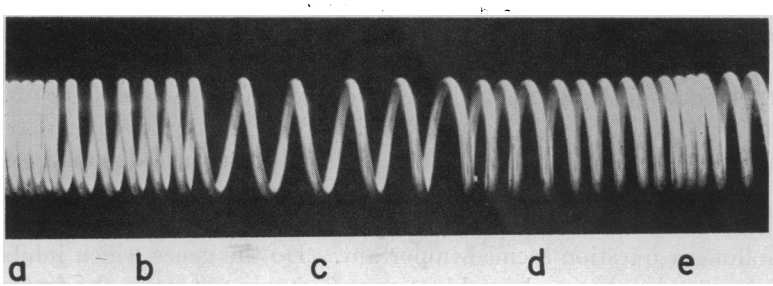

FIG. 4.—Model of a single helix with variable distances between the coils to illustrate the possible similarity to the giant chromosomes.

resolving power of the microscope and are thus invisible. This point will be brought out in a later paper that will consider the multiple-strand nature of the chromosome and of their hierarchy of helices.

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*HETEROCARYOSIS AND PROTOPLASMIC INCOMPATIBILITY IN NEUROSPORA CRASSA**

BY LAURA GARNJOBST AND JAMES F. WILSON

DEPARTMENT OF BIOLOGICAL SCIENCES, STANFORD UNIVERSITY, STANFORD, CALIFORNIA

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Introduction.—The physiological basis of incompatibility between organisms is a subject of considerable interest. Although incompatibility reactions are known in less complex forms, they have been studied most intensively in the higher organisms—for example, the researches concerned with outbreeding in flowering plants and blood types in animals. Among the micro-organisms, certain incompatibilities described in the past are related to sex, e.g., the mating-type factors of some Protozoa and fungi. In contrast, incompatibilities recently described in strains of *Neurospora crassa* appear to be related primarily to the vegetative stages. This

type of incompatibility is manifested by the failure of certain strains of like mating type to form successful heterocaryons.

Several biochemical mutant strains of *N. crassa* differing in ability to form heterocaryons have been investigated genetically.[1, 2, 3] Isolates from crosses made in this laboratory with strains of the *CD* and *cd* heterocaryon genotypes provided suitable material for further study of heterocaryosis. Since hyphal fusion ordinarily is a prerequisite for the experimental production of heterocaryons, an answer to the following question seemed important. Do the genes which inhibit heterocaryosis in certain unisexual combinations affect some steps in the fusion process, or do they affect some subsequent stage? Microscopic observation of growing hyphae showed that hyphal fusions do occur between all pairs of isolates that fail to form stable heterocaryons, as well as between all pairs that form stable heterocaryons. In the former pairs, however, fusion between hyphae is followed by a series of visible changes whereby the cells involved are destroyed. It is the purpose of the present paper to describe this "incompatibility reaction."

Materials and Methods.—Isolates of the *inositolless* (37401, *inos*) and *riboflavinless* (Y30539, *rib*-2) strains obtained in the genetic analysis previously described[2, 3] were used for most of the microscopic observations. The heterocaryon genotypes of the isolates of each biochemical mutant strain are *CD*, *cD*, *Cd*, or *cd*. Pairs of isolates (*inos* + *rib-2*) which give wild-type rate of growth on minimal medium (*het*$^+$ response) have the same heterocaryon genotype, i.e., *CD* + *CD*, *cD* + *cD*, *Cd* + *Cd*, and *cd* + *cd;* those which give no growth or delayed erratic types of growth on minimal medium (*het*$^-$ response) have unlike heterocaryon genotypes, i.e., *CD* + *cD*, *CD* + *Cd*, *CD* + *cd*, *cD* + *Cd*, *cD* + *cd*, and *Cd* + *cd*. Other strains and isolates will be mentioned in connection with the particular tests concerned.

