JOURNAL OF BACTERIOLOGY, July 1976, p. 135-140

Vol. 127, No. 1
Printed in U.S.A.

Isolation and Identification of the Conidial Germination Factor of *Neurospora crassa*

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Received for publication 13 February 1976

The germination-essential substance (germination factor [GF]) that is lost from conidia of *Neurospora crassa* on exposure to solutions of low water activity has been isolated and identified as a group of iron-transport compounds, or siderochromes. The principal siderochrome of conidia is ferricrocin, a cyclic hexapeptide. A closely related substance, ferrichrome C, is tentatively identified as a minor constituent. The same substances are also present in extracts of mycelium along with small amounts of a third siderochrome, which has not been identified. The GF activity of culture filtrates is due to coprogen, the only siderochrome previously identified with *N. crassa*.

When conidia of *Neurospora crassa* are exposed to media whose water activity, a_w, has been brought to 0.95 or lower by the addition of solutes, either electrolytes or non-electrolytes, they lose a substance that is essential for their germination (3). The germination factor, GF, can be shown to be present in cell-free filtrates of the medium. Release of GF is apparently caused by an alteration of the plasma membrane induced by low a_w (4). GF activity is present in mycelial extracts of *N. crassa* and various other filamentous ascomycetes, and it is also found in the culture medium ($a_w = 0.995$) in which growth of *N. crassa* has taken place.

We report here the isolation of the germination factor from mycelial extracts of *N. crassa* and its identification as a group of iron-transport compounds, or siderochromes. The germination factors present in conidia and in spent culture medium are also identified.

MATERIALS AND METHODS

N. crassa wild-type strain 74A was used in all experiments. For production of mycelium, it was grown for 48 h at 25 C in carboys or fermenters, with aeration, in Vogel's medium N (19) containing 2% sucrose as carbon source. For production of conidia, medium N with 2% sucrose, 0.25% casein hydrolysate, 0.5% yeast extract, and 1.5% agar was used, and the previously described procedure was followed (3). The GF assay medium consisted of medium N with the following additions per kilogram of water: sucrose, 20 g; casein hydrolysate, 1 g; yeast extract, 2.5 g; $MgSO_4 \cdot 7H_2O$, 5 g; and NaCl, 103.1 g. The resulting water activity is 0.932 (17). In the assay, the dry weight of mycelium produced as a function of GF concentration is measured, following the procedure described previously (4). Conidial counts were made by hemocytometer.

A rapid bioassay procedure was used for qualitatively identifying active fractions during the isolation of GF. The same medium as for the quantitative assay was used, but with the NaCl and supplementary $MgSO_4 \cdot 7H_2O$ omitted and with 0.0075% disodium ethylenediaminetetraacetic acid added. Inoculation and incubation were as in the standard assay, but the incubation time was reduced to 24 to 48 h.

Thin-layer chromatography was performed with silica gel chromatogram sheets (Eastman Kodak Co.). Early in this investigation we found that phenol reagents such as the ferric chloride-ferricyanide reagent and, especially, the Folin-Ciocalteu reagent are sensitive indicators of GF activity on chromatograms. Later, after identifying the GFs as siderochromes, we learned that Subramanian et al. (18) had previously used the Folin-Ciocalteu reagent for estimating these compounds. To locate GF activity, we sprayed chromatograms with Folin-Ciocalteu reagent (Sigma) diluted with 3 parts of water and then air-dried the sheets and oversprayed with 10% aqueous sodium carbonate. Alternatively, a spray made by mixing equal volumes of 1% $FeCl_3 \cdot 6H_2O$ and 1% potassium ferricyanide was used. Both tests give blue spots. (It should be noted that, despite these tests, the GFs are not phenols.) Iron (III)-containing spots were detected on chromatograms by spraying with 2 N HCl, followed by 1% aqueous potassium ferrocyanide.

