GENETICS AND ANTIBIOTIC PRODUCTION OF EMERICELLOPSIS SPECIES[1]

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THE genus Emericellopsis, comprising five species and one variety, belongs in the order Eurotiales. All species have a perfect or cleistothecial stage, an imperfect (Cephalosporium) stage, and all are homothallic. A comparison of the various species has recently been published (DURRELL 1959). All members of the genus have been reported to produce the antibiotic substance synnematin B, a penicillin (GROSKLAGS and SWIFT 1957; KAVANAGH, TUNIN and WILD 1958). Only two species, *E. salmosynnemata* and *E. terricola* var. *glabra*, however, are reasonably good producers of the antibiotic, the remaining species showing weaker activity. Synnematin B has also been described as cephalosporin N in England; the two substances have been shown to be identical (ABRAHAM *et al.* 1955). Chemical analysis has shown synnematin B to be essentially a penicillin with an α-amino adipic acid side chain (ABRAHAM *et al.* 1953; NEWTON and ABRAHAM 1954; ABRAHAM, NEWTON and HALE 1954).

As pointed out by RAPER (1959), the application of the breeding techniques used by geneticists in producing better varieties in higher plants have frequently been looked upon with great interest by microbiologists engaged in improving microbial strains of economic and industrial significance. This type of approach has been barred to them by the absence of a sexual stage in the penicillia, aspergilli, and actinomycetes commonly employed in industrial fermentations. However, following ROPER's description (1952) of a technique for the production of strains with heterozygous diploid nuclei in filamentous fungi, PONTECORVO (1952, 1953) and PONTECORVO and ROPER (1952) have demonstrated a system by which recombinant types could be obtained in fungi lacking a sexual stage. In another paper, PONTECORVO, ROPER and FORBES (1953) suggested the practical application of somatic recombination to industrial fermentations.

The genus Emericellopsis, an antibiotic producer with both an imperfect and a sexual stage, offers an opportunity for investigating the effects of the application of heterokaryosis, diploidy, mitotic recombination, and meiotic recombination on antibiotic yields.

On the other hand, the genus is far from ideal for a thorough investigation of

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the above genetic facets. The fungus is homothallic and sexual recombination appears to occur only rarely. Conidiation also is rather poor, making it difficult to detect somatic recombinants which normally have a low order of incidence.

In general, however, the results obtained are in agreement with the information reported on heterokaryosis and somatic recombination and present new and unreported data on the effect of sexual crosses on antibiotic yields.

MATERIALS AND METHODS

Essentially two Emericellopsis species, supplied by J. H. GROSKLAGS of the University of Wisconsin, were used in these studies: *E. salmosynnemata* and *E. terricola* var. *glabra*. For the interspecific crosses, the following, supplied by L. W. DURRELL of Colorado State University, were also employed: *E. terricola* (Colorado isolate), *E. terricola* (Indiana isolate), and *E. mirabilis*. The characteristics of the latter cultures have been described by DURRELL (1959).

Mutants: The morphological and physiological mutants employed in these experiments were obtained by ultraviolet irradiation of conidia, followed by the concentration technique of FRIES (1947). The identification of physiological mutants was accomplished by employing the auxanographic method of PONTECORVO (1949). All auxotrophs were found to be quite stable following a two-step isolation of single conidia, and no revertants were recovered from any platings where viable counts averaged 10^6 conidia/ml. Except for a purineless mutant, all auxotrophs obtained had an amino acid requirement.

The nutritional mutants used in these experiments are designated by the conventional symbols and will be fully described when mentioned for the first time; the number following the requirement refers to separate mutational origin.

Cytology: A few cytological preparations were made in order to determine the number of nuclei in mycelial cells and in the conidia of *E. salmosynnemata* and *E. terricola* var. *glabra*. The Azure A-Eosin y staining technique of D. KLEIN (private communication cited in CARR and OLIVE 1958) was used with moderate success. Some difficulty was encountered because of the small size of hyphae and nuclei. The mature hyphal cells were found to contain from three to five nuclei, while the conidia were uninucleate. This suggested, cytologically at least, that heterokaryons could be formed; the observation of occasional hyphal anastomosis supported this view further.

Media—defined medium: A series of previously described defined media were investigated, such as those of WESTERGAARD and MITCHELL (1947), PISANO, OLSON and SAN CLEMENTE (1954), PONTECORVO (1953), and CZAPEK-DOX (1910). PONTECORVO's defined medium was found to be quite satisfactory for Emericellopsis and was used routinely with two percent Difco Bacto-agar or Difco Special Agar (Noble). This medium will be designated hereafter as Pt^-.

