Syntrophic growth of auxotrophic strains of *Neurospora crassa* by cross- feeding

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Vigorous growth occurred when conidia of two heterokaryon-incompatible auxotrophic strains of *N. crassa* were mixed on minimal medium. The syntrophic growth could often, but not always, be propagated through several subcultures using minute conidial inocula. We demonstrate that growth of auxotrophic strains occurred without hyphal fusion by cross-feeding.

The phenomenon of cross-feeding has been used to recognize biosynthetic intermediates excreted by auxotrophic mutants in bacteria (Davis 1950 Experientia **6**:41; see Stent 1971 Molecular Genetics, pp 139- 140, W.H. Freeman, San Francisco). Cross- feeding is commonly invoked to explain growth of strains of different nutritional capabilities in mixed cultures although, surprisingly, there is an apparent lack of published information, particularly in fungi. We report here on a case of growth of auxotrophic mutants of *Neurospora crassa* resulting from cross- feeding which was initially assumed to result from complementation of nutritional deficiencies due to heterokaryon formation. This example demonstrates a potential pitfall in assuming an initial growth response of an auxotrophic mutant with a heterokaryon-compatibility tester to indicate heterokaryon compatibility. The syntrophic culture showed an unpredictable loss of growth potential in subcultures. This observation is similar to senescence in some field isolates of Neurospora for which no explanation has been found.

Strains. The mutants used for construction of strains were *tre* (trehalase, FGSC 4509), *inv* (invertase, FGSC 1857), *pab-1* (p-aminobenzoic acid, FGSC3863), *nic-1* (nicotinic acid, FGSC 765) and *mcm* (microcycle microconidiation,FGSC 7455). The genotypes of constructed strains were *mcm;tre+;nic-1+;pab-1;inv;a* (A97-17) and *mcm;tre;nic-1;pab-1+;inv+;a* (RM146-9). The strains were tested for their ability to form heterokaryon using the tester strain *am1;ad-3B;cyh-1;het-Cde*(FGSC 4564) (Perkins 1984 Neurospora Newsl. **31**:41-42). RM146-9 macroconidia (referred throughout as conidia) produced a vigorous growth with a^{m1} conidia which was subculturable. Strain A97-17 conidia also formed a vigorous growth with a^{m1} conidia but such growth was subsequently found not capable of surviving more than 2-3 subcultures.

Syntrophic growth. Equal numbers (10^7) of conidia of A97-17 and RM 146-9 in small volumes of distilled water were mixed and the pellet was placed on sorbose minimal medium. A vigorous mycelial growth (SG) developed within two days at 34 C and conidiated profusely in 3- 4 days. We assumed that this growth resulted because of heterokaryon formation between the two strains. The growth rates of the auxotrophic strains on vitamin- supplemented media and of SG on minimal medium supplemented with glucose, sucrose or trehalose as carbon source (0.5%), measured in race tubes, were comparable (4 mm/h).

Conidial test of heterokaryosis. To study whether hyphal fusion followed by nuclear mixing occurred in SG, it was grown on Vogel's N agar medium supplemented with sucrose or trehalose (1.5%). Samples of conidia were plated on differentially supplemented media to determine their genotype. Colonies were formed only in medium supplemented with nicotinic acid or p-aminobenzoic acid. The absence of colonies on minimal medium implied that heterokaryotic conidia were not produced on either of the carbon sources. The ratio of the homokaryotic *nic* and *pab* conidia varied in the two carbon source tested; being 1.4:1 in sucrose and 1:4.4 in trehalose, respectively.

Hyphal tip analysis. To further check the absence of nuclear mixing in SG, it was grown for 18-24 h on Vogel's N medium with 4% agar and 0.5% sucrose or trehalose. The individual hyphal tips were excised from the front edge of the mycelium and their genotypes determined by growing them individually in differentially supplemented media. In sucrose-grown culture all hyphal tips were of *pab* genotype. In trehalose- grown culture, 82% of the hyphal tips were *pab* and 18% *nic.* The results showed that the mycelial front of a young agar- grown SG comprised only of homokaryotic hyphae.

Stability of SG. To check the stability of SG, twenty replicate subcultures from the original plate culture were made. For subculturing, a minute quantity of conidia was transferred onto slants of fresh medium each time. More than 50% of the cultures remained healthy after six subcultures, indicating that SG is quite stable in subcultures. However, the observation that SG could not be propagated in all replicate cultures required an understanding of the nature of syntrophic growth.

Disproportionate conidial types. The unpredictable loss of growth potential of SG in some subcultures could result from the disproportionate numbers of *pab* and *nic* conidia produced. Consequently, in minute inocula, as is the usual practice, both types of conidia may not be present leading to cessation of growth. To determine their proportions, conidia from SG cultures were sampled by two methods: (1) Minute quantity of conidia taken from three replicate cultures were suspended in water and plated after suitable dilution. (2) Total conidia from the same three cultures were suspended in water and plated after suitable dilution. In sucrose-grown cultures, the *nic* conidia were 1% (method 1) to 12% (method 2) only; the majority were *pab* conidia. In another experiment, A97- 17 and RM146-9 conidia were inoculated at one end on minimal medium (1.5% glucose, sucrose or trehalose as carbon source) in race tubes. A mycelial growth developed across the tubes. After 10 days, conidiation had occurred at both ends. Samples of conidia from the two ends of the race tubes were plated on differentially supplemented media. After 10-20 days, in most of the race tubes conidia produced at the mycelial front were only of one type: either *pab* (8 race tubes) or *nic* (3 race tubes) regardless of the carbon source used. In only 5 race tubes both conidial types were found, either *pab* or *nic* being in excess. However, at the inoculation end the two conidial types were always recovered.