Absorption spectra were determined with a Cary 15 recording spectrophotometer. Proton magnetic resonance spectra were obtained with a JEOL model PS-100 spectrometer at 100 MHz in methanol-d_4. ^{13}C-nuclear magnetic resonance spectroscopy was done with a JEOL model FX-60 instrument at 15.03 MHz in D_2O.

Samples for amino acid analysis were hydrolyzed in constant boiling HCl or HI at 110 C overnight in sealed, evacuated tubes. Qualitative amino acid analyses were made by high-voltage paper electrophoresis (5). Quantitative analyses were obtained

with a Beckman amino acid analyzer, model 120-C. Iron was determined by the *o*-phenanthroline method (2).

RESULTS

Isolation of GF from mycelium. The following isolation method was used for each 2.5 to 3 kg (wet weight) of mycelium. This procedure was worked out empirically without prior knowledge of the identity of GF. With GF now identified, a more direct procedure could be designed.

The mycelium, washed free of medium, was homogenized in a large blender with sufficient boiling water to produce a slurry. This was stirred for 15 min in a beaker at 85 C and was then filtered through cheesecloth and allowed to cool. The pH of the turbid filtrate was adjusted to 5.0 with 1 N HCl, and the suspension was refrigerated overnight to precipitate proteins. The precipitate was removed by filtration through a layer of Celite 535 on a Büchner funnel. The pH of the filtrate was adjusted to 6.5 to 7.0, and its volume (3 to 4 liters at this stage) was reduced to about 300 ml by evaporation under reduced pressure. The concentrated extract was poured slowly into 6 liters of cold 95% ethanol, and the precipitate was removed by centrifugation and discarded. The ethanol was removed by evaporation, the volume of the resulting concentrate was brought to 150 to 200 ml with water, and a 25% solution of neutral lead acetate was added until no further precipitate formed. The precipitate was removed by centrifugation and discarded. Excess lead was precipitated from the solution with saturated Na_2HPO_4 and was removed by centrifugation.

Pb-free solutions prepared as above were concentrated to a dry weight of 0.25 to 0.5 g/ml (containing the GF equivalent of 25 to 50 g of the starting mycelium). A volume of this concentrate containing approximately 100 g of dry matter (equivalent to 10 kg of mycelium) was then placed on a column (11 by 8 cm) of washed Amberlite XAD-2 (Rohm and Haas Co.), followed successively by 16 bed volumes of water and 6 volumes of 10% methanol. GF activity was then eluted with 5 bed volumes of 40% methanol. The methanolic solution was evaporated to dryness, and the residue, weighing about 1 g, was dissolved in 20 ml of 0.05 M NH_4HCO_3 and applied to a column (90 by 3.6 cm) of 200- to 400-mesh Bio-Gel P2 (Bio-Rad Laboratories). The column was eluted with the same solvent, and 10-ml fractions were collected. Two peaks of GF activity were detected—a minor one, not further investigated, and, following it, a large, orange-colored peak containing almost all the activity. The solvent and buffer were removed from the pooled fractions of this peak by evaporation. The residue, weighing approximately 300 mg, was then chromatographed on a column (20 by 5 cm) of Silica Gel 60, particle size <0.063 mm (E. Merck), with a solvent consisting of chloroform-propanol-methanol-water, 18:7:2:1 (vol/vol/vol/vol). The activity emerged from the column in three orange-red bands; these were numbered GF I, -II, and -III, respectively, in the order of their elution.

Use of the Folin-Ciocalteu reagent with silica gel thin-layer chromatography showed that GF I was homogeneous with respect to Folin-reacting material. GFs II and III showed cross-contamination, however, and in addition contained a Folin-reacting, biologically inactive impurity. Both kinds of contamination were eliminated by rechromatographing GFs II and III on silica gel with chloroform-benzyl alcohol-methanol, 2:1:1 (vol/vol/vol).

Homogeneity and yield. The GFs prepared as described above showed no Folin-reacting contaminants in several solvent systems. In addition, they gave a negative ninhydrin reaction and showed no absorption peak at 260 nm. All of these tests had been positive at earlier stages of purification. Since additional chromatography produced no further increase in their specific activity, the GFs were considered to be homogeneous, although amorphous.

