INTER-ISOLATE HETEROKARYOSIS IN *THANATEPHORUS CUCUMERIS*

I. BETWEEN ISOLATES OF SIMILAR PATHOGENICITY

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Abstract

Previous investigations in this laboratory of heterokaryosis in *T. cucumeris* have utilized mutant progeny of one field isolate. The work has now been extended to include four field isolates, of different geographic origin, yet of similar host specificity in their pathogenic reactions (viz. all pathogenic to crucifers only among the hosts tested). Mutant single-spore cultures derived from these four have been induced to form inter-isolate heterokaryons, regardless of their differences (e.g. in origin, cultural appearance, fruiting ability, survival ability). Complementation for pathogenicity and fruiting behaviour was observed in these heterokaryons and wild-type progeny showed no segregation in pathogenic reactions. This suggests genetic similarity between all four isolates in regard to the factors controlling pathogenicity. However, wild-type progeny exhibited considerable variation in cultural appearance, the extent varying with the mutants used. Cultural differences may be a reflection of different saprophytic abilities.

The results of these heterokaryon tests do not appear to support the hypothesis that a bipolar compatibility system controls heterokaryosis.

I. INTRODUCTION

Field isolates of *Thanatephorus cucumeris* (Frank) Donk are usually heterokaryotic for a range of characters, e.g. cultural appearance (Exner and Chilton 1943; Whitney and Parmeter 1963), growth rate (Flentje and Stretton 1964), virulence (Garza-Chapa and Anderson 1966), survival ability (Papavizas 1964; Baker *et al.* 1967). Homokaryotic cultures can be obtained from uninucleate single basidiospores. Mutants derived from one such culture designated 48–11–14–56 (Flentje, Stretton, and McKenzie 1967) have been used in previous studies on heterokaryosis and genetic recombination in this fungus (McKenzie *et al.* 1969). Pathogenicity was shown to be restored when two different non-pathogenic mutants from this culture were anastomosed to form a heterokaryon. In subsequent recombination studies, the genetic control of pathogenicity was demonstrated by the recovery of the two non-pathogenic mutants, the non-pathogenic double mutant, and the pathogenic wild type. Further detailed physiological studies on the pathogenic reactions of a number

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of different mutants derived from this one culture have shown that in pathogenesis a series of steps is involved, any one of which may be blocked as the result of a single gene mutation (Flentje, Stretton, and McKenzie 1967).

Investigation of heterokaryosis and of factors determining pathogenicity in *T. cucumeris* has been confined previously to the use of sibling progeny. These studies have now been extended to include crosses between progeny of field isolates of different origin, yet of similar host specificity. Studies on the genetic relationship of these isolates obtained from widely separated locations and different soil types, are reported in this paper.

II. Materials and Methods

Four field isolates of *T. cucumeris* "solani" type, all of which are pathogenic to stems of Cruciferae only when tested against four host families, were used in these studies and are listed in the following tabulation:

Isolate No.	Source	Locality	Soil type
48	Soil	Waite Institute, Adelaide, S.A.	Heavy red-brown silt loam, pH 6·5
68	Cabbage stem lesion	Hitchin, England	Clay-loam over chalk
69	Cabbage stem	Clare, S.A.	Heavy red-brown silt loam, pH 6·5
106	Crucifer stem	Slough, England	Medium red-brown alluvial loam, pH 5·3

These isolates correspond with anastomosis group 2 (Parmeter, Sherwood, and Platt 1969). All four cultures were fertile, though the English isolates only sparingly so, suggesting sterility factors were present preventing profuse development of hymenia. Mutant single-basidiospore cultures derived from these four isolates were obtained as a result of either spontaneous mutation or ultraviolet irradiation (Flentje, Stretton, and McKenzie 1967). Cultures were grown on *Rhizoctonia* medium (R.M.),* soil-extract agar, or potato–Vegemite–dextrose–agar (P.V.D.A.), the latter two having been described previously (Flentje 1956; McKenzie *et al.* 1969), as have the methods used for determination of anastomosis and heterokaryon formation (McKenzie *et al.* 1969), pathogenicity (Flentje, Stretton, and McKenzie 1967), and fruiting (Stretton *et al.* 1964).