Material for examination was prepared under sterile conditions as follows. The cultures to be tested were grown at 30° C. in Petri plates containing a thin layer of glycerol complete medium[4] with 2.5 per cent agar. When the mycelium from a central inoculation of conidia had reached about halfway toward the periphery of the plate, small squares were cut along the advancing hyphal front for transfer to slides. Previous to the transfer, a small rectangle of glycerol complete agar (2.5 per cent) from an uninoculated plate had been put on each slide within a moistened Petri plate. The squares of agar with hyphae from each of the two cultures to be tested were then placed at opposite ends of the rectangle, so that the hyphal tips pointed toward the central "bridge." Such slide preparations, six for each pair, were incubated at 34° C. until the hyphae growing toward one another on the surface of the agar were about 0.3 mm. apart. A drop of minimal medium and a cover slip were then added for observation under the microscope at room temperatures (20°–28° C.).

The best results were obtained when the agar bridge and squares with hyphae were equal in thickness. A narrow bridge facilitated tracing the fused hyphae to their origins and allowed diffusion of oxygen. To support the cover slip and reduce evaporation, small squares or strips of agar were added to one or both sides of the bridge.

Both phase-contrast and ordinary compound microscopes were used. A magnification of 645× was found to be adequate for the observations presented.

Results.—As a basis for this study, the wild type SY7A was examined first.

Many types of intrastrain fusions were observed from the time of contact until protoplasmic flow took place through the new opening. The types of fusions (Figs. 1 and 2) are quite similar to those described in *Neurospora* and in other incompletely septate fungi.[5, 6]

The interstrain hyphal fusions of the *het*$^+$ pair *CD inos* and *CD rib-2* (in $a + a$ or $A + A$ mating types), as well as the intrastrain fusions of each of these isolates, were found to be indistinguishable from the intrastrain fusions observed in the wild type, as might be expected. The same was true of the intrastrain fusions of each member of the *het*$^-$ pair of isolates, *CD rib-2* and *cd inos* (in $a + a$ or $A + A$ mating types, and in the reciprocals, *CD inos* and *cd rib-2*), and also of the interstrain fusions up to and including formation of an "interseptum." Thereafter, a series of visible, irreversible changes was initiated.

To facilitate a description of these changes, one hypha is termed the "attacking hypha" and the other the "quiescent hypha." However, in most of the fusions observed the quiescent hypha responded by forming a protuberance (Figs. 1 and 9), and both hyphae often were equally active, i.e., visibly advancing toward one another on the agar surface. The subsequent series of changes occurred in the following order.

1. Shortly after contact (from 2 to 30 minutes or more at room temperatures of from 25° to 28° C.) protoplasmic movement took place through the new opening. The flow in some instances lasted about 45 or 60 seconds, occasionally somewhat longer. However, a mere start of movement or no perceptible movement at all were also common.

2. With or without movement, the protoplasm in the vicinity of the fusion, characteristically in both hyphae, sooner or later became gradually more granular, then finely vacuolated (Fig. 6). The vacuoles increased in size, often becoming quite large, as shown in Figures 7, 8, and 9. At the same time the intervacuolar portion became dense (Fig. 9). The conspicuous, affected region varied in extent from one to several cells in each hypha and usually was sharply delimited by septa, each containing a distinct opaque disk or plug (Figs. 5, 6, and 10) in place of an opening. At other times two or three consecutive septa in the outer cells were plugged. When this occurred, the outermost cells frequently showed a delayed effect or a graded effect, i.e., less granulation and vacuolation distally. The plugs were most prominent at the height of the vacuolation.

3. Finally, the vacuoles faded from view, leaving the contents of the affected cells pale and disorganized. Many hours later shrunken protoplasmic remnants could still be seen within the old hyphal walls.

During the late stage (stage 3) of the incompatibility reaction, the normal cells immediately behind the outermost plugged septa often developed new growing points (Figs. 10 and 11). These points formed new branches or instead grew within the old cell wall, pushing aside the plug, now partly resorbed, as well as the disintegrating protoplasm. When the latter course was taken, two growing tips within the wall of the old quiescent hypha were observed to approach one another and fuse, thus reconstituting this hypha (Fig. 12). New hyphae growing within attacking hyphae discontinued growth or broke out through the wall before reaching the point of union. Figures 11 and 12 illustrate the two types of regeneration observed.

The important result of the fusion and interaction of the two protoplasms *appears*

to be that the affected region in each hypha is effectively destroyed. However, the methods employed in this study do not rule out the possibility that some of the components of the invading protoplasm may escape into the adjoining unaffected region.