The yield from 10 kg of mycelium containing about 100 mg of GF was 80 to 85 mg. Of this amount, 80% was GF II. The rest was divided approximately equally between GFs I and III. Since the specific activities of the GFs are the same within the precision of the bioassay, this distribution holds for the biological activity as well.

Identification of GF II. When GF II was submitted to proton magnetic resonance spectroscopy, the sample was found to be strongly paramagnetic. On analysis, 7.2% iron was found, a value lying within the range reported for various fungal siderochromes (reviewed in 6, 10, and 13). The iron was reversibly removable with 8-hydroxyquinoline by the method of Winkelmann et al. (21), as is the case with siderochromes. The absorption spectrum of GF II (Fig. 1) closely resembles that of ferrichrome (12) and related siderochromes. Consistent with a presumptive role of GF in iron transport, we find that inorganic iron is active in the GF bioassay but with an efficiency only 10^{-3} to 10^{-4} that of GF II per iron atom.

A 100-MHz proton magnetic resonance spectrum was obtained of iron-free GF II. It bore a close resemblance to the 60-MHz spectrum re-

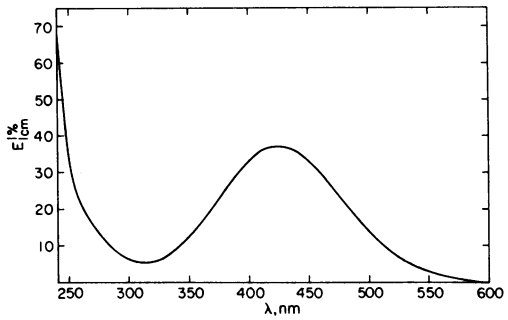

FIG. 1. *Absorption spectrum of GF II in methanol. The spectrum of ferricrocin is identical.*

ported for ferricrocin (8), a siderochrome from various aspergilli. HCl hydrolysis of iron-free GF II, followed by amino acid analysis, yielded ornithine, glycine, and serine in the ratio 0.14:2:0.92, together with an unidentified amino acid, presumably δ-*N*-OH-ornithine. Reductive hydrolysis of GF II with HI (23) yielded ornithine, glycine, and serine plus alanine in the ratio 2.8:2:0.88. These are typical results for ferricrocin, a cyclic peptide containing three residues of δ-*N*-acetyl-δ-*N*-OH-ornithine, two of glycine, and one of serine (Fig. 2a).

An authentic sample of ferricrocin was compared with GF II biologically, spectrally (optical and ^{13}C-nuclear magnetic resonance spectra), and chromatographically. The iron-free compounds were used for nuclear magnetic resonance spectroscopy, with the results shown in Table 1. For chromatography, we used silica gel thin-layer chromatography with the solvents described above, and paper chromatography with system B of Zähner et al. (23). The resulting R_f values are shown in Table 2. The biological findings are shown in Fig. 3c. We conclude from this evidence that ferricrocin and GF II are identical.

Ferrichrome, ferrichrome C, and ferrichrysin, structural analogues of ferricrocin, show the same biological activity as ferricrocin in the GF assay. Ferrichrome A is 0.5% as active on a molar basis. Rhodotorulic acid is inactive.

Identification of GF I. GF I is indistinguishable from GF II in its biological activity and absorption spectrum but it differs chromatographically. Insufficient GF I was available for nuclear magnetic resonance spectroscopy, but analysis of acid hydrolysates was possible. HCl hydrolysates of iron-free GF I contained alanine but not serine; otherwise they resembled GF II hydrolysates. HI hydrolysis gave ornithine, glycine, and alanine in the ratio 2.75:2:1.03. On the basis of this analysis and its other properties, GF I appeared to be similar to ferrichrome C, an alanine-containing siderochrome isolated from *Cryptococcus melibiosum* by Atkin et al. (1). When GF I and a sample of ferrichrome C were compared, they were found to be indistinguishable biologically (Fig. 3b), spectrally, and chromatographically (Table 2). HCl hydrolyses carried out in parallel on the iron-free compounds confirmed that the same amino acids are present in both. We conclude that GF I is probably identical with ferrichrome C.

