patulin (the 96-h yield was taken as 100% in each case).

[c] *m*-Hydroxybenzyl-alcohol dehydrogenase (EC 1.1.1.97).

[d] Below the limit of accurate detection (see text).

TABLE 3. *Mutants of P. urticae (NRRL 2159 A) used in this study*

Mutant	Mutagen	Characteristics
M1	UV (254 nm)	Late stage oligosporogenous ($Sp^{\pm}$, Pat^+)
M3	EMS	Early stage asporogenous (Sp^-, $Pat^{\pm}$)
M4	EMS	Early stage asporogenous (Sp^-, $Pat^{\pm}$)
J1	EMS	Late patulin pathway negative (Pat^-, Sp^+)
J2	EMS	Late patulin pathway negative (Pat^-, Sp^+)
S11	EMS	Late patulin pathway negative (Pat^-, Sp^+)
S15	EMS	Early patulin pathway negative (Pat^-, Sp^+)

and, if grown in glucose-nitrate medium, some 6-MSA (Fig. 2). In contrast to this mutant, mutant S15 produced only 6-MSA. The parent strain (NRRL 2159A) produced gentisyl alcohol, *m*-hydroxybenzyl-alcohol, large amounts of patulin (i.e., 9 to 12 μmol/ml), one of the unknowns produced by mutant J1, and a little toluquinol and gentisic acid.

For all of the above strains, the intracellular levels of patulin pathway metabolites were examined by preparing extracts from 30 mg of 96-h-old cells. Subsequent TLC did not show detectable amounts of any pathway metabolite. Griseofulvin was, however, present in appreciable quantities.

Secondary metabolism and conidiogenesis in mutant S15. The effect of calcium on mutant S15 was examined in an experiment identical to that described for NRRL 2159A (Table 2). Cultivation of mutant S15 in glucose-nitrate medium supplemented with increasing concentrations of calcium (Table 4) showed only a slight retardation of growth, a marked decrease in 6-MSA production, and a large increase in conidia production. No detectable levels of patulin (Table 4), other post-6-MSA metabolites (Fig. 3), or *m*-hydroxybenzyl-alcohol dehydrogenase (Table 4) could be detected in any cultures of mutant S15. When grown in the absence of added calcium, mutant S15 accumulated larger amounts of 6-MSA and griseofulvin in the culture medium than did NRRL 2159A (Table 2), whereas in the presence of 10 g of $CaCl_2 \cdot 2H_2O$ per liter even these two metabolites were undetectable (Table 4). Despite this essentially complete absence of patulin pathway metabolites, mutant S15 produced an equivalent or greater number of conidia ($\sim 3.7 \times 10^7$/mg of dry cells) than did NRRL 2159A ($\sim 1.8 \times 10^7$/mg of dry cells) in the presence of 10