As reported by others (BHUYAN and JOHNSON 1958), *E. terricola* var. *glabra* was found to have a requirement for biotin, while *E. salmosynnemata* is independent of this requirement.

Complete medium: The defined medium of PONTECORVO was used, supple-

mented with 0.25% casamino acids and 0.25% yeast extract. This will be designated as Pt^+.

Conidiation medium: Originally honey-peptone was employed as described by GROSKLAGS and SWIFT (1957); however, since it was later found that one of the lysineless auxotrophs grew very poorly on this medium, Pt^+ was used routinely for this purpose also.

Conidiation by *E. terricola* var. *glabra* was found to be so poor that no somatic recombination experiments were attempted with it.

Medium for production of fruiting bodies: As reported by GROSKLAGS and SWIFT (1957), corn meal agar supplemented with 0.1% yeast extract was found to be satisfactory for production of cleistothecia and for sexual crosses. Mature ascospores are produced on this medium after a period of 2–3 weeks of incubation at 28°C by both *E. salmosynnemata* and *E. terricola* var. *glabra*, the latter usually fruiting after the shorter period of time.

Fermentation media: Both defined and complex media reported by BHUYAN and JOHNSON (1958), NARA and JOHNSON (1959), HARVEY and OLSON (1958), and OLSON, JENNINGS, PISANO and JUNEK (1954) were investigated for antibiotic production. Under our rather limited fermentation facilities, the results obtained were reasonably comparable. The medium of NARA and JOHNSON (1959) was chosen and was used routinely for antibiotic production in these studies. The yields obtained from repeat runs were found to be quite consistent, and this was satisfactory for the purposes of this investigation. At no time under the fermentation conditions available in our laboratory, were the higher yields reported by the Wisconsin group achieved (cottonseed oil was not added to our medium, however).

Fermentations were run at 28° to 30°C on a Brunswick gyrotory shaker, at 200 r.p.m.

Synnematin assay: Antibiotic yields were assayed by a slightly modified SCHMIDT and MOYER (1944) technique, using agar wells rather than porous cups, all other conditions, however, being the same. The test organism was a sensitive strain of *Bacillus subtilis*, and a synnematin preparation supplied by the Lilly Research Laboratories was used as the standard. Since the purity of the preparation was low, the synnematin unit in milligrams was determined for the standard against *Salmonella typhimurium* (GOTTSHALL, ROBERTS, PORTWOOD and JENNINGS 1951).

Sexual crosses: Mycelial inocula of appropriate genotypes were placed approximately one inch apart on corn meal agar plates. After an appropriate period of incubation, single cleistothecia formed at the line of contact between the two parental strains were removed and single spores dissected out either by means of a De Fonbrune micromanipulator or by free-hand breaking of the cleistothecium. In the latter case, the cleistothecium, resting on the surface of a small cover-slip fragment, was broken with a fine glass needle; addition of a few drops of sterile water and spreading with a glass rod was successful in producing well dispersed colonies.

It should be mentioned at this point that only in immature cleistothecia are the

asci discernible as discrete round structures containing eight ascospores. As the cleistothecia and ascospores become fully mature, the ascus walls break down, releasing the spores within the cleistothecial cavity. Microscopic examination of the fruiting body at this time will reveal a mass of loose spores within the cleistothecium and no asci. Spore dissections were done on Pt^+ medium, one cleistothecium per plate, and colonies appearing after four days of incubation were analyzed for recombination on suitably supplemented defined media. Conidial contamination did not prove to be a problem, as demonstrated by the overwhelming majority of plates showing colonies of only one of either parental genotype from any one cleistothecium.

Parasexual crosses: The techniques employed here have been described at length by several investigators (PONTECORVO 1953; ROPER and PRITCHARD 1955; ISHITANI, IKEDA and SAKAGUCHI 1956; SERMONTI 1959). Briefly, the process consists of mixing and plating auxotrophs of suitable genotypes on defined media, and analyzing any resulting prototrophs to determine whether they may be heterokaryons, heterozygous diploids, somatic recombinants, or revertants.

EXPERIMENTAL RESULTS

Heterokaryosis: Preliminary experiments designed to synthesize heterokaryons in *E. salmosynnemata* showed that the heterokaryotic condition is not easily achieved in this organism. With mycelial inocula of two different auxotrophs on unsupplemented defined media, results have been either negative or of doubtful value. Conclusive proof of heterokaryosis was obtained, however, by employing germinating conidia on defined medium. All of these experiments were run with *E. salmosynnemata.*

Filtered and washed conidia from two auxotrophs of *E. salmosynnemata* were first plated on defined and supplemented media, respectively, in order to determine the frequency of back mutation and the viable count. Viable counts as a rule approximated 10^6 conidia/ml; in none of these experiments were revertants recovered.