III. Experimental Details and Results

(a) *Heterokaryon Formation between Mutant Progeny of Different Field Isolates*

Four to seven morphological mutants were obtained from each of the four field isolates (Fig. 1) used in this study. Each mutant had a characteristic morphology and differed from its parent isolate in growth habit on agar media; half were non-pathogenic to stems of Cruciferae, but they differed in the stages at which the infection process failed. None of the mutant cultures fruited. Brief descriptions of their cultural characteristics and pathogenic reactions on crucifer stems are listed in Table 1.

The mutants were macerated in pairs in all possible combinations, the macerated pulp in each case being transferred to P.V.D.A. plates in order to observe possible

* Composition as follows: KH_2PO_4 0·68 g, K_2HPO_4 0·87 g, $MgSO_4.7H_2O$ 0·12 g, sucrose 17·10 g, Bacto-peptone 7·50 g, distilled water 1 litre, agar 15 g.

heterokaryon formation. As the mutants represented four field isolates, the total of macerated combinations represented six combinations of field-isolate gene pools, viz. 48×68, 48×69, 48×106, 68×69, 68×106, 69×106.

Wild-type growth was obtained from representatives of each of the six possible combinations of parent gene pools, and approximately 75% of the total combinations produced wild-type growth (Table 2) similar to one or other of the parent field isolates.

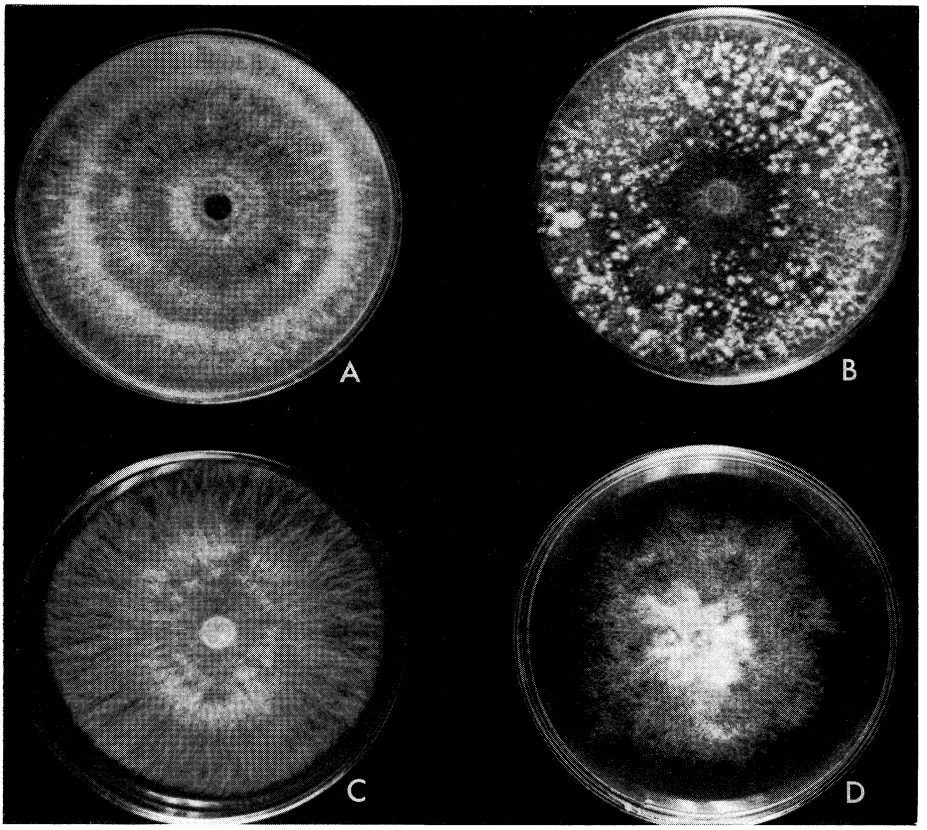
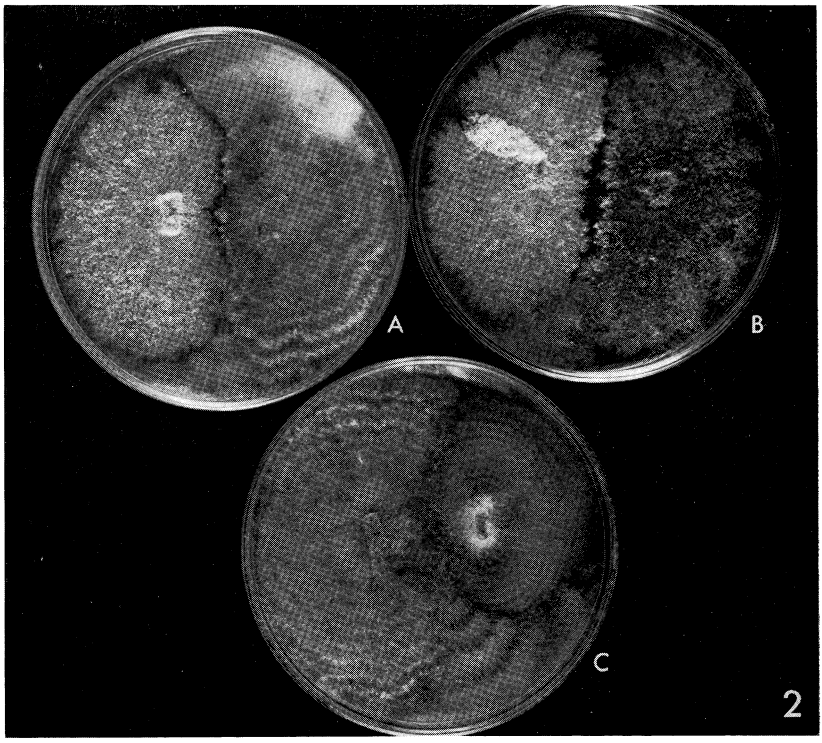