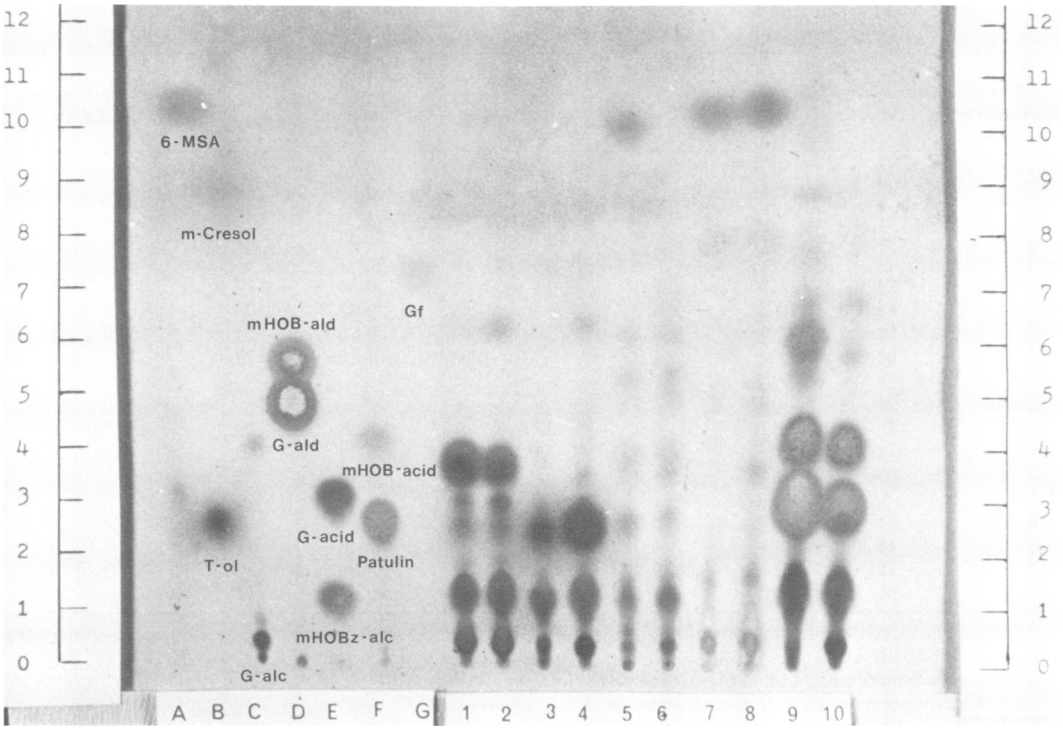

FIG. 2. *TLC of secondary metabolites produced by mutants J1, J2, S11, and S15 and by the parent strain NRRL 2159A. Shake culture samples (48 h) were extracted and chromatographed on TLC plates with chloroform-glacial acetic acid (9:1, vol/vol) as described in the text. The standards spotted were: A, 6-MSA (purple); B, m-cresol (diffuse, purple) and toluquinol (T-ol, red-orange); C, gentisyl alcohol (G-alc, red); D, m-hydroxybenzaldehyde (mHOB-ald, violet with creme center) and gentisaldehyde (G-ald, light brown); E, gentisic acid (G-acid, brown) and m-hydroxybenzyl-alcohol (mHOBz-alc, purple); F, m-hydroxybenzoic acid (mHOB-acid, gray) and patulin (bright yellow); G, griseofulvin (Gf, poorly detectable). The culture extracts spotted were 1 and 2 (mutant J1), 3 and 4 (mutant J2), 5 and 6 (mutant S11), 7 and 8 (mutant S15), and 9 and 10 (NRRL 2159A). Cultures yielding samples 1, 3, 5, 7, and 9 and 2, 4, 6, 8, and 10 were grown in glucose-nitrate and glucose-yeast extract medium, respectively.*

g of $CaCl_2 \cdot 2H_2O$ per liter. The viability (i.e, the ability to grow spherically and germinate) of conidia harvested from agar slants of *P. urticae* NRRL 2159A and S15 was examined as described above, and 98% of the conidia obtained from both strains were found to be viable. Thus, *P. urticae* conidiogenesis does not appear to depend upon the simultaneous or prior production of any post-6-MSA patulin pathway metabolites, and the calcium effect suggests that this is also true for 6-MSA and the related polyketide griseofulvin. In fact, since the number of conidia per milligram of dry cells increases in this mutant (Table 4) at a faster rate than in the parent (Table 2), some patulin pathway metabolites may inhibit conidiogenesis.

Characterization of conidiation-deficient mutants. Conidiation-deficient mutants that were blocked at early (M3, M4) and late (M1) stages of conidiogenesis were also isolated (Table 3). In contrast to the normal submerged-culture conidiation of the parent strain NRRL 2159A (I in Fig. 4), mutant M1 (II in Fig. 4) produced an immature "conidiophore" from which conidia were released very poorly. Mutants M3 (III in Fig. 4) and M4 produced no recognizable conidiophore-like structures. Differences in gross colony morphology allowed early and late mutants to be readily distinguished from each other and from their parent.