The two auxotrophic conidial suspensions were then combined and plated on defined medium. After 24–48 hours incubation, a thin layer of defined medium was added. Any heterokaryons that may be formed are capable of growing through the second layer of medium; but this is not so with any residual growth of the auxotrophs, and isolation of the former is then facilitated. Conidia obtained from hyphal tip isolations from the heterokaryons were then plated on suitable media and their genotype determined.

The uninucleate condition of the conidia was found to be a useful tool in these experiments, since the heterokaryotic state cannot be carried on through conidial platings. Conidia from presumptive heterokaryons then will normally give rise to colonies belonging to the parental genotypes which had been used in synthesizing the heterokaryon. Recovery of other than the two original genotypes employed would suggest possible revertants, diploids, or somatic recombinants. These can usually be distinguished by further plating on suitable media.

From a conidial mixture of the auxotrophs *aspg⁻–1 me⁺–1* (asparagine requiring) and *aspg⁺ –1 me⁻–1* (methionine requiring), 23 prototrophic colonies were obtained from a total of 24 plates used. Hyphal tips from these colonies were tested on defined media and only two appeared to be balanced heterokaryons. Questionable growth by the other 21 may have been due to unbalanced nuclear ratios, or to cross-feeding by the component genotypes after heterokaryon breakdown. Following a suitable period of incubation of the balanced heterokaryons, conidia were harvested, washed, and plated on defined medium, defined plus asparagine, defined plus methionine, and defined plus both supplements. No growth was observed on any of the defined unsupplemented plates; the number of colonies appearing in the plates containing both supplements were about equal to the total number of colonies in the plates supplemented with asparagine plus the methionine supplemented ones. The number of methionineless to asparagineless nuclei was in the ratio of 4.7 to 1.

A fermentation was run with the two balanced heterokaryons, and antibiotic yields were compared with those of the parent auxotrophs, as well as with those of the original wild-type strain (average from eight fermentations 625 units/ml). In a first fermentation, both heterokaryons yielded 500 units of the antibiotic per milliliter, while the parental auxotrophs showed no antibacterial activity. A second fermentation run a week later gave 30 and 75 units/ml for the two heterokaryons, respectively, and again nothing for the parents.

This would suggest that the auxotrophs which made up the heterokaryons were segregating out with time, resulting in a decrease in antibacterial activity. The remaining 21 unbalanced heterokaryons exhibited yields which ranged from 0 to 300 units/ml when first tested. Similar results have been reported by YUILL (1953) for members of the *Aspergillus niger* group.

Comparable data were observed in balanced and unbalanced heterokaryons obtained by using the auxotrophs *lys⁺–2 me⁻–1* (methionine requirement) and *lys⁻–2 me⁺–1* (lysine requirement). Of 22 potential heterokaryons isolated, four proved to be balanced heterokaryons. Antibiotic yields by the entire group of isolates ranged from zero to 400 units/ml, the parentals being a nonproducer and a low producer (75 units/ml).

In all cases, plating of conidia from presumptive heterokaryons resulted in the recovery of the parental genotypes. This of course would be expected in view of the uninucleate condition of the conidia.

From the above results and from similar observations by other investigators (SERMONTI 1956, 1959; CIEGLER and RAPER 1957) it may be concluded that a practical application of heterokaryosis to fermentation may be of only limited value. Its potential usefulness, however, should not be disregarded if newly synthesized balanced heterokaryons can readily be made available for each fermentation.

On the other hand, the variations in antibiotic yields described above for heterokaryons have never been observed in any of the recombinants obtained from sexual crosses.

It was observed during the course of these experiments that heterokaryons

formed by an *al–4 lys⁻–1 me⁺–1* and *al⁺–4 lys⁺–1 me⁻–1* would show a predominantly white color when grown on a lysine supplemented medium and a predominantly salmon color (wild-type pigmentation) when grown on a methionine supplemented medium. The color of these heterokaryons on Pt^- and Pt^+ media was essentially a light tan. This would suggest that the single supplements gave a selective advantage to the nuclei with the particular requirement, and that this was expressed by the pigmentation of the colony.

Diploidy and somatic recombination: The above studies with heterokaryosis had actually been designed for the primary purpose of obtaining heterozygous diploids. Several different combinations of auxotrophs were tried in an attempt to synthesize diploids. Treatments with camphor (ROPER 1952) or ultraviolet light (ISHITANI *et al.* 1956) proved to be inconclusive when used with this organism. In addition it would have proved more desirable to employ mutants with several markers. Obtaining doubly auxotrophic mutants of *Emericellopsis salmosynnemata*, however, proved to be difficult, as will be explained later.