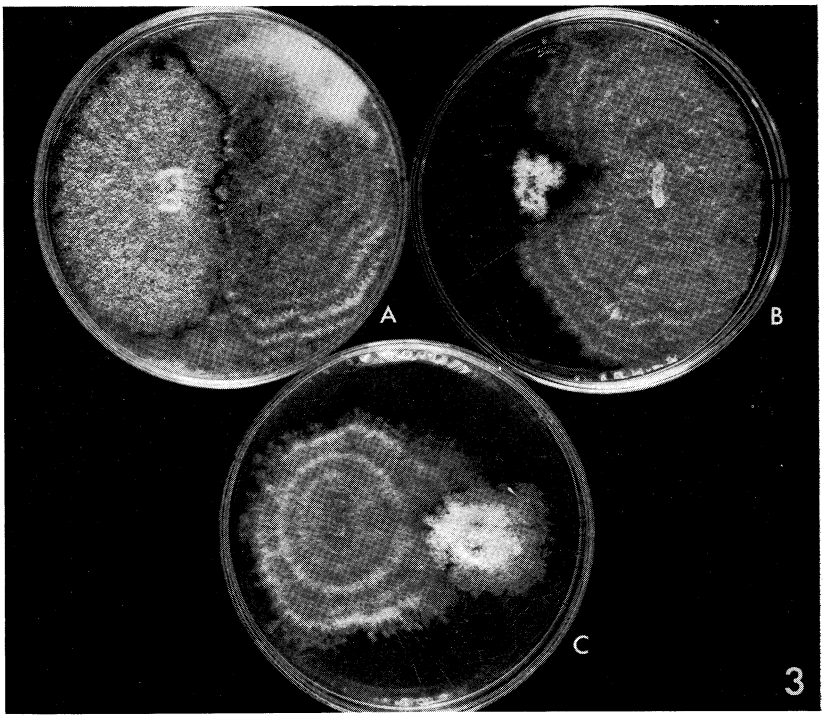
Fig. 1.—Five-day-old cultures of the four field isolates used in these studies, grown on *Rhizoctonia* medium. *A*, isolate 48; *B*, isolate 68; *C*, isolate 69; *D*, isolate 106.

Wild-type refers to both a cultural appearance and growth rate. At least one putative heterokaryon from each of these six groups was selected for detailed investigation of heterokaryosis, namely:

48 G_4 13 × 68 G_1 4	68 G_1 4 × 69 G_4 51
48 G_4 13 × 69 G_4 6	68 G_1 3 ×106 G_1 2
48 G_1 25 ×106 G_1 1	69 G_4 39 ×106 G_1 10

Each of these six putative heterokaryons was tip-cultured and the resulting wild-type cultures were stable on all subsequent vegetative transfers, showing no sectoring. Approximately 100 basidiospores were isolated from each heterokaryon.


A
B
C
2


A
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C
3

TABLE 1

CULTURAL CHARACTERISTICS AND PATHOGENIC REACTIONS OF MUTANT PROGENY USED IN HETEROKARYON TESTS

Mutant progeny (generation and mutant number)	Cultural characteristics	Stage reached in infection process on crucifer stems*
Isolate 48		
G_1 25	*curly* II—light brown; curling of hyphae, loss of apical dominance	originally I‡
G_4† 13	*curly* I—dark brown on agar, aerial hyphae white; curling of hyphae, loss of apical dominance	V
G_4† 150	*ropy*—off-white, irregular-shaped colony, deeply dentate periphery	II§
G_4† 9	*stumpy*—off-white, gradually browning, short side branches	I
Isolate 68		
G_1 3	*curly* IV—light brown	II, later VI
G_1 4	wavy, defined concentric rings	VI
G_1 6	wavy–curly, densely branched	VI
G_1 10	sparse, irregular periphery	II, later VI
G_1 11	sparse, feathery appearance	VI
G_1 14	wavy–curly, zonate rings	V
G_2 5	similar to wild-type parent but slower	VI
Isolate 69		
G_4 6	slow, compact, yellowish	II
G_4 39	fluffy, areas of dense branching on agar	V, later VI
G_4 51	*curly* III—brown	II
G_4 56	fluffy, aerial hyphae curly	VI
G_4 60	dark, rings, not very slow	VI
Isolate 106		
G_1 1	uneven edge on colony	II
G_1 2	slow, apical dominance pronounced	VI
G_1 8	"coral-like" appearance	II
G_1 10	"feathery" runner hyphae, brownish	III

* The following stages have been described previously (Flentje, Stretton, and McKenzie 1967) and are now numbered thus: I, growth inhibited on stems; II, growth on stems, but no reaction; III, attachment of fungal hyphae to stems; IV, cushion formation but no penetration; V, hypersensitive reaction; VI, pathogenesis.

† These mutants were previously referred to as "curly", "ropy", and "stumpy" (Flentje, Stretton, and McKenzie 1967) but extension of this designation to all mutants obtained since has been discontinued.

‡ Later testing showed some V.

§ With occasional superficial IV.

Fig. 2.—Growth of wild-type heterokaryons from junction of two mutants opposed on Cellophane overlying agar. *A*, 48–13 × 68–6; *B*, 48–13 × 69–51; *C*, 68–6 × 69–6.

Fig. 3.—Varying reactions of mutants when paired on Cellophane overlying agar. *A*, 48–13 × 68–6 : heterokaryon produced; *B*, 48–13 × 68–4 : repulsion; *C*, 106–2 × 68–3 : stimulation of 106–2 towards 68–3.

TABLE 2

HYPHAL GROWTH PRODUCED WHEN MUTANT SINGLE-BASIDIOSPORE CULTURES WERE MACERATED IN PAIRS AND PLATED ON P.V.D.A.

W, wild type; I, intermediate; X, mutant only; —, not tested: mutants died before testing completed

	Isolate 48				Isolate 68							Isolate 69					Isolate 106			
Mutant No.:	25	9	13	150	3	4	6	10	11	14	5	6	39	51	56	60	1	2	8	10
Isolate 48																				
25																				
9	W																			
13	W	W																		
150	X	W	W																	
Isolate 68																				
3	W	X	X	X																
4	W	X	W	W	X															
6	W	X	W	W	X	X														
10	W	X	W	W	W	W	W													
11	W	X	W	W	W	W	W	X												
14	W	X	W	W	X	X	X	W	W											
5	W	X	W	W	I	W	I	X	X	W										
Isolate 69																				
6	W	W	W	X	X	W	W	W	W	X	W									
39	W	W	W	W	W	W	W	—	—	W	W	W								
51	W	W	W	W	X	W	W	W	W	W	W	W	W							
56	X	W	W	X	W	W	W	—	—	W	W	W	W	W						
60	W	W	I	X	W	W	W	—	—	W	W	W	X	W	W					
Isolate 106																				
1	W	W	W	X	W	W	W	—	—	W	W	W	W	W	W	W				
2	W	X	W	W	W	W	W	—	—	W	W	X	X	X	X	X	X			
8	W	W	W	X	W	W	W	—	—	W	W	X	X	X	X	X	X	I		
10	W	W	W	X	W	W	W	—	—	W	W	W	W	W	W	W	W	W	X	

The progeny from each heterokaryon could be separated into four cultural groups which fitted the expected pattern for recombination and segregation of two independent genes, viz. wild type, one or other of the two original mutants, and double mutant recombinants. In general the grouping was straightforward because of the obvious similarity in cultural characters between the original cultures and the recovered progeny for the wild types and single mutants, and because of the very slow growth of the double mutants. This grouping, based on cultural morphology, was confirmed genetically in the case of single and double mutants by establishing the identity of the mutant marker genes involved using heterokaryon tests.

Although the progeny cultures placed within any one group were similar, they still exhibited some variation. The variation was greater in the progeny from some heterokaryons than from others, e.g. progeny from 68 G_1 3×106 G_1 2, two isolates from England, showed more variation than progeny of 69 G_4 39×106 G_1 10, isolates from Australia and England respectively.

The number of progeny in each of the four groups from each heterokaryon were not always in a 1 : 1 : 1 : 1 ratio. For example, heterokaryon 68 G_1 4×69 G_4 51 produced a very high proportion of 68–4 types suggesting a high percentage of diploidization of 68–4 nuclei in basidia, which may be the result of preferential selfing, or an unequal nuclear ratio in the stable heterokaryon. Yet, because 68 G_1 4 mutant culture is apparently self-sterile, these results suggest that heterokaryosis is necessary to trigger fruiting even if two genetically identical nuclei subsequently take part in karyogamy.

Sectors appeared at low frequency in single-spore cultures and may represent reversion of a mutant nucleus. Dissociation of nuclei, in a thallus originating from a binucleate spore, could not account for these sectors, since complementation would presumably operate from the time of germination of such spores.

Some of the macerations between the original mutants produced cultures unlike either of the mutants involved or the wild type. Their growth rate was faster than that of either mutant but not as fast as in the wild type, nor was their cultural appearance typical of wild type. For convenience these cultures were designated "intermediate". As hyphal tip cultures maintained this intermediate growth rate and cultural morphology it seemed probable that heterokaryosis had occurred. However, attempts to induce the perfect state in these cultures failed.

Macerations which did not produce either wild-type or intermediate growth usually showed each of the two mutants growing independently or one mutant dominating the plate. In all these cases the original macerated pulp was transferred to new P.V.D.A. plates and from some of these, wild-type growth was produced. Parmeter (personal communication) has demonstrated that violent incompatibility reactions between two mutants macerated in this way can prevent heterokaryon growth even after anastomosis and nuclear transfer has occurred. Removal of the plug from the site of extensive cell death to a new agar plate occasionally enables a newly formed heterokaryon to grow away and become established.

Attempts were made to analyse the results regarding heterokaryon formation set out in Table 2, in terms of a bipolar compatibility system as suggested by Garza-Chapa and Anderson (1966) in their work, but no overall pattern could be established.

However, results of intra-isolate heterokaryon tests between the six G_1 mutants of 68 do fit such a compatibility system with the G_2 mutant (68 G_2 5) fitting also if intermediate growth is considered as a positive heterokaryon. But this is the only group where such a compatibility system could be considered from these results. Results of mutant pairings in all other groups (both intra- and inter-isolate crosses between mutants of similar or differing generations) do not fit a bipolar compatibility system. This is consistent with earlier heterokaryon tests with six G_4 progeny of 48, discussed in a previous paper (McKenzie *et al.* 1969), three of which are included in these present studies.

(b) Anastomosis Studies between Mutant Progeny of Different Field Isolates

Anastomosis reactions between pairs of mutants opposed on cellophane overlying agar were studied in representative crosses from each of the six combinations of field isolates to determine compatibility [i.e. whether successful (S), wall fusion (W.F.), or killing (K) reactions—(Flentje and Stretton 1964)]. The anastomosis reactions varied in degree of success, but in 9 of the 12 pairings there was evidence of incompatibility resulting in repulsion or cell death or both, even though all these pairings gave

TABLE 3

ANASTOMOSIS REACTIONS AND HETEROKARYON FORMATION ON CELLOPHANE OVERLYING AGAR, OF PAIRS OF MUTANTS DERIVED FROM DIFFERENT FIELD ISOLATES AND KNOWN FROM MACERATION TESTS TO BE CAPABLE OF HETEROKARYON FORMATION

Gene pools	Mutant pairs	Anastomosis reaction	Heterokaryon formation
48 × 68	48–13 × 68–4	Repulsion, fusion, no killing†	−
	48–13 × 68–6	Fusion, line of killed cells*	+
48 × 69	48–13 × 69–51	Repulsion, fusion, some killing*	+
	48–25 × 69–51	Repulsion	−
48 × 106	48–25 × 106–1	Fusion, some killing	−
	48–13 × 106–2	Some fusion, some killing	−
68 × 69	68–4 × 69–51	Fusion and killing	−
	68–6 × 69–6	Fusion, no obvious killing*	+
68 × 106	68–3 × 106–2	Fusion, no killing, apparent stimulation of 106–2†	−
	68–4 × 106–2	Fusion, some killing	−
69 × 106	69–39 × 106–10	Attachment, fusion	−
	69–51 × 106–10	Attachment, killing	−

* See Figure 2. † See Figure 3.

positive heterokaryons by the maceration technique. This confirms earlier evidence that cell death does not necessarily preclude heterokaryon formation (McKenzie *et al.* 1969). The anastomosis tests are summarized in Table 3. Out of 12 anastomosis pairings studied, only three produced heterokaryons from the areas of anastomosis at the junction of the two mutant cultures (Fig. 2).

On no occasion were "tufts" observed at the line of interaction between pairs of mutants as described by Whitney and Parmeter (1963) and Garza-Chapa and

Anderson (1966) in their anastomosis studies, and as observed in this laboratory with *T. cucumeris* "praticola" type isolates (Stretton and Flentje, unpublished data).

(*c*) *Pathogenicity Reactions of Heterokaryons on Cruciferae Stems*

Pathogenic reactions of the mutants on crucifer stems are described in Table 1. Whenever two non-pathogenic mutants (i.e. those showing reactions I → V inclusive) produced a heterokaryon, the resulting wild-type culture was pathogenic showing that complementation had restored the ability to attack crucifer stems.

(*d*) *Fruiting Ability of Heterokaryons*

All wild-type heterokaryons which were tested for fruiting produced hymenia and basidiospores, demonstrating that fruiting ability had been restored when a heterokaryon was produced from two self-sterile mutants.

IV. Discussion

The above studies demonstrate that inter-isolate heterokaryons can be formed between mutant progeny of different field isolates.

The four field isolates used were similar in pathogenicity, being specific to Cruciferae among the hosts tested, and exhibiting the same infection process. The fact that the heterokaryons resulting from pairs of non-pathogenic mutants and all the wild-type progeny of these heterokaryons were similarly pathogenic to Cruciferae with no evidence of segregation regarding pathogenicity, indicates a high degree of genetic similarity between all four field isolates with respect to this character. This may be expected as the host is the same, irrespective of the geographic origin of the isolate. Complementation for fruiting behaviour was also observed in these heterokaryons.

However, the four field isolates were dissimilar with regard to cultural characteristics. The heterokaryons, although all stable, differed culturally, and the wild-type progeny of these heterokaryons differed in cultural appearance with evidence of segregation for such characters as colour, zonation, and sclerotial production. This segregation indicates genetic dissimilarity with respect to these characters. These cultural differences do not reflect pathogenic differences (since these are lacking) but they may reflect different saprophytic abilities. The conditions for saprophytic growth and survival may well differ in different soils, thus exercising different selection pressures on the isolate. This hypothesis is supported by the fact that the Australian isolates showed different survival abilities when tested in Urrbrae loam (Stretton and Flentje, unpublished data).

Cultural and other differences between the four isolates used, e.g. diversity of origin in terms of geographic locality and soil type, fertility, and survival ability (Stretton and Flentje, unpublished data), though not necessarily preventing heterokaryosis may influence the frequency of it. In this regard, we suggest that the intermediate-type cultures resulting from some pairs of mutants may be the result of genetic differences between isolates, giving a vegetative incompatibility such that growth rate and cultural appearance of the heterokaryon is affected. However, there

are other possible explanations such as incomplete complementation, but evidence so far does not favour any one possibility.

Detailed studies of anastomosis between cultures derived from different isolates show varying degrees of repulsion and subsequent killing. Previous studies (Flentje and Stretton 1964), however, have shown that these variations occur also between G_1 progeny from one isolate, and in the present studies 106–8 with 106–10 for example results in a killing reaction, preventing in this case a heterokaryon from forming. Incompatibility is therefore not necessarily dependent on different geographic origin of isolates. But when identical progeny from a homokaryotic isolate are paired, no incompatibility is observed. It would seem then that genetic differences are responsible for incompatibility reactions. The extent of these differences, however, is not known. The frequency and severity of the reaction appears to increase with widening genetic differences between the two cultures being opposed, but there is no clear pattern of the genetic factors involved.

Other workers (Whitney and Parmeter 1963; Garza-Chapa and Anderson 1966) have suggested a simple genetic control of compatibility leading to heterokaryon formation on the basis of a bipolar compatibility system but these workers were using isolates from anastomosis group 1 where the tuft phenomenon operates also. Our results do not appear to fit a bipolar system but instead support the hypothesis of an inbreeding system operating, as do previous results (McKenzie *et al.* 1969) in which six mutant progeny from one homokaryotic isolate, when paired in all possible combinations, produced heterokaryons from every pairing.

In the present studies, 9 out of 12 pairings made on cellophane overlying agar did not yield heterokaryons, but attachment was achieved in every pairing but one, followed in almost every case by some cell death. Yet when these same pairs were macerated, all twelve pairs yielded heterokaryons, a phenomenon that was common with intra-isolate heterokaryon studies also. Thus the method of opposing isolates is important, as any one method alone may not yield the total possible number of heterokaryons that can be produced between isolates.

Earlier studies (McKenzie *et al.* 1969) on heterokaryosis involved progeny of one isolate, differing by a single gene or single gene block only. The present investigations show that heterokaryosis is also possible between progeny of different isolates which, in these studies, were pathogenic specifically to the same host. Therefore, the role of heterokaryosis in the biology of this organism may be to preserve by outbreeding the genetic factors which influence saprophytic growth, survival, virulence, and fruiting ability. On the other hand, the extent of the outbreeding may be limited by the killing reaction which at least in some instances appears to prevent heterokaryosis. Evaluation of this can only be made after much more thorough investigation of the nature and extent of the killing reaction.

V. Acknowledgments

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