Mutants M1, M3, and M4 were cultivated in glucose-nitrate medium supplemented with increasing concentrations of calcium. In the absence of added calcium, the late-conidiation-stage mutant M1 produced about half as much patulin and 6-MSA (Table 5) as did the parent (Table 2), whereas in the presence of calcium, patulin and 6-MSA were produced in quantities similar to those of the parent. In contrast to the parent, however, mutant M1 produced some-

TABLE 4. *Effect of calcium on the production of secondary metabolites, m-hydroxybenzyl-alcohol dehydrogenase, and conidia by submerged cultures of mutant S15*

$CaCl_2 \cdot 2H_2O$[a] (g/liter)	Culture time (h)	Cell dry wt (mg/ml)	Medium pH	Patulin (μmol/ml)	6-MSA (μmol/ml)	Dehydrogenase (U/mg of dry cells)	Conidia (no./mg of dry cells)
0	24	4.98	7.4	–[b]	–	–	–
	48	10.3	5.5	–	2.12	–	–
	72	10.3	5.2	–	5.82	–	–
	96	11.9	5.4	–	4.12	–	
0.2	24	2.78	7.5	–	–	–	1.6×10^6
	48	8.42	6.8	–	0.34	–	4.7×10^6
	72	10.2	5.5	–	0.60	–	1.2×10^7
	96	11.5	5.4	–	0.41	–	
2	24	2.96	7.0	–	–	–	1.9×10^7
	48	5.94	6.0	–	–	–	3.2×10^7
	72	7.89	4.5	–	0.27	–	3.2×10^7
	96	9.45	4.4	–	–	–	
10	24	2.38	7.0	–	–	–	1.6×10^7
	48	5.52	5.4	–	–	–	3.4×10^7
	72	8.07	4.3	–	–	–	3.7×10^7
	96	9.02	4.1	–	–	–	

[a] Shake cultures were grown in glucose-nitrate medium that was supplemented with various concentrations of $CaCl_2 \cdot 2H_2O$.

[b] Below the limit of accurate detection (see text).