What appeared to be a heterozygous diploid was finally obtained from one combination with the use of the auxotrophs *al–4 lys⁻–1 me⁺–1* (albino, lysine requiring) and *al⁺–4 lys⁺–1 me⁻–1* (wild-type color, methionine requiring). Plating of conidia from a heterokaryon synthesized from the above auxotrophs, produced an albino prototrophic colony. When conidia from the latter were then plated on suitable media, the following genotypes were recovered: *al⁺–4 lys⁺–1 me⁺–1*, *al⁺–4 lys⁺–1 me⁻–1*, *al–4 lys⁺–1 me⁺–1*, *al–4 lys⁻–1 me⁺–1*. In addition, 17 of 25 albino prototrophic colonies (68%) were observed to sector to *al⁺* after about four weeks of incubation on Pt^- medium. Since the conidia are uninucleate, a high frequency of sectoring such as this can be explained only by a diploid condition of the albino whose genotype must then be $\frac{al–4\ lys^-–1\ me^+–1}{al^+–4\ lys^+–1\ me^-–1}$. Albino then appears to be epistatic to *al⁺*.

The high frequency of sectoring plus the recovery of *al* and *al⁺* prototrophs is strongly suggestive of diploidy and mitotic recombination in this organism.

Isolation of an *al–4 lys⁺–1 me⁻–1* and/or *al⁺–4 lys⁻–1 me⁺–1* colony would make the occurrence of the parasexual cycle in *E. salmosynnemata* much more convincing. Since only a small number of markers is involved, however, the nonrecovery of these recombinant types should not disprove the above inference that the *al* and *al⁺* prototrophs recovered are recombinants from a heterozygous diploid.

Any attempt to explain the genotypes recovered in terms of back mutations would have to imply concurrent reversions for too many factors. This would seem to be very unlikely in view of a back mutation frequency of less than 10^{-6} for the mutants employed in this experiment, particularly when compared to the high frequency of sectoring (68%) observed in the presumptive diploid colonies.

Fermentation studies were carried out with the diploid and the various recombinants obtained from it. When first fermentation-tested, the diploid produced a zone of inhibition of 8 mm; subsequent testing of diploids obtained from conidial plating of the original diploid gave zones of 6 mm. While an improve-

ment in antibiotic yields over that of the auxotrophs used in synthesizing the diploid was obtained, the higher yields (except for the original diploid) were not as good as those of the wild-type strain (625 units/ml; average of eight fermentations). Table 1 summarizes the fermentation results.

Sexual crosses: Since the fungus is homothallic, no ready criterion is available for detecting whether a cleistothecium is the result of cross-fertilization or self-fertilization. In view of the advantages shown by spore color mutants for the detection of hybrid perithecia in Sordaria (OLIVE 1954, 1956), all colonies recovered from UV treatments of conidial suspensions were examined with this end in mind. While no spore color mutants were found, one asparagine-requiring mutant of *E. terricola* var. *glabra* was recovered which possesses peanut-shaped ascospores (*aspg⁻–1 p*). The wild-type ascospores are elliptical and have typical longitudinal wings or flanges (3 to 5) which seem to extend around the spore. The spores measure 7.5–8.5 μ in length and 4–5 μ in width, and have an olive to brown color.

A cross on corn meal agar between *aspg⁻–1 p* and the wild-type strain produced mostly nonhybrid cleistothecia. A small number of fruiting bodies, however, were observed which possessed both *p* (peanut-shaped) and p^+ (wild-type ascospores) as shown in Figure 1. This of course was not evidence that sexual recombination had occurred, but was at least suggestive that the potential for recombination did exist in this homothallic fungus.

A series of crosses was then made, using both morphological and biochemical markers, for the purpose of demonstrating sexual recombination. Two species were employed for most of these studies: *E. salmosynnemata* and *E. terricola* var. *glabra*. A few interspecific crosses were also tried; the results will be reported separately for each species.

While the induction and identification of mutants by the technique described

TABLE 1

Antibiotic yields of diploid $\frac{\text{al–4 lys}^-\text{–1 me}^+\text{–1}}{\text{al}^+\text{–4 lys}^+\text{–1 me}^-\text{–1}}$ *and somatic recombinants*

Genotype	No. fermented	Radius of inhibition (mm)	Synnematin units/ml	Type
al–4 lys⁻–1 me⁺–1	1	3.5	75	Parental
al⁺–4 lys⁺–1 me⁻–1	1	3.5	75	Parental
al–4 lys⁻–1 me⁺–1	1	8	800	Diploid
al⁺–4 lys⁺–1 me⁻–1				
al–4 lys⁺–1 me⁺–1	5	0	0	Recombinant
*al–4 lys⁺–1 me⁺–1 f**	1	2	30	Recombinant
al⁺–4 lys⁺–1 me⁻–1	7	6	400	Recombinant
al⁺–4 lys⁺–1 me⁺–1	1	6	400	Recombinant
*al⁺–4 lys⁺–1 me⁺–1 f**	1	0	0	Recombinant
al–4 lys⁻–1 me⁺–1†	1	6	400	Diploid
al⁺–4 lys⁺–1 me⁻–1				

* *f* flat mycelium; both original auxotrophs had fluffy (*f⁺*) aerial mycelium.
† diploid obtained from conidial plating of original diploid.