Syntrophic growth without hyphal fusion. The syntrophic growth of auxotrophic strains could have resulted from cross- feeding without involving hyphal fusion. The growth of one auxotrophic strain was tested in the diffusate of the other auxotrophic strain without their cells having come into contact. Approximately 10^6-10^7 dry conidia of one strain (feeder conidia) were suspended in liquid minimal medium containing 0.5% sugar and incubated at 34 C. After 24 h conidia (germination 17-37%) were aseptically removed by filtration through Millipore filters (0.45 um) and the diffusate was tested for its ability to support growth of conidia of the other strain (test conidia). Although the auxotrophic strains did not grow individually, A97-17 grew on the diffusate of a minute quantity (10^6-10^7) of RM146- 9 conidia (Table 1). However, RM146-9 did not grow in the 24 h diffusate of A97- 17 conidia. Since physical contact between the two auxotrophic strains was not necessary for growth of A97-17 conidia, the minimal number of RM146-9 conidia required to satisfy the growth factor required by A97-17conidia was determined. A suspension of RM146-9 (feeder) conidia was prepared in distilled water and their concentration determined by hemocytometer counting. Different samples of the conidial suspension were dispensed in flasks containing 20 ml Vogel's N medium supplemented with 0.5% sucrose. After incubation for 24 h at 34 C, the conidia were aseptically removed by filtration. The diffusate was tested for its ability to provide the growth requirement of A97-17 test conidia (Table 2). A minimum of 10^6 conidia of RM146- 9 could provide the growth factor required for the optimal growth of A97-17 conidia. It was postulated that growth of A97-17 + RM146-9 conidia results from cross-feeding in which each strain supplemented the growth factor required by the other strain without hyphal fusion. However, growth of RM146- 9 occurs subsequent to the growth of A97-17. To test this possibility the delayed growth of RM146- 9 was tested in culture filtrates of A97-17 taken at different times of growth (Table 3). Growth of RM146-9 conidia occurred in culture filtrates after A97-17 had produced some mycelial growth.

In summary, the following observations need to be accounted for: (1) mostly pab hyphal tips were recovered from the edge of 18-24 h SG mycelium; (2) only homokaryotic pab or nic conidia were recovered from a conidiating SG culture; (3) SG could not always be propagated in subcultures; (4) the A97-17(*inv;pab*) conidia germinated and produced mycelial growth in the diffusate of RM146-9 (*tre;nic*) conidia; and (5) conidia of RM146-9 did not grow in the 24 h diffusate of A97-17 but grew in 48-96 h culture filtrates of A97-17. These observations may be explained as follows. When suspended in the medium containing either sucrose or trehalose, the RM146- 9 conidia apparently leach out a growth factor that allows A97-17 to begin growth. When A97- 17 has produced some mycelium, it leaches out sufficient growth factor and some carbon compounds that allow growth of RM146-9. The occasional loss of growth potential A97-17 + RM146-9 culture on serial subculturing appears to result from the progressive loss of a particular conidial type from the disproportionate mixture of the conidial types when small quantity of inoculum is taken for subculturing. This was similar to unpredictable loss of growth potential in some naturally occurring strains of Neurospora (Maheshwari *et al*. 1994 Fungal Genet. Newsl.**41**:60) and may be relevant to senescence in some strains of Neurospora for which no explanation has been found (Griffiths and Yang 1993 Mycol. Res. **97**:1379-1387). It might be that these wild strains are mixtures of cross- feeding mutants that become unbalanced during conidial transfers.

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Table 1. Cross-feeding between auxotrophic strains A97-17(*inv;pab*) and RM146-(*tre;nic*).

Carbon source	Genotype		Growth of test strain after 5 days (mg dry wt)[2]
	Feeder conidia<sup1< sup="">	Test conidia	
Sucrose	tre;nic	tre;nic	0.65
	inv;pab	inv;pab	1.70
	tre;nic + inv;pab3	nil	50.00
	tre;nic	inv;pab	31.00
	inv;pab	tre;nic	0.50
Trehalose	tre;nic	tre;nic	1.60
	inv;pab	inv;pab	2.55
	tre;nic + inv;pab[3]	nil	53.30

tre;nic	inv;pab	34.34
inv;pab	tre;nic	4.35

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[1]Approximately 10^6-10^7 conidia incubated for 24 h.

[2]Average of two independent experiments.

[3]Conidia of two strains were allowed to grow continuously for 5 days.

Table 2. Quantity of *tre;nic* conidia required to satisfy the growth requirement of *inv;pab*.1

Number of tre;nic conidia used for diffusate obtaining diffusate	Growth of inv;pab conidia in after 5 days), mg dry weight)2
Nil	1.25
102	1.20
103	1.20
104	0.70
105	2.40
106	14.55
107	19.70
108	14.90

[1]Data were average values of two separate experiments.
[2]10^3 test conidia were added.

Table 3. Delayed growth of *tre;nic* in the spent medium of *inv;pab*.

Days of growth of inv;pab in in the diffusate of of tre;nic[1] weight)[2]	Growth of inv;pab (mg dry weight)	Growth of tre;nic culture filtrates inv;pab (mg dry
1	4.3	3
2	15.7	25
3	19.5	52
4	15.3	41
6	18.2	12

1Approximately 106 conidia were incubated for 24 h in Vogel's N medium + 0.5% trehalose for obtaining diffusate.
2Measurement after 5 days of incubation.

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