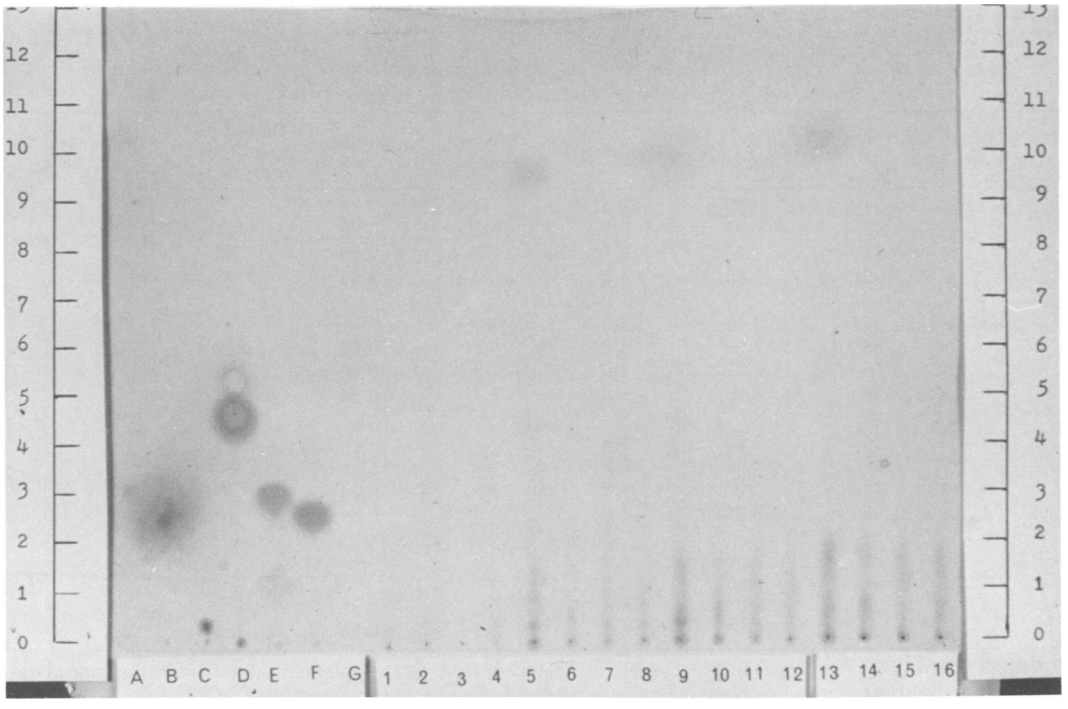

FIG. 3. *TLC of secondary metabolites produced by mutant S15. Shake culture samples were extracted and chromatographed on TLC plates with chloroform-glacial acetic acid (9:1, vol/vol) as described in the text. The samples spotted were A to G (standards as described in the legend to Fig. 2), 1 to 4 (24-h samples), 5 to 8 (48 h), 9 to 12 (72 h), and 13 to 16 (98 h). Cultures yielding samples 1, 5, 9 and 13; 2, 6, 10, and 14; 3, 7, 11, and 15; and 4, 8, 12, and 16 were grown in glucose-nitrate medium supplemented with 0, 0.2, 2, and 10 g of $CaCl_2 \cdot 2H_2O$ per liter, respectively.*

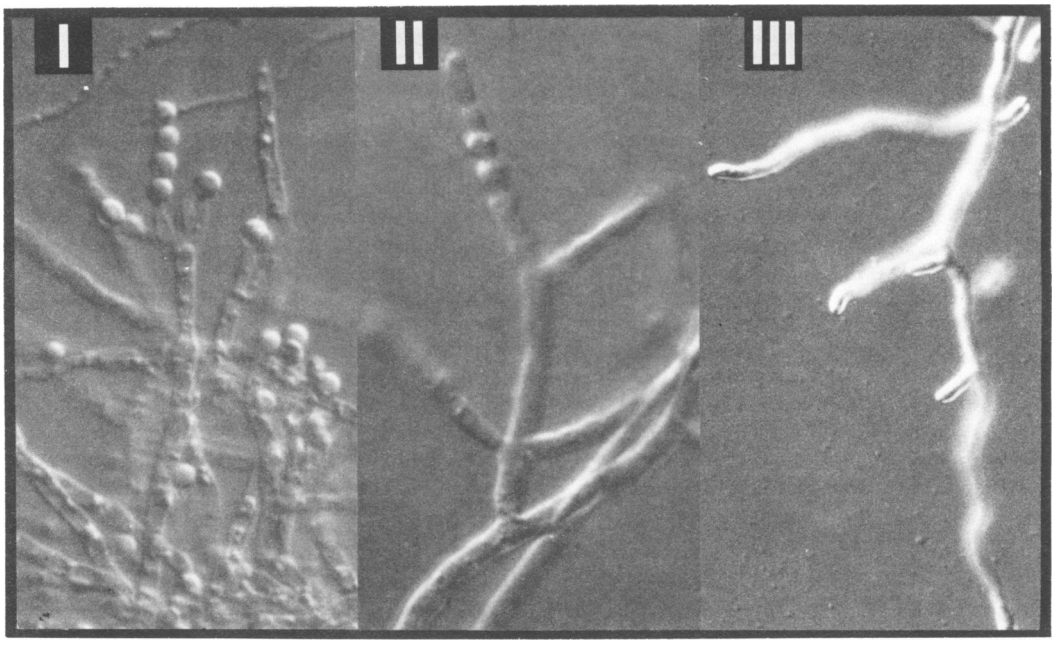

FIG. 4. *Light micrographs (×435) of submerged-culture "conidiophores" of NRRL 2159A (I), mutant M1 (II), and mutant M3 (III). Samples were obtained during the stationary phase (48 to 72 h) of cultures grown in calcium-supplemented glucose-nitrate medium.*

TABLE 5. *Effect of calcium on the production of secondary metabolites, m-hydroxybenzyl-alcohol dehydrogenase, and conidia by submerged cultures of oligosporogenous mutant M1*