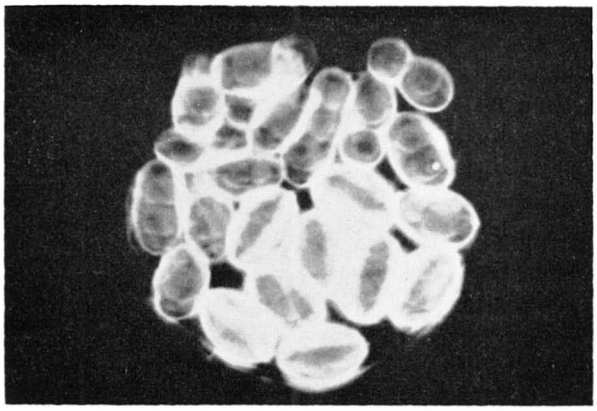

FIGURE 1.—Peanut and wild-type ascospores in a cleistothecium of *Emericellopsis terricola* var. *glabra*.

under Materials and Methods presented no great difficulties, the two following problems were encountered: (1) introduction of a biochemical requirement resulted in most cases in a lowering or complete absence of antibiotic yields; and (2) irradiation of a biochemical mutant for the purpose of obtaining a double auxotroph has resulted in the induction of 25% dwarf mutants among the survivors, and no double auxotrophs were recovered. A few morphological markers were added in this manner, however.

E. salmosynnemata *sexual crosses:* Of a total of 98 cleistothecia in which single-spore analysis was performed, only nine showed conclusive recombinant types; this gives an incidence of 9.2% crossed cleistothecia. Analysis of colonies resulting from single-spore isolates from individual fruiting bodies was performed on suitable media. In most cases all of the expected products of meiosis were not recovered.

From a cross between *al–2 aspg*$^{+}$*–1 r* (albino with restricted growth, antibiotic yield 675 units/ml) and *al*$^{+}$*–2 aspg*$^{-}$*–1 r*$^{+}$ (wild-type color, asparagine requiring, nonrestricted growth, antibiotic yield zero units/ml), five *al*$^{+}$ unrestricted prototrophs and three *al aspg*$^{+}$ restricted colonies were recovered. Antibiotic yields by the prototrophic recombinants were quite low (75 units/ml). Obviously, evidence for meiotic recombination from the above cross is not very convincing since only one recombinant type was recovered. A total of 15 other cleistothecia from this same cross were analyzed, but no further evidence of cross-fertilization was obtained.

In another cross, 21 cleistothecia were analyzed; the parental genotypes involved were *al–2 lys*$^{+}$*–1 r* (albino with restricted growth) and *al*$^{+}$*–2 lys*$^{-}$*–1 r*$^{+}$ (wild-type color, lysine requiring, normal growth). In this case, four hybrid cleistothecia were found, and the evidence for meiotic recombination was more conclusive. Table 2 shows the genotypes obtained, and their antibiotic yields. The parental genotype *al–2 lys*$^{+}$*–1 r* recovered from the mating showed no antibacterial activity; since the parental strain of the same genotype was a good

TABLE 2

Progeny from two hybrid cleistothecia resulting from cross al–2 lys$^+$–1 r *(600 units/ml)** × al$^+$–2 lys$^-$–1 r$^+$ *(0 units/ml)**

Genotype	No. recovered	Radius of inhibition (mm)	Synnematin (units/ml)
al–2 lys$^-$–1 r	13	6.5	500
al–2 lys$^-$–1 r$^+$	6	3.5	75
al–2 lys$^+$–1 r$^+$	4	5	200
al–2 lys$^+$–1 r	1	0	0
al$^+$–2 lys$^+$–1 r$^+$	6	3.5	75
al$^+$–2 lys$^+$–1 r	1	0	0
al$^+$–2 lys$^-$–1 r$^+$	5	2.5	40
al$^+$–2 lys$^-$–1 r	5	2.5	40

* Antibiotic yield of parental auxotroph.

producer, this would suggest recombination of unselected markers resulting in a complete loss of antibiotic-producing ability. On the other hand, the lysineless *al–2 lys$^-$–1 r* gave 500 units/ml. As a whole, however, antibiotic yields by recombinants covered the general range between the zero and 600 units/ml of the parental strains.