GF III. Spectrally and chromatographically,

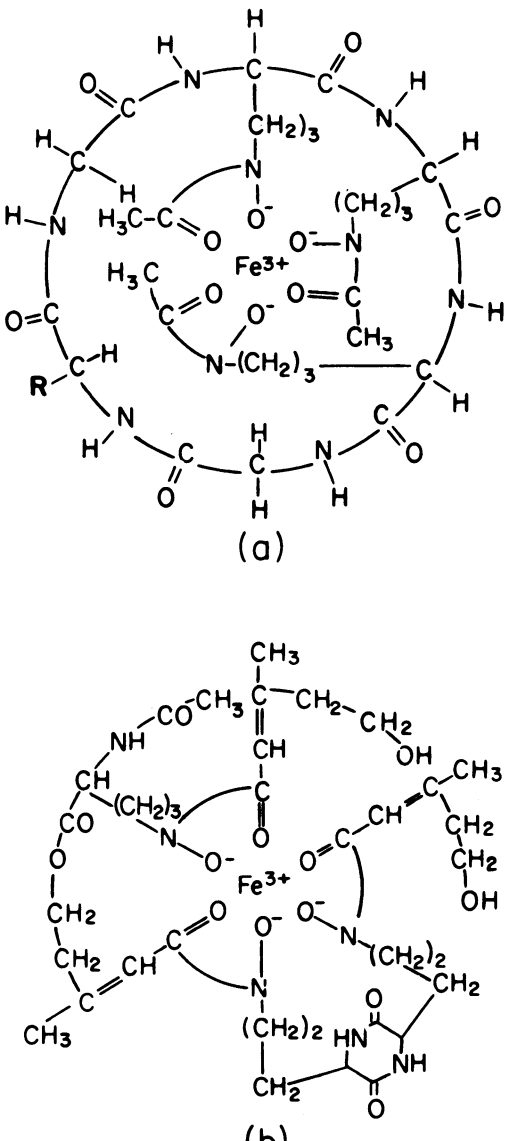

FIG. 2. *(a) Ferricrocin, R = —CH_2OH. Ferrichrome C, R = —CH_3. (b) Coprogen.*

TABLE 1. *^{13}C chemical shifts of desferriferricrocin and desferri-GF II*[a]

Carbon assignment	Desferri-ferri-crocin[b]	Desferri-GF II[b]
C=O	174.5[c]	174.5[c]
	174.1	174.0
	172.6	172.6
	172.3	172.3
	172.0	171.9
Serine C—β	61.3	61.3 (tr)
Serine C—α	56.0	56.1 (d)
δ-*N*-OH-ornithine C—α	54.6[d]	54.9 54.7 (d)
δ-*N*-OH-ornithine C—δ	48.0[d]	48.0 (tr)
Glycine C—α	43.6	43.7 43.6 (tr)
δ-*N*-OH-ornithine C—β	29.2	29.3
	28.3	29.1
		28.2
δ-*N*-OH-ornithine C-γ	23.3	23.3
		23.1
Acetyl methyl	20.1	20.2

[a] Noise-decoupled, 15-MHz Fourier transform spectra; values are given in parts per million downfield from tetramethyl silane. Solvent: D_2O with ~5% dioxane as internal reference. Tr (triplet) and d (doublet) are observed multiplicities on single-frequency off-resonance decoupling (SFORD). Assignments are in accord with the SFORD data and with applicable published values (7, 11).

[b] GF II spectra were determined in a 10-mm tube (~20 mg/0.4 ml); desferriferricrocin spectra were determined on a more concentrated solution (~5 mg/35 μl) in a 1.8-mm tube. Resolution in the 10-mm tube was slightly better; the small differences in chemical shifts are probably concentration effects.

[c] Signal intense and broad relative to the other C=O signals.

[d] Assignment confirmed by selective proton decoupling.