Representatives of all other *het*$^+$ and *het*$^-$ pairs of isolates (*inos* + *rib-2*) previously described were inspected microscopically. The results obtained with all *het*$^+$ pairs were essentially the same; that is, the intra- and interstrain fusions observed were indistinguishable and like the intrastrain fusions of the wild type SY7A. The difference between the intra- and interstrain fusions, as described in detail for the *het*$^-$ pair *CD rib-2* and *cd inos*, also holds for all other *het*$^-$ pairs tested; that is, all interstrain fusions induced the incompatibility reaction. No perceptible difference was observed in the reaction between pairs of strains differing by one or by two heterocaryon genes.

In a previous paper[3] it was shown that the effect of the genes concerned with heterocaryosis is independent of the mutant genes *inos* and *rib-2* and of all other biochemical mutant genes examined. Thus it seemed logical to assume that the incompatibility reaction would take place following fusion between two isolates of the *same* biochemical mutant strain differing in heterocaryon genotype. This was actually found to be the case. Representatives of all types of *het*$^-$ pairs of isolates previously described were tested.

The results of heterocaryon tests (unpublished) made in our laboratory with certain biochemical mutants suggested that the wild types from which they were derived differ in heterocaryon genotype. These wild types are SY7A or SY4a and ST A or ST a (St. Lawrence strains).[7] Since heterocaryon formation of nutritionally wild-type strains cannot be determined by the usual methods, it was thought that a difference might be detected microscopically. To test this possibility, the following pairs of wild types were observed: (1) SY4a f_9 + Lindegren (L) 25a; (2) ST a + SY4a f_9; (3) ST a + L25a; (4) *pe*^{m} *fl a* (Y8743-21 (13-7), *microconidial fluffy*) + SY4a f_9; (5) *pe*^{m} *fl a* + ST a; (6) *pe*^{m} *fl a* + L25a; and (7) SY4a f_9 + ST a. Consistent results were obtained as follows: (*a*) All strains formed functional intrastrain fusions in all preparations. (*b*) In preparations including a St. Lawrence strain, the interstrain fusions were of two types—completed fusions initiating an incompatibility reaction and uncompleted fusions apparently resulting from failure to produce an opening at the point of contact. (*c*) All other pairs were found to be compatible according to the new test.

The mating-type factors *A* and *a* in *Neurospora* have also been called incompatibility factors.[8] Since the function of these allelic genes is not well understood,

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FIGS. 1–12.—Photomicrographs of living, unstained hyphae of *Neurospora crassa* growing on nutrient agar. ×600. Arrows indicate fusions not obvious in photomicrographs.

FIGS. 1 and 2.—Intrastrain fusions.

FIGS. 3 and 4.—Interstrain fusions, *het*$^+$ pairs.

