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Germination-Defective Mutant of *Neurospora crassa* That Responds to Siderophores

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A conditionally germination-defective mutant of *Neurospora crassa* has been found to be partially curable by ferricrocin and other siderophores. The mutant conidia rapidly lose their membrane-bound siderophores when suspended in buffer or growth media. Germination is consequently delayed unless large numbers of conidia are present (positive population effect). This indicates that the mutant has a membrane defect involving the siderophore attachment site.

Neurospora crassa produces two principal iron transport peptides, or siderophores: coprogen, which is secreted into the medium of growing cultures, and ferricrocin, which is retained in the cells. Considerable evidence indicates that a fraction of the total ferricrocin of conidia is located in the plasma membrane, where it functions in iron transport. This ferricrocin is lost from the membrane in media of low water activity (a_w), resulting in a prolonged lag in conidial germination; a sensitive bioassay for siderophores is based on this effect (1-3).

This note reports experiments on a temperature-sensitive, germination-defective mutant that responds to siderophores. The mutant, JS 134-9, was the gift of J. C. Schmit and S. Brody (manuscript in preparation).

For all experiments, strain JS134-9 was grown on Vogel's minimal medium N (5) agar for 5 or 6 days at 25°C under light. Conidia were harvested in distilled water, filtered through four layers of cheesecloth, centrifuged, washed once, and suspended in water. Counts were made with a hemocytometer. Experiments to determine growth at different temperatures and response to siderophores were carried out with 25 ml of liquid minimal medium N (plus 2 drops of Tween 80 to prevent aerial growth) in 125-ml Erlenmeyer flasks. Incubation was at 25, 30, or 34°C, without shaking, for 72 h. Although the strain responds equally well to several siderophores, ferricrocin was used in most experiments.

Population effects. The results in Table 1 show that the amount of growth of the mutant in 72 h at any temperature is dependent on the initial conidial density. For example, when one flask was inoculated with 10^5 conidia per ml and 10 flasks received the same total number of conidia or 10^4 conidia per ml, the mycelial dry weights after incubation at 34°C for 72 h were 38.4 mg for the single flask and 1.7 mg for the 10 flasks combined. We have called this a positive population effect, and, in this strain, the effect increases with increasing temperature.

Under the same conditions, wild-type 74A exhibits a negative population effect. Thus, 10^3 conidia per ml inoculated into one flask produced much less growth (86 mg) than did the same number distributed into 10 flasks at 10^2/ml (73.2 mg/flask, or 732 mg for 10 flasks combined). This is the effect expected when growth is limited by the availability of nutrients. The final amount of growth in each flask is nearly the same regardless of the size of the inoculum.

In our study of the germination and growth of *N. crassa* at low a_w, we showed that a positive population effect results from the reversible loss of ferricrocin from conidia at low a_w. At low conidial densities, the ferricrocin is essentially lost from the cells, and germination is long delayed. At high conidial densities, however, sufficient ferricrocin accumulates in the medium to allow some conidia to germinate. When these conidia begin to grow, they secrete coprogen into the medium. Since coprogen is also an effective siderophore for *Neurospora*, the rest of the conidial population now germinates (1-3). Mutant JS 134-9 behaves in a similar manner in media of normal a_w.

When growth is very slow there is no population effect; i.e., the mycelial dry weight collected from 10 flasks with a diluted inoculum is the same as that of the single flask with the same total number of conidia. An example of this can also be found in Table 1. At 25°C one flask with 10^4 mutant conidia per ml produced 10 times the dry weight of one flask with 10^3 conidia per ml.

Mutant response to ferricrocin. The re-

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TABLE 1. *Growth of JS 134-9 at different incubation temperatures as a function of inoculum size*

Strain	Conidial concn (no./ml)	Dry wt (mg) at: 25°C	30°C	34°C
JS 134-9	10^6			42.9 (1)[a]
	10^5		63.4 (1)	38.4 (1)
	10^4	42.9 (1)	43.7 (1)	1.7 (10)
	10^3	4.2 (1)	3.8 (10)	0 (10)
	10^2	Tr (10)	0 (10)	
	10^1	0 (10)		
74A (wild type)	10^3	56.4 (1)	79.3 (1)	86.0 (1)
	10^2	42.2 (1)	66.1 (1)	73.2 (1)
	10^1	20.4 (1)		

[a] Number in parentheses indicates number of flasks combined to give the dry weight.