GF III resembles coprogen, the only siderochrome previously known from *Neurospora* (see below). Acid hydrolysates, however, showed the presence of glycine and serine, as well as ornithine, in GF III; glycine and serine are not found in coprogen. Unfortunately, our sample of GF III was degraded by drying at 100 C before the tests were completed. Insufficient material remained for further characterization studies.

GFs of conidia. Bioassays showed the GF content of conidia to be 1.43 mg/g (dry weight), or 25 to 30 times higher than that of 48-h mycelium. To identify the conidial GFs, 7.9 g (dry weight) of washed conidia, filtered through cheesecloth to remove hyphal fragments, was extracted by shaking with chloroform-saturated water for 5 h at 30 C (4). After evaporating the chloroform, 16 mg of $FeCl_3 \cdot 6H_2O$ was added to the cell-free supernatant to convert desferri-siderochromes to their Fe-chelate forms. The solution was then chromatographed on Amberlite XAD-2 and silica gel as described above. Two GFs were found and were identified as GFs I and II by thin-layer chromatography. The respective yields were 1.65 and 8.0 mg.

GF of spent medium. It is known that *N. crassa* grown under iron-deficient conditions secretes desferricoprogen, the iron-free form of coprogen, into the medium (9, 14) (Fig. 2). We have confirmed this finding, following the growth and isolation procedures described by Zähner et al. (23). The crude coprogen was further purified by chromatography on Celite 545 (Johns-Manville Co.) as described by Pidacks et al. (16). Purified coprogen is fully active in the GF assay (Fig. 3a), but it is distinguishable from GFs I and II spectrally, chromatographically, and by its amino acid content.

The iron content of medium N is 143 μg/liter. Iron does not limit growth on this medium through at least 24 h of incubation under our conditions. GF activity is nevertheless detectable in the medium within 6 h after inoculation; by 12 h, sufficient GF is present for identification. We extracted the GF activity from 12-, 24-, 36-, and 48-h culture filtrates by the method of Zähner et al. (23), a general one for siderochromes. Identification was made by chromatography and amino acid analysis. Coprogen was the only siderochrome found.

The amounts of coprogen found in medium N vary considerably depending on the age of the culture and the amount of growth. The range in

TABLE 2. *R_f values of siderochromes and GFs in three chromatographic systems*

Substance	R_f values in chromatographic system:		
	1[a]	2[b]	3[c]
GF I	0.38	0.65	0.43
GF II	0.23	0.36	0.21
GF III	0.20	0.61	0.62
Ferrichrome C	0.38	0.66	
Ferricrocin	0.22	0.36	0.21
Coprogen	0.20	0.61	0.63

[a] Ascending silica gel thin-layer chromatography using chloroform-propanol-methanol-water, 18:7:2:1 (vol/vol/vol/vol).

[b] The same as in footnote *a* but using chloroform-benzyl alcohol-methanol, 2:1:1 (vol/vol/vol).

[c] Descending paper chromatography using system B of Zähner et al. (23). R_f values obtained with this system are not reproducible from one day to another, in our experience. Within any one experiment, however, the values are highly reproducible.