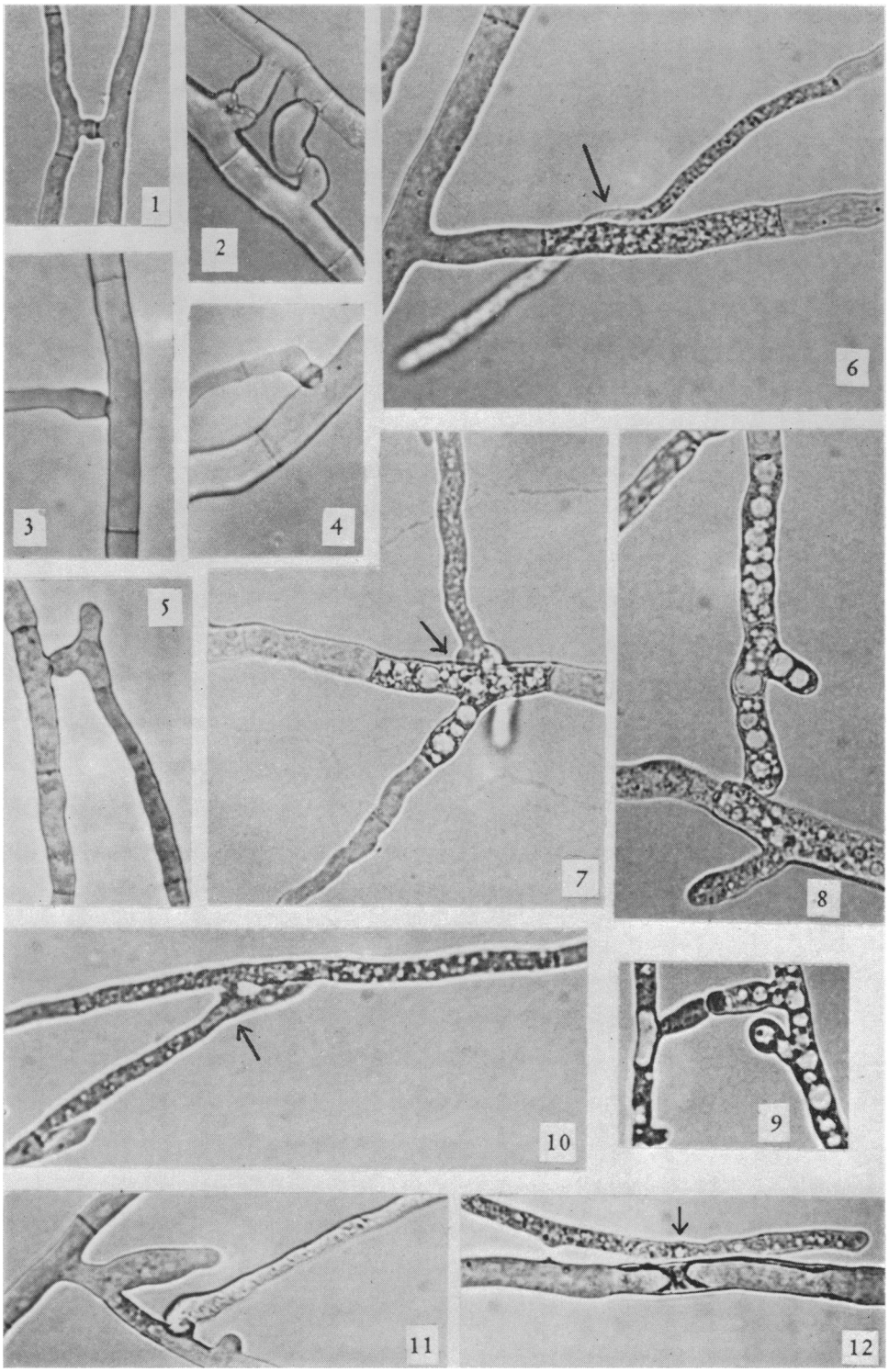
FIGS. 5 and 6.—Early stages of the incompatibility reaction, *het*$^-$ pairs. Plugs visible in septa delimiting affected regions.

FIGS. 7, 8 and 9.—Later stages of reaction showing prominent vacuolation: Fig. 7, in wild-type pair differing in mating type; Fig. 8, in pair differing in both mating type and heterocaryon genotype; Fig. 9, in pair differing in heterocaryon genotype.

FIG. 10.—Later stage of reaction in which vacuoles remained small. New growing point at lower left.

FIGS. 11 and 12.—Types of regeneration.

PLATE 1



it seemed of interest to extend the new test to strains differing in mating type. The pairs selected for these tests were *CD rib-2 a + CD inos A*, *CD inos a + CD inos A*, and *CD inos A + cd inos a*. In these tests interstrain fusions were infrequent in some slide preparations but common in other preparations of the same pair. Whenever the fusions did occur, an incompatibility reaction was initiated. The series of changes in most instances was similar in all respects to that described for *het*$^-$ pairs of the same mating type, but in the first two pairs listed an occasional difference was noted. This difference consisted of an unusually long period of protoplasmic flow through the interconnection, followed by a delay in the appearance of the reaction.

This unexpected evidence of incompatibility led to still further tests, this time with pairs of wild-type strains differing in mating type, as follows: (1) SY4a f_9 + SY7A; (2) SY7A + L25a; (3) SY7A + ST a; (4) ST A + ST a; and (5) ST A + L25a. As in the tests mentioned above, the interstrain fusions observed between these *A* and *a* strains also initiated an incompatibility reaction (Fig. 7). The variability described above was also found in pairs 1, 2, and 4. In addition, in pairs 3 and 5 a few fusions appeared to be uncompleted.

