JOURNAL OF BACTERIOLOGY, May 1982, p. 785–787
0021-9193/82/050785-03$02.00/0

Vol. 150, No. 2

Extracellular Siderophores of Rapidly Growing *Aspergillus nidulans* and *Penicillium chrysogenum*

GISELA CHARLANG,[1] ROBERT M. HOROWITZ,[2] PETER H. LOWY,[1] BRADFORD NG,[1] STEPHEN M. POLING,[2] AND NORMAN H. HOROWITZ[1]*

Division of Biology, California Institute of Technology, Pasadena, California 91125[1] and Fruit and Vegetable Chemistry Laboratory, U. S. Department of Agriculture, Pasadena, California 91106[2]

Received 27 October 1981/Accepted 5 December 1981

The highly active extracellular siderophores previously detected in young cultures of *Aspergillus nidulans* and *Penicillium chrysogenum* have been identified as the cyclic ester fusigen (fusarinine C), and its open-chain form, fusigen B (fusarinine B).

It has been known for some time that fungi and other microorganisms secrete into their environment a variety of low-molecular-weight chelating agents, called siderophores, that solubilize and transport ferric iron (9). More recently, it was found that besides these extracellular agents, fungi also produce specific cellular siderophores (2, 6, 10). The cellular siderophores are important in conidial germination, and they may have other functions. When exposed to solutions of low water activity (a_w), conidia lose a fraction of their cellular siderophores and thereby become siderophore dependent. Subsequent germination fails, or is greatly delayed, unless a suitable siderophore is supplied. This response is the basis for a sensitive bioassay procedure. The assay is species specific—i.e., it detects cellular and extracellular siderophores (and some of their structural analogs) produced by the assay organism, but not siderophores in general (2, 6).

When *Aspergillus nidulans* and *Penicillium chrysogenum* were used to assay their own culture filtrates by this method, it was found that both species secrete a number of different siderophores during growth (2). Of these, the most active by far were four unidentified substances—two in each species—that appear in filtrates of young cultures, reach their peak concentrations at 3 to 6 days of growth, and then disappear. They are replaced in older cultures by less active or inactive ligands (2). The highly active siderophores from *A. nidulans* were designated A-α and A-β, respectively; those from *P. chrysogenum* were called P-α and P-β. We report here the identification of these substances.

MATERIALS AND METHODS

Organisms used were *A. nidulans* (Glasgow wild type no. 4, Fungal Genetics Stock Center, Arcata, Calif.), *P. chrysogenum* (ATCC 9480), and *Fusarium roseum* (ATCC 12822, supplied by Thomas Emery). Media for culturing *A. nidulans* and *P. chrysogenum* and for assaying siderophores by use of these species have been described, together with details of the bioassay procedure (2). *F. roseum* was maintained on slants of Vogel medium N (3), with the addition of 0.5% yeast extract and 0.25% casein hydrolysate. For the production of extracellular siderophores, we used the Fe-deficient "Normalmedium" of Kappner et al. (7).

Methods for siderophore isolation and for thin-layer chromatography, paper electrophoresis, amino acid determination, and ^{13}C-nuclear magnetic resonance spectroscopy were as previously described (2, 6). Mass spectrometry was performed with a VG 7070 F instrument operating in the chemical ionization mode and using ammonia.

Formol titrations were carried out as described by Bersin and Mayer (1). Acetylation was done in acetic anhydride-methanol-pyridine at room temperature (5). For acetyl determinations, 5-mg samples in 0.1 ml of 1 N NaOH were heated overnight at 110°C in sealed tubes and then dried in vacuo. After being cooled, the tubes were acidified with 0.1 ml of 1.2 N HCl, and 5-μl samples were immediately injected into a Hewlett-Packard 5700A gas chromatograph (with integrator), equipped with a Poropak N column operating at 180°C. Triacetylfusigen (2) was used as a control.

RESULTS

The previous findings suggested that the α's and β's are closely related compounds. All four were found to be cations, and they were alike in their high biological activity (2). Subsequent tests showed that they are all ninhydrin positive and unstable compared with other siderophores we have worked with. On reductive hydrolysis with HI, ornithine was the only amino acid found. The compounds did not lose iron to EDTA, and no shift in their visible spectra occurred after acidification of an aqueous solution to pH 2; these findings are characteristic of ferric trihydroxamates (8). Analysis showed no significant amounts of acetyl in A-α, P-α, or P-β

(A-β was not tested). The α's migrated faster than the β's in an electric field (Table 1); Formol titrations showed that whereas the β's contain one free carboxyl group, the α's contain none. No differences were found between the two α's or the two β's.