$CaCl_2 \cdot 2H_2O$[a] (g/liter)	Culture time (h)	Cell dry wt (mg/ml)	Medium pH	Patulin (μmol/ml)	6-MSA (μmol/ml)	Dehydrogenase (U/mg of dry cells)	Conidia (no./mg of dry cells)
0	24	0.28	6.3	–[b]	–		
	48	2.95	6.4	0.75	0.78	111	
	72	7.56	5.8	2.2	0.79	86	
	96	7.03	4.5	6.0	0.64	73	–
0.2	24	0.46	5.9	–	–		
	48	7.32	4.2	5.1	0.24	229	
	72	8.37	4.0	7.3	0.50	123	
	96	8.28	5.4	7.7	0.64	42	–
2	24	0.50	5.8	–	–		
	48	4.06	5.2	–	–	–	–
	72	7.00	4.2	1.5	0.30	78	1.8×10^5
	96	8.28	4.2	7.0	0.34	73	1.7×10^5
10	24	0.36	5.2	–	–		
	48	2.65	5.9	–	–	–	–
	72	6.00	4.6	–	–	–	4.8×10^5
	96	8.60	4.5	2.3	0.43	24	4.2×10^5

[a] Shake cultures were grown in glucose-nitrate medium that was supplemented with various concentrations of $CaCl_2 \cdot 2H_2O$.

[b] Below the limit of accurate detection (see text).

what higher levels of dehydrogenase and much lower numbers of conidia (i.e., 1 to 3% of the number produced by the parent) in the presence of high concentrations of calcium (Table 5). In both the absence and presence of added calcium, the early stage mutant M3 grew poorly and produced low levels of dehydrogenase, particularly small amounts of patulin, and no co-

nidia (Table 6). Whereas other pathway metabolites were also produced in very low yields, unusually large quantities (i.e., 11 to 12 μmol/ml) of 6-MSA were produced in medium containing low concentrations (i.e., 0 or 0.2 g of $CaCl_2 \cdot 2H_2O$ added per liter) of calcium (compare Tables 2, 4, and 6). In a medium more favorable to secondary metabolite production (i.e., glucose-yeast extract), cultures of mutant M3 grew more rapidly (i.e., possessed a shorter lag) and attained the same ultimate dry weight and patulin yields, but a much lower yield of 6-MSA and a significantly higher level of dehydrogenase (compare Tables 6 and 7). As with the parent NRRL 2159A, mutants M1 and M3 also exhibited the antagonistic effect of high calcium concentrations on secondary metabolism (Tables 5 and 6). Under culture conditions identical to those used for mutants M1 and M3 (Tables 5 and 6), the early stage mutant M4 grew poorly and produced no detectable amounts of 6-MSA, patulin, dehydrogenase, or conidia. Growth of M4 in glucose-yeast extract medium did, however, result in somewhat better growth yields and definitely better (i.e., detectable) levels of patulin, 6-MSA, and dehydrogenase (Table 7).

TABLE 6. *Effect of calcium on the production of secondary metabolites and m-hydroxybenzyl-alcohol dehydrogenase by submerged cultures of conidiation-deficient mutant M3*

$CaCl_2 \cdot 2H_2O$[a] (g/liter)	Culture time (h)	Cell dry wt (mg/ml)	Medium pH	Patulin (μmol/ml)	6-MSA (μmol/ml)	Dehydrogenase (U/mg of dry cells)
0	24	0.314	6.2		–[b]	–
	48	1.98	4.8	–	–	–
	72	4.85	5.4	–	1.57	21
	96	4.63	4.5	2.0	11.0	24
0.2	24	0.359	6.2		–	–
	48	2.62	4.5	–	–	–
	72	7.22	5.2	1.8	6.95	80
	96	4.93	4.6	2.4	12.4	31
2	24	0.423	4.9		–	–
	48	2.33	5.9	–	–	–
	72	5.05	4.4	–	1.39	40
	96	4.73	4.3	0.36	6.32	36
10	24	0.725	4.6		–	–
	48	1.83	5.7	–	–	–
	72	3.14	5.2	–	0.67	–
	96	4.74	5.0	–	1.35	–

[a] Shake cultures were grown in glucose-nitrate medium that was supplemented with various concentrations of $CaCl_2 \cdot 2H_2O$.

[b] Below the limit of accurate detection (see text).