TABLE 2. *Response of JS 134-9 to ferricrocin at different incubation temperatures*

Ferricrocin concn (μg/25-ml flask)	Dry wt (mg)[a] at: 25°C[b]	30°C[b]	34°C[b] Expt I	Expt II
20	14.7	14.6	19.0	15.1
10	11.9	12.0	15.7	19.1
5	12.4	7.7	13.9	12.8
2.5	11.7	3.2	12.4	5.7
1	10.6	0.8	7.8	
0.5	7.1	Tr	6.2	
0.25	3.2			
0.1	1.5			
None	0.7	0.0	9.0	2.2

[a] Average of two flasks.

[b] Inoculum size: 25°C, 5×10^2 conidia per ml; 30°C, 10^3 conidia per ml; 34°C, 5×10^4 conidia per ml.

sults presented in Table 2 are representative of several experiments and clearly show a response by the mutant to ferricrocin at all incubation temperatures. The wild type shows no response to ferricrocin under the same conditions (1-3). The positive population effect shown by the mutant at the higher temperatures is only partially prevented by ferricrocin, and larger inocula have to be used to obtain measurable growth. At 34°C the response is more variable than at lower temperatures, but the amount of ferricrocin needed for germination remains essentially the same. Adding more does not enhance germination or growth at any temperature.

Since the population effect observed here is similar to, though more severe than, that found in wild type in media of low a_w (1), it seemed that ferricrocin and other substances might be lost by the mutant conidia when they first contact water or aqueous media. To test this, conidia were dry-harvested into buffer ($a_w = 0.998$) at room temperature (ca. 24°C) and at 35°C, incubated for 15 min, and filtered off. The buffer was then bioassayed for siderophore activity (2), and the 260-nm absorbance and amount of ninhydrin-positive material were measured. The results (Table 3) confirm that siderophores were rapidly lost from the mutant conidia on contact with the buffer.

The loss of siderophores from the mutant conidia is similar at both temperatures and is considerably greater than that lost by wild type under the same conditions (Table 3). However, when wild-type conidia are subsequently placed into buffer whose a_w has been lowered to 0.938 with NaCl, an additional 5 to 10% of the conidial siderophore is lost (2, 3). Thus the total amount of siderophore that can be released by both types of conidia is very similar. We have postulated previously (3) that the ferricrocin released from wild type at low a_w represents the active and essential pool located in the plasma membrane. Apparently the conidia of strain JS 134-9 have a defective membrane which is unable to hold membrane-bound ferricrocin even at high a_w.

Other substances are also released as indicated by the 260-nm-absorbing and ninhydrin-positive materials detected in the buffer. Here temperature shows some effect; more material is released at the higher temperature. The total amount of 260-nm-absorbing and ninhydrin-positive materials lost from the mutant conidia is, however, much less than that lost by the wild-type conidia. Therefore, the mutation does not seem to involve an indiscriminately leaky membrane, but to have a more specific effect on whatever holds ferricrocin in the membrane. However, since siderophores cannot completely "cure" the mutation, i.e., eliminate the popula-

TABLE 3. *Amounts of siderophores, 260-nm-absorbing, and ninhydrin-positive materials released from 10^8 conidia per ml into 0.067 M sodium phosphate buffer ($a_w = 0.998$) at different temperatures*

Strain	Temp (°C)	Siderophores[a]	OD_{260}[b]	Ninhydrin[c]
JS 134-9	24	13-18	0.233	0.075
	35	13-18	0.273	0.088
74A (wild type)	24	3-7	0.330	0.312
	35	3-7	0.370	0.369

[a] Percentage of total siderophores extractable from conidia by chloroform-saturated buffer. Siderophores were assayed with the wild-type strain, 74A, and the low a_w medium described previously (2).

[b] OD_{260}, Optical density at 260 nm. Peak absorbance in the 250- to 260-nm range was read from spectral curves obtained on a Cary 15 spectrophotometer.

[c] Expressed in millimolar amino acid equivalents based on a leucine standard, using the method of Moore and Stein (4).

tion effect, especially at the higher temperatures, something else must be lost from the conidia. Experiments to isolate and identify such a compound are being planned.

In summary, then, the behavior of JS 134-9 in our experiments suggests that the mutation affects the siderophore attachment site on the plasma membrane. It appears, however, that more than this attachment site is involved. For example, both conidial germination and vegetative growth of the mutant are temperature sensitive, whereas the loss of siderophore from the conidia is not. Further, Schmit and Brody (personal communication) have shown that the mutant is sensitive to aerobic conditions, especially at elevated temperatures, and that it expresses a strong circadian conidiation rhythm at the permissible temperature (21°C). All of these properties are revertable simultaneously, indicating that a single mutational event is involved (Schmit and Brody, personal communication). We suggest that the mutation affects the plasma membrane, possibly a lipid component. Further studies are in progress to determine the nature of the membrane alteration.

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