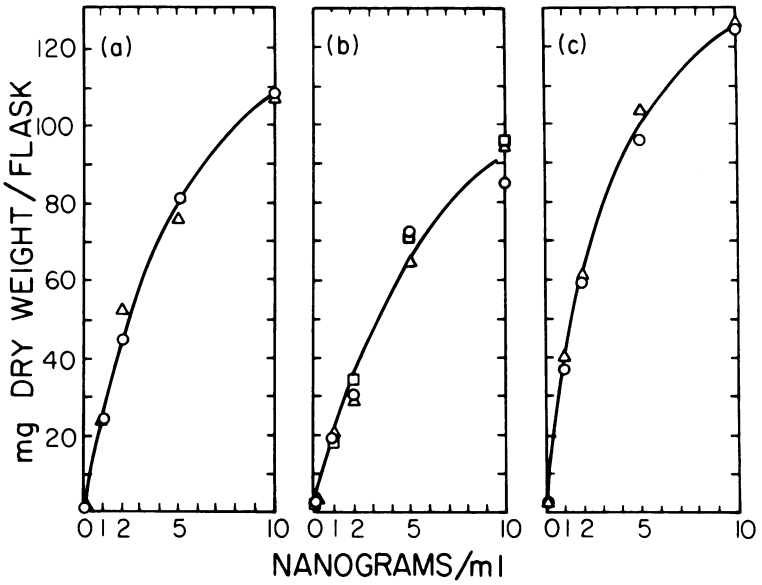

FIG. 3. *Growth of N. crassa as a function of siderochrome concentration under the conditions of the GF assay. Results from three experiments are shown: (a) GF II (○), coprogen (△); (b) GF II (○), GF I (□), ferrichrome C (△); (c) GF II (○), ferricrocin (△).*

our experiments was 0.5 to 50 μg/ml. This is distinctly less than the concentration produced in iron-deficient media, but it is a biologically important quantity. The failure of Padmanaban and Sarma (15) in their early study to detect coprogen in the medium of iron-sufficient cultures of *N. crassa* was almost certainly due to the insensitivity of their assay method.

DISCUSSION

Identification of the *Neurospora* GFs as siderochromes raises a number of questions concerning the functioning of these substances. First, since a requirement for iron is not unique to germinating conidia, why do siderochromes behave as GFs in our experiments, rather than as general growth factors? The answer appears to be that newly shed conidia have no mechanism for concentrating siderochromes from the environment. They acquire such a mechanism during the first hour after inoculation into minimal medium, or about 2 h before germination occurs (unpublished data from our laboratory). Conidia at low a_w, having lost their siderochromes and lacking an active transport mechanism, depend for iron on the diffusion of iron-carrying siderochrome molecules from the environment. If the external siderochrome concentration is very low, an iron deficiency results, and the germination process (including acquisition of the iron transport mechanism) is delayed or inhibited. Hyphal cells have the transport mechanism, however, and in addition they secrete substantial quantities of siderochromes into the medium. They are, therefore, immune to the iron-depriving effect of reduced a_w. The kinetics and specificity of the hyphal transport mechanism have been studied by Winkelmann and co-workers (20–22).

When the quantity of GF activity released at low a_w is compared to the total GF activity of conidia, it is found that the GF released is only a small fraction of the total. This is evident in our previously published data (4), and it has been confirmed in more recent experiments, which show that loss of less than 10% of the conidial GF is sufficient to render conidia siderochrome dependent. How can this discrepancy be explained? The hypothesis we propose is that there are two pools of conidial siderochromes: active and inactive. The smaller, active pool is located on or in the plasma membrane, where it functions in iron transport. The larger, inactive pool is stored in the cell, perhaps to be incorporated into newly forming plasma membranes after germination begins. Because of the well-known asymmetry of membranes, the inactive siderochromes are not readily emplaced in the completed conidial membrane; consequently, they cannot take the place of siderochrome molecules lost in solutions of low a_w.

Other questions less readily answered at the present time concern the molecular mechanisms that underlie (i) the release of sidero-

chromes into solutions of low a_w and (ii) the physical separation of the two major siderochromes, ferricrocin in the cells and coprogen in the medium. These mechanisms are presumably related to the structure of the plasma membrane. Answers to these questions must await further study.

ACKNOWLEDGMENTS

We are indebted to the Merck, Sharp & Dohme Research Laboratories and T. H. Stoudt for the gift of GF-containing concentrates made from *N. crassa*. We thank G. Winkelmann for a sample of ferricrocin, W. Keller-Schierlein for samples of ferricrocin, ferrichrysin, and ferrichrome, and J. B. Neilands for samples of ferrichrome C, ferrichrome A, and rhodotorulic acid. We also acknowledge the invaluable help of Robert M. Horowitz, who performed and interpreted the NMR spectroscopy.

This research was supported by a grant from the National Aeronautics and Space Administration (NGR 05-002-121).

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