TABLE 7. *Optimal production of secondary metabolites and m-hydroxybenzyl-alcohol dehydrogenase by submerged cultures of conidiation-deficient mutants M3 and M4*

Mutant	Culture time[a] (h)	Cell dry wt (mg/ml)	Medium pH	Patulin (μmol/ml)	6-MSA (μmol/ml)	Dehydrogenase (U/mg of dry cells)
M3	24	3.99	4.9	–[b]	1.03	92
	48	4.55	4.5	2.1	2.08	152
	72	4.67	4.3	2.0	1.76	77
	96	4.96	4.1	2.3	1.45	36
M4	24	2.77	5.5	–	–	10
	48	5.86	4.7	0.95	2.24	152
	72	8.42	4.6	2.0	0.74	139
	96	9.10	4.5	2.8	0.71	154

[a] Shake cultures were grown in glucose-yeast extract medium without added $CaCl_2 \cdot 2H_2O$.

[b] Below the limit of accurate detection (see text).

DISCUSSION

The first report of submerged-culture sporulation of a *Penicillium* sp. (i.e., *P. notatum*) was that of Foster et al. (12), who noted the requirement for $CaCl_2$ (>45 mM). This requirement for calcium was confirmed and further examined by Hadley and Harrold (15), who noted that calcium was not required for vegetative growth of *P. notatum* and that at an $NaNO_3$ concentration of 6 g/liter, calcium concentrations of greater than 1 mM gave maximum yields of conidia (i.e., 10^7 spores/mg [dry weight] of cells). They also noted that strontium and barium were much less effective than was calcium and that lower concentrations of the nitrogen nutrient markedly enhanced the capacity of the culture to sporulate and lowered the minimum concentration of calcium required for maximum sporulation. Similar results were obtained by Morton et al. for *P. griseofulvum* (26, 27), which is closely related to *P. urticae*. Thus, submerged cultures sporulated only when the nitrogen nutrient (i.e., 2.3 g of KNO_3 per liter) was exhausted, some oxidative glucose metabolism remained, and high concentrations of either calcium (i.e., >5 mM) or nonmetabolized polyols such as mannitol (i.e., 100 g/liter) were present. Replacement cultures in nitrogen-free medium containing 9 mM calcium could be prevented from sporulating if nitrate or ammonium ions were added within 7 to 8 h after replacement. Of particular interest was the finding that surface cultures sporulate profusely in the absence of calcium, polyols, or an external energy source. Since the sporulation ability of aerial hyphae was inde-

pendent of the availability of CO_2, O_2, or water, the ability of an air-water interface, polyols, or calcium to stimulate conidiogenesis was ascribed to their ability to effect structural changes in the cell envelope (26). This suggestion that calcium can alter the structure and hence the function of the fungal cell envelope was supported by the later observation that the fresh water fungus *Achlya* possesses an absolute requirement for calcium (9) and that removal of calcium immediately arrests amino acid transport and protein synthesis (8). A calcium-binding glycoprotein in the cell envelope has been implicated in this effect (21). Calcium has also been shown to induce stalk formation in the slime mold (25). The effect of calcium on fungal differentiation is, however, not ubiquitous since calcium is not an effective sporulant for submerged cultures of *Aspergillus nidulans* or *A. niger* (27) and since surface cultures of *P. clavigerum* and *P. claviforme* require manganese for conidiation (36).

In this study, submerged cultures of *P. urticae* grown on a glucose-nitrate medium (i.e., 2.5 g of $NaNO_3$ per liter) exhibited detectable and maximum levels of sporulation at calcium concentrations of about 1 and 10 mM, respectively, and concentrations as high as 68 mM did not decrease the yield of conidia. This closely resembles the response reported for *P. griseofulvum* (27). The fact that of the divalent cations examined, Ca^{2+} and to a much lesser extent Sr^{2+} were effective in stimulating conidiogenesis is similar to the finding for *P. notatum* (15) and undoubtedly reflects the differences in the ionic radii of these ions (i.e., $Ca^{2+} < Sr^{2+}$, etc.).