Although no constant difference in the incompatibility reaction was observed in *a + A* pairs as compared to *a + a* or *A + A* pairs, the results of the preliminary tests with strains differing in mating type suggest that the incompatibility reaction in these combinations is more readily affected by environmental conditions.

Discussion.—These findings raise certain questions regarding heterocaryosis, mating-type factors, and particularly regarding the nature of the incompatibility reaction. It has been concluded from a variety of experiments that functional anastomoses do not occur between mycelia of different species.[6, 9] The *Neurospora* species, *crassa*, *tetrasperma*, and *sitophila*, were among those tested.[6] The incompatibility reaction described in this paper, then, might be looked upon as similar to one of the many possible genetically controlled barriers leading to the evolution of sexual differentiation or speciation. This irreversible reaction appears to be an effective mechanism to prevent heterocaryosis in either unisexual (*het*$^-$) or bisexual[10, 11] combinations, but in the *A + a* combinations the stimulus inducing the reaction might be present only during the vegetative stages.

Numerous observations concerning hyphal fusion in fungi during vegetative growth and reproduction have been reported in the literature. Some of the findings described in this report are not new—for example, the open "pore"[12] in the center of each hyphal septum, the "pore-plug"[12] which appears as a result of injury to one or more cells in a hypha, and the growth of new hyphae within the old hyphal walls.[13] Most recently Gerdemann[14] described, in a phycomycetous fungus, a method of wound-healing which differs slightly from that observed in a number of species by Buller[12] and in *Neurospora* during the present study. These injury reactions resemble the incompatibility reaction but differ from it in one important respect—the absence of interhyphal fusion and subsequent intermingling of incompatible protoplasm.

In Protozoa there is at least one recorded observation of protoplasmic incompatibility not related to sex. Reynolds[15] investigated a "shattering reaction" in *Arcella polypora* and concluded that this reaction was a transitory change in phenotype induced by environmental differences. In contrast, the heterocaryon phenotypes of the *Neurospora* strains used in this study have remained consistent with their genotypes.

In comparing the *C* and *D* (or *c* and *d*) genes to the incompatibility factors of some higher fungi, as seems inevitable, it should be borne in mind that the strains of *Neurospora* obtained in the genetic analysis[2, 3] are either of the *a* or *A* mating type as tested with SY7A and SY4a (or ST A and ST a) and form perithecia when and only when the *a* and *A* strains are crossed, regardless of the heterocaryon genotype.

At this stage of the investigation in *Neurospora*, little is known of the relationship between the incompatibility reactions occurring (1) in pairs composed of strains of the same mating type and (2) in pairs composed of strains of different mating types. However, the results obtained with the various het^+ and het^- pairs appear clear cut. The agreement between the heterocaryon tests previously made and the occurrence of the incompatibility reaction in all het^- pairs is complete. Furthermore, the observations indicate that the cause of the incompatibility reaction lies in the protoplasmic portion of the mycelium.

Summary.—The genes *C* and *D* and the alternate alleles *c* and *d* in *N. crassa* are concerned with heterocaryon formation, as previously reported. Heterocaryon tests with pairs of isolates of the same mating type proved that all pairs of the same heterocaryon genotype form compatible heterocaryons, whereas all other pairs are incompatible, i.e., fail to grow or grow erratically on minimal medium. These two classes of pairs were termed het^+ and het^-, respectively.

The present experiments indicate that the reaction between members of all het^- pairs involves protoplasmic incompatibility. Representative isolates of both het^+ and het^- pairs of the *riboflavinless* (Y30539, *rib-2*) and the *inositolless* (37401, *inos*) strains were observed microscopically during vegetative growth, with results as follows: In the het^- pairs all interstrain hyphal fusions initiated an irreversible incompatibility reaction. Upon exchange of protoplasm through the new opening between the hyphae, the cells in the vicinity of the fusion gradually disintegrated. Since no exception was found in any het^- pair and all interstrain hyphal fusions in all het^+ pairs were normal, it is concluded that the incompatibility reaction is the mechanism whereby heterocaryotic growth in the het^- pairs is inhibited or prevented.

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