These findings suggested the possible identity of the unknowns with the cationic siderophores fusigen (fusarinine C) and fusigen B (fusarinine B), which are produced by *Fusarium* species. The structures and properties of these substances were elucidated by Diekmann and Zähner (5) and by Sayer and Emery (11). Both are trihydroxamates composed of three molecules of fusarinine, a derivative of ornithine:

$$^{-}OOC—CHNH_3^{+}—(CH_2)_3—NOH—CO—CH=C(CH_3)—(CH_2)_2—OH$$

In fusigen, the three subunits are cyclized through ester bonds between the carboxyl group of one fusarinine molecule and the hydroxyl group of the next. In fusigen B, one of the bonds remains open, giving a linear trimer. Fusigen, with three net positive charges, migrates faster in an electric field at pH 5.2 than fusigen B, which has two. Both compounds bind iron strongly, and both are unstable, owing to their aminoacyl ester bonds (8).

Fusigen and fusigen B were isolated from cultures of *F. roseum* and compared with the four unknowns. Biological activities were measured in the low a_w test with *P. chrysogenum* and *A. nidulans*. The data of one such test are shown in Fig. 1. Despite some scatter attributable to slow growth at a_w 0.874 combined with instability of the compounds, the results show close similarity in biological activity among all of these siderophores. There is a suggestion that fusigen and the α's are slightly more active than fusigen B and the β's.

Electrophoretic and chromatographic comparisons are shown in Tables 1 and 2. The α's and β's are clearly different substances; the former are indistinguishable from fusigen, and the latter are indistinguishable from fusigen B.

Further evidence for the structure of α was obtained as follows. (i) Acetylation yielded a derivative which was chromatographically the same as triacetylfusigen (Table 2) and the Fe-free form of which gave a ^{13}C nuclear magnetic resonance spectrum identical with that of triacetyldesferrifusigen (see reference 2 for the spectrum of this compound). (ii) P-α was heated to 60°C for 2 h in methanol or deuteromethanol (d_4-methanol), and the trimer underwent methanolysis to the monomer fusarininemethyl ester (or d_3-methyl ester). The monomer was identified by mass spectrometry after acetylation to the N,O,O-triacetyl or N,O,O-tri-d_3-acetyl derivative. The calculated and experimentally determined values of M + 1 for N,O,O-triacetylfusarinine methyl ester,

$$X_3COOC—CH(NYCOCZ_3)—(CH_2)_3—N(OCOCZ_3)—CO—CH=C(CH_3)—(CH_2)_2—OCOCZ_3,$$

were 401 (X = Y = Z = H), 405 (X = Y = D; Z = H), and 410 (X = Y = H; Z = D). The results prove the structure of the monomer from which α is constructed and confirm that α contains no acetyl groups.

TABLE 1. Paper electrophoresis of siderophores[a]

Siderophore	Migration toward cathode (cm)
A-α	4.3
P-α	4.3
Fusigen	4.3
A-β	3.1
P-β	3.1
Fusigen B	3.0

[a] Electrophoresis at 200 V for 2 h in pyridine-acetate buffer (pH 5.2), using Whatman 3MM paper strips.

DISCUSSION

Fusigen was identified in young cultures of *A. fumigatus* and *P. chrysogenum* by Diekmann (4). It was later shown by Wiebe and Winkelmann (12) to act as an iron donor for *Aspergillus* species at micromolar concentrations, and the authors suggested that it might play a transport

TABLE 2. Thin-layer chromatography of siderophores

Siderophore	R_f value in solvent system:[a]				
	1	2	3[b]	4	5
A-α	0.65	0.38	0.56		
P-α	0.64	0.38	0.57		
Fusigen	0.64	0.38	0.57		
A-β	0.45	0.10	0.38		
P-β	0.45	0.10	0.39		
Fusigen-B	0.45	0.10	0.39		
Acetylated A-α	0.71			0.68	0.56
Acetylated P-α	0.69			0.71	0.56
Triacetylfusigen	0.71			0.69	0.56

[a] Eastman silica-gel sheets with the following solvent systems: (1) *N*-propanol-water-ammonium hydroxide, 15:5:1 by volume; (2) ethanol-water-ammonium hydroxide, 18:1:1 by volume; (3) *N*-butanol-pyridine-water-ammonium hydroxide, 1:1:1:1 by volume; (4) chloroform-*N*-propanol-methanol-water, 18:7:3.3:1 by volume; (5) *N*-butanol-acetic acid-water, 6:1:1 by volume.

[b] Streaked.