Although calcium had a marked stimulatory effect on conidiogenesis of the parent strain and mutants S15 and M1 (Table 3), it had an opposite, inhibitory effect on polyketide biosynthesis in the parent strain and in mutants S15, M1, M3, and M4 (Fig. 5). Thus, in the parent, for example, an increase in calcium from 0 to 68 mM decreased the yield of patulin from about 13 to 3 μmol/ml and also decreased the rate of patulin biosynthesis. Calcium also inhibited the production of the pathway enzyme *m*-hydroxybenzyl-alcohol dehydrogenase. These latter observations confirm an earlier observation that 20 g of calcium carbonate per liter inhibits patulin production in surface cultures of *P. urticae* (2). Since this calcium effect may act by modifying the cell envelope and since the patulin pathway metabolite pools are essentially extracellular (see below), it is possible that calcium interferes with the transport and hence the biosynthesis of these secondary metabolites. Since an increase in *B. licheniformis* sporulation was reported to decrease antibiotic production (4), it was also possible that calcium inhibited secondary metabolism via its stimulatory effect on sporulation. This was tested by examining mutants M1, M3, and M4 (Table 3, Fig. 5). These conidiation-deficient mutants, especially early stage mutants M3 and M4, generally exhibited a diminished rather than an increased patulin pathway activity, and this decrease was independent of added calcium. Since both conidiogenesis and secondary metabolism are generally derepressed by depletion of nitrogen nutrients, it is certainly possible that a single mutation at this level could effect both biosynthetic phenomena (Fig. 5).

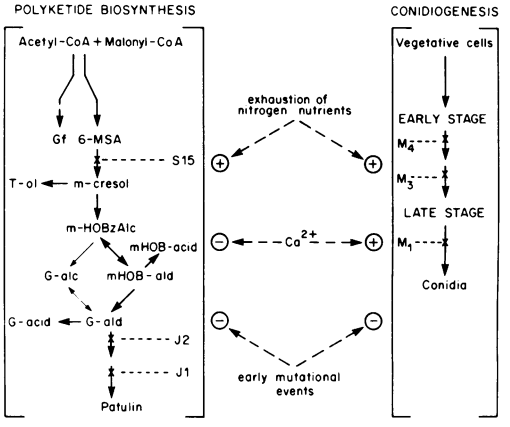

FIG. 5. *Indirect relationships between secondary metabolism and conidiogenesis in P. urticae. See the legend to Fig. 2 and the text for an explanation of the abbreviations used in this figure.*

Patulin-minus mutants J1 and J2 appear to be mutants that have lost enzymes which occur in the post-gentisaldehyde part of the patulin pathway. The block in J1 occurs after the block in J2 since two previously undetected metabolites accumulate in cultures of J1 but not in J2 (Fig. 5). The normal sporulation of these two mutants clearly indicates that patulin and some of its immediate precursors are not required for sporulation. The patulin-minus mutant S15 is blocked immediately after the first pathway metabolite, 6-MSA, and thus the only polyketides detected were 6-MSA and griseofulvin. As the calcium concentration of the medium was increased, the yield of these two metabolites decreased, and at a calcium concentration of 68 mM both metabolites were undetectable, whereas the yield of viable conidia remained maximal. The intracellular levels of various patulin pathway metabolites are very low in both parent and mutant (i.e., J1, J2, and

S15) strains. An approximate calculation based on the detection limits given above and on a 30-ml volume for 30 mg of dry cells indicates that, for the metabolites that are produced, their intracellular concentration must be less than 10^{-6} to 10^{-8} M. In addition, the absence of the pathway enzyme *m*-hydroxybenzyl-alcohol dehydrogenase in mutant S15 suggests that most, if not all, of the enzymes which catalyze post-6-MSA steps are absent and, thus, even undetectable, intracellular concentrations of these metabolites must also be absent. Therefore, conidiogenesis in *P. urticae* does not appear to require the biosynthesis of any patulin pathway metabolite or probably griseofulvin. It is still possible, however, that an extremely small intracellular concentration of 6-MSA, griseofulvin, or some other secondary metabolite is both required and sufficient for conidiogenesis. Finally, it should be noted that various other functions, such as growth inhibition, were not tested in this study and therefore cannot be eliminated at present.

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