More interesting and more convincing evidence of sexual recombination in *E. salmosynnemata* was obtained from the cross *al–4 lys$^-$–1 ser$^+$ f$^+$* (albino, lysine requiring mutant with fluffy mycelium) and *al$^+$–4 lys$^+$–1 ser$^-$ f* (wild-type color, serine requiring mutant with flat mycelium) (FANTINI and OLIVE 1960). Of six cleistothecia analyzed, three showed recombinant types for one or more of the markers involved. In addition to the fact that unusually high numbers of hybrid cleistothecia were recovered (50%), this cross proved to be interesting in that twelve recombinant types were identified. Unfortunately, because of the low antibiotic activity of both parent strains, a more critical evaluation of the effect of recombination on yields was not possible. An examination of the recombinant types obtained suggests that genetic reconstitution to a prototrophic state by meiotic recombination is not sufficient to establish an antibiotic-yielding capacity comparable to that of the wild-type strain (average zone of inhibition 7 mm). The results from this particular cross indicate that possibly several factors active in the synthesis of the antibiotic may be linked to the chromosome carrying the gene for fluffy mycelium (*f$^+$*); when other factors are brought together in a prototroph with *f$^+$*, the proper gene combination may be achieved and the result is antibiotic production. Mycelial color appears to have no effect on antibiotic yields.

Comparable results were obtained from a cross between two other low antibiotic-producing auxotrophs. The mutants used were *al–4 lys$^-$–1 me$^+$–1* and *al$^+$–4 lys$^+$–1 me$^-$–1*. In this case, all prototrophic recombinants produced antibiotic yields higher than the parental strains; however, both parents had fluffy (*f$^+$*) mycelia. As expected, no flat (*f*) recombinants were recovered. Once again mycelial color seemed to have no effect on yields of the prototrophs. The results are summarized in Table 3.

TABLE 3

Progeny from a hybrid cleistothecium resulting from cross
al–4 lys$^-$–1 me$^+$–1 *(75 units/ml)** × al$^+$–4 lys$^+$–1 me$^-$–1 *(40 units/ml)**

Genotype	No. recovered	Radius of inhibition (mm)	Synnematin (units/ml)
al–4 lys$^-$–1 me$^+$–1	3	3	50
al–4 lys$^-$–1 me$^-$–1	4	0	0
al–4 lys$^+$–1 me$^+$–1	1	6	400
al$^+$–4 lys$^+$–1 me$^-$–1	4	0–2	0–30
al$^+$–4 lys$^-$–1 me$^+$–1	4	2	30
al$^+$–4 lys$^-$–1 me$^-$–1	3	0	0
al$^+$–4 lys$^+$–1 me$^+$–1	1	5.5	300

* Antibiotic yield of parental auxotroph.

A similar cross involving *al$^+$–4 lys$^+$–1 me$^-$–2* as one of the parents produced what appeared to be recombinant types; however, in view of the poor growth of the above methionineless mutant on supplemented media, a clear-cut interpretation of the recovered genotypes could not be made.

A few attempts were made to increase the antibiotic-yielding capacity of some of the auxotrophs by crossing with a high-yielding dwarf mutant. Recovery of recombinant types, however, was unsuccessful. Since the increase of productivity was not the primary purpose of this problem, experiments along this line were not pursued further.

An analysis of single-spore isolates from several different matings showed no other recombinant types.

Mutant S 66, *al–1 aspg$^-$–2 st* (albino, asparagine-requiring, sterile), was recovered from one of the early irradiation treatments. When crossed with other auxotrophs in the usual manner, the latter generally showed stunted development, and a line almost lacking in mycelial growth formed between the two colonies. Since this appeared to be an inhibition, the filtrate from a fermentation of S 66 was assayed against *B. subtilis* and against germinating ascospores of the fungus *Sordaria fimicola*. Activity against the bacterium was negative, but a clear zone of inhibition was obtained against the fungus. This experiment was repeated several times, and in addition other auxtrophs were tested against *S. fimicola*. None of the other mutants, however, showed activity against the fungus, but S 66 gave an activity of 9 to 10 mm (radius) against the fungus and no antibacterial effect.

Further attempts were then made to cross S 66 with other auxotrophs, and to obtain heterokaryons. All proved to be unsuccessful, except for a questionable heterokaryon which showed a slight radius of inhibition (3.5 mm) against *S. fimicola*.

While the colonial morphology of S 66 differs from that of the wild-type *E. salmosynnemata*, there is no doubt that it is a mutant of this species, since a few fruiting bodies containing typical ascospores of the species were produced on a medium containing soya bean extract.

It should prove interesting to determine whether the chemical nature of the substance produced by S 66 is that of a penicillin (as is synnematin B), or whether a new compound is involved.

E. terricola *var.* glabra *sexual crosses*: Attempts to obtain recombinants by sexual crosses in this species were on the whole rather unsuccessful. Of 152 cleistothecia analyzed, only two showed recombinant types.

In one of the crosses, involving the mutants *leu⁻ aspg⁺–2 st* (leucine requiring, sterile) and *leu⁺ aspg⁻–2 st⁺* (asparagine requiring, fertile), only two prototrophic recombinants were recovered. Their antibiotic activity, however, was better than that of the original wild-type strain (360 units/ml). The results are summarized in Table 4. Nonrecovery of a recombinant type with double requirement is probably due to lack of viability or formation of abortive spores.

Interesting data were obtained from the cross *aspg⁻–1 ad⁺ p st⁺* (asparagine requiring, peanut-shaped ascospores, fertile) by *aspg⁺–1 ad⁻ p⁺ st* (adenine requiring, sterile). Of 41 cleistothecia analyzed only one gave rise to what appears to be a prototrophic recombinant type. Seven single ascospore isolates were obtained from this one cleistothecium. When tested on suitable media, all seven isolates proved to be prototrophic. In addition, all had a fluffy aerial mycelium, rather than flat as the parents, all fruited abundantly (*st⁺*), and all had normally shaped spores (*p⁺*). When fermented and assayed, zones (radius) of inhibition against *B. subtilis* ranged from 8 to 9 mm (785 units/ml), whereas the parents gave no activity whatsoever.

Since neither parentals nor other recombinant types were recovered from this cleistothecium, it may be unwise perhaps to conclude that these prototrophs are recombinant types. On the other hand, it does not seem plausible to explain them as revertants since several mutant genes are involved and it would assume back mutations for all factors in one or the other parent. This would seem unlikely, particularly since no revertants have ever been observed in either of the parental strains used in this cross. In addition, the change in antibiotic yield from zero to 785 units/ml would also have to be explained in terms of further mutation. The above prototrophs then, are probably not revertants.

As these prototrophs appeared to be generally more vigorous than the original wild-type strain, they were further investigated to determine whether they might be diploids. This was done by testing colonies from 64 single conidia and from 89 single ascospores on suitable media. In no case were auxotrophic or recom-

TABLE 4

Progeny from a hybrid cleistothecium resulting from cross
leu⁻ aspg⁺–2 st *(0 units/ml)** × leu⁺ aspg⁻–2 st⁺ *(0 units/ml)**

Genotype	No. recovered	Radius of inhibition (mm)	Synnematin (units/ml)
leu⁻ aspg⁺–2 st	2	0	0
leu⁺ aspg⁻–2 st⁺	7	0	0
leu⁺ aspg⁺–2 st⁺	2	8	785

* Antibiotic yield of parental auxotroph.

binant types recovered, other than the original prototrophic *aspg*$^+$*–1 ad*$^+$ *p*$^+$ *st*$^+$.

In attempting to explain the origin of this prototroph, some thought has been given to the possibility of a parasexual mechanism involving somatic karyogamy and recombination, followed by the incorporation of prototrophic recombinant nuclei in the formation of a fruiting body. This would produce the type of data described above, where no parental nor other recombinant types were recovered.

Results from several other different sexual crosses were completely negative and no further recombinant types were recovered for *E. terricola* var. *glabra.*

Attempts at interspecific sexual crosses: A total of 54 cleistothecia from interspecific matings were analyzed; examination of single-spore isolates from these cleistothecia revealed that in almost all instances the colonies obtained were of one species or the other. In only two instances were spores belonging to two species recovered from a single fruiting body. However, no evidence of meiotic recombination, at least for the selected markers employed, was obtained.

E. terricola var. *glabra* was paired with *E. terricola* (Colorado isolate), *E. terricola* (Indiana isolate), and *E. mirabilis.* The Colorado and Indiana isolates of *E. terricola* differ only slightly in pigmentation. Ascospore sizes of the two *E. terricola* isolates and *E. terricola* var. *glabra* do not differ significantly. This was considered as indicating close relationship and therefore an advantage in interspecific crossing. On the other hand the ascospores of *E. mirabilis* are about twice as large.

Four different auxotrophic mutants of *E. terricola* var. *glabra* were paired with wild-type *E. mirabilis.* All single-spore isolates from any single cleistothecium analyzed proved to be entirely of one or the other parental species, indicating that only selfing had occurred.

Mating of the Colorado isolate of *E. terricola* with *E. terricola* var. *glabra* gave essentially negative results. However, one cleistothecium was found to contain spores of both species, as determined by the morphology and physiology of the colonies obtained. Of the 19 colonies isolated from single spores of this cleistothecium, nine were *E. terricola* (Colorado) and ten *E. terricola* var. *glabra.* No recombination was observed, however, for the selected markers, nor was any change in antibiotic yields effected by the association.

A change in the antibiotic producing ability of *E. terricola* var. *glabra* (*aspg*$^-$*–1 p*) was obtained, however, by a cross of the latter with *E. terricola* (Indiana isolate). While still asparagineless, five colonies obtained from single spores of one of the cleistothecia, produced spherical rather than peanut-shaped ascospores. In addition, these colonies were now capable of producing some antibacterial activity (200 units/ml). The parental *aspg*$^-$*–1 p* used in this cross had never, in 11 separate fermentations, produced any antibiotic.

Fermentations were repeated several months later to determine whether this antibacterial activity was due to a meiotic recombination of unselected markers, or to a residual cytoplasmic effect, the latter finding expression in an improved antibiotic producing strain. Results from these later fermentations were essentially the same as those reported above. This would suggest that the original yields were probably due to meiotic recombination of unselected markers.

DURRELL (1959) suggests that possibly all Emericellopsis species fall into two main groups: *E. mirabilis* and *E. terricola*. All species described, other than *E. mirabilis*, he considers to belong to the species *E. terricola*. Evidence based on amino acid content of the mycelia of the two species would also tend to support this view (MAAG, DURRELL and PAYNE 1959).

With this in mind, recombination within the members of *E. terricola* may be expected to be a definite possibility. Actually, the recombinant types obtained from intraspecific crosses between mutants of *E. terricola* var. *glabra*, such as *leu*$^+$ *aspg*$^+$*–2 st*$^+$ and *aspg*$^+$*–1 ad*$^+$ *p*$^+$ *st*$^+$, resemble morphologically the Colorado and Indiana isolates of *E. terricola* more than they do the parental strains or wild type of their own species (i.e. *E. terricola* var. *glabra*). Further work along this line may well support DURRELL's concept (1959) of two known species, *mirabilis* and *terricola*, in the genus Emericellopsis.

DISCUSSION

While the application of mutagenic treatment and selection (BACKUS and STAUFFER 1955) has undoubtedly contributed a great deal to the improvement of antibiotic yields, it seems probable that the application of hybridization and recombination to this field may prove to be an invaluable complement. This would apply, perhaps not so much to the more obvious problem of yield improvement, but to that of modifying the chemical nature of the metabolic products under investigation. This would seem to be a definite possibility based on new gene combinations within recombinant types. Whether this is brought about by a sexual or parasexual system should not matter. It is felt, however, that the recombination potential by means of the parasexual cycle will probably be limited in comparison to that of organisms possessing a true sexual cycle (BONNER 1959). But, it should be kept in mind that the number of organisms investigated along these lines has also been limited.

A number of papers have appeared in recent years on the genetics of antibiotic-producing microorganisms (Ann. N. Y. Acad. Sci. Vol. 81 art. 4, 1959). Further interest in this field is rapidly gaining momentum, spurred by developments in the field of viral, bacterial, and fungus genetics achieved during the past decade.

LINDEGREN and ANDREWS (1945), in the early days of penicillin production, suggested the use of cytoplasmic hybrids for improvement of yields. Their results were not too encouraging, but further work with this approach by other investigators did produce some positive data. LINDEGREN and ANDREWS further suggested that a search for penicillia with a sexual stage may prove to be a worthwhile approach.

Fermentation studies by various investigators (BONNER 1947; YUILL 1953; CIEGLER and RAPER 1957; SERMONTI 1956) involving mixed cultures of mutants of penicillia or aspergilli have shown that in general antibiotic or organic acid yields from mixed cultures are very low. On the other hand, yields by heterokaryons (CAGLIOTI and SERMONTI 1956; SERMONTI 1956) show a good deal of variation, which appears to be dependent on the degree of segregation of the genotypes involved during the fermentation. Yields by balanced heterokaryons

are usually high, and SERMONTI finds them to be perfectly comparable to those of heterozygous diploids. Breakdown of these heterokaryons usually results in low yields.

Similar results have been obtained, as reported above, with heterokaryons synthesized from low-producing or nonproducing strains of *E. salmosynnemata*. A heterokaryon synthesized from two nonproducing auxotrophs gave 500 units/ml of the antibiotic. With time, however, segregation of component nuclei occurs within the heterokaryon and antibiotic yields drop