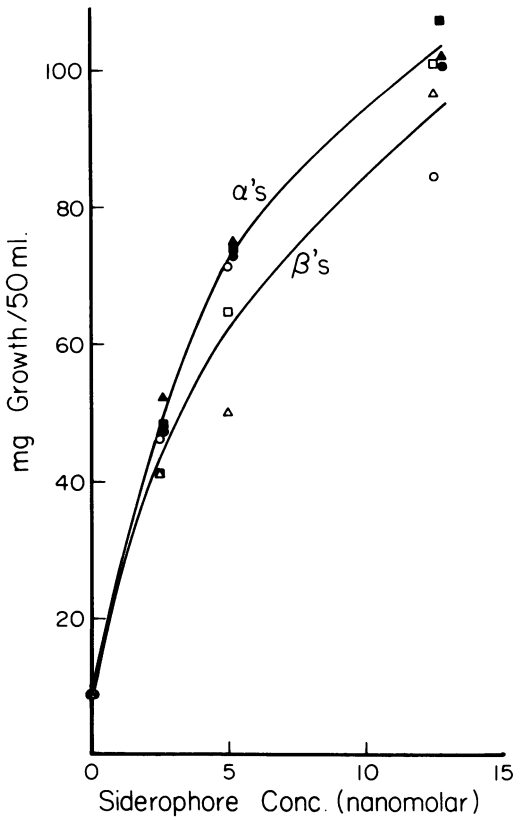

FIG. 1. Growth (dry weight) of *P. chrysogenum* as a function of siderophore concentration under the conditions of the siderophore assay (2); 6 days of growth in 50-ml medium at a_w 0.874 and 26°C. Inoculum, 10^3 conidia per ml. Points are averages of duplicates. Curves are drawn through the means of the corresponding three points. A-α (▲), A-β (△), P-α (●), P-β (○), fusigen (■), fusigen B (□).

role in young cultures. The instability of the fusarinines, however, led Moore and Emery (8) to question the biological role of these compounds. Our findings show that both fusigen and fusigen B are produced by *A. nidulans* and *P. chrysogenum*, and both are active biologically at nanomolar concentrations. In our view, there can be little doubt that these substances are functional siderophores in these species.

A point of special interest is that, despite their instability, the fusigens are more active in the low-a_w test than any of the other siderophores produced by *A. nidulans* or *P. chrysogenum* (2 and unpublished data). They are secreted only during rapid mycelial growth and are replaced in older cultures by stable, but less active, structural relatives—triacetylfusigen in *A. nidulans* and coprogen in *P. chrysogenum*. (Coprogen is a rearrangement of the subunits of fusigen, with *cis-trans*-isomerization of the anhydromevalonic acid component.) Instability is not a necessary condition for high activity, however. *Neurospora crassa* produces only one significant extracellular siderophore, coprogen, and two cellular siderophores which are cyclic peptides. These compounds are all stable in solution, yet they are also active at nanomolar concentrations (6).

ACKNOWLEDGMENTS

This work was supported by Public Health Service grant AI15739.

We thank Thomas Emery for supplying *F. roseum* and J. Roger Shelton for amino acid analyses.

LITERATURE CITED

1. **Bersin, T., and H. J. Mayer.** 1945. Über die Electrometrische Formoltitration mit einer Antimon-Kleinelectrode. Hoppe-Seyler's Z. Physiol. Chem. **282**:21–28.
2. **Charlang, G., B. Ng, N. H. Horowitz, and R. M. Horowitz.** 1981. Cellular and extracellular siderophores of *Aspergillus nidulans* and *Penicillium chrysogenum*. Mol. Cell. Biol. **1**:94–100.
3. **Davis, R. H., and F. J. De Serres.** 1970. Genetic and microbiological research techniques for *Neurospora crassa*. Methods Enzymol. **17**:79–143.
4. **Diekmann, H.** 1967. Stoffwechselprodukte von Mikroorganismen. 56. Mitteilung. Fusigen—ein neues Sideramin aus Pilzen. Arch. Mikrobiol. **58**:1–5.
5. **Diekmann, H., and H. Zähner.** 1967. Konstitution von Fusigen und dessen Abbau zu Δ^2-Anhydromevalonsäurelacton. Eur. J. Biochem. **3**:213–218.
6. **Horowitz, N. H., G. Charlang, G. Horn, and N. P. Williams.** 1976. Isolation and identification of the conidial germination factor of *Neurospora crassa*. J. Bacteriol. **127**:135–140.
7. **Kappner, M., A. Hasenböhler, and H. Zähner.** 1977. Stoffwechselprodukte von Microorganismen. 166. Mitteilung. Optimierung der Desferri-Ferricrocinbildung bei *Aspergillus viridi-nutans* Ducker & Thrower. Arch. Microbiol. **115**:323–331.
8. **Moore, R. E., and T. Emery.** 1976. N^{α}-Acetylfusarinines: isolation, characterization, and properties. Biochemistry **15**:2719–2723.
9. **Neilands, J. B.** 1981. Microbial iron compounds. Annu. Rev. Biochem. **50**:715–731.
10. **Ong, S. A., and J. B. Neilands.** 1979. Siderophores in microbially processed cheese. J. Agric. Food Chem. **27**:990–995.
11. **Sayer, J. M., and T. F. Emery.** 1968. Structures of the naturally occurring hydroxamic acids, fusarinines A and B. Biochemistry **7**:184–190.
12. **Wiebe, C., and G. Winkelmann.** 1975. Kinetic studies on the specificity of chelate-iron uptake in *Aspergillus*. J. Bacteriol. **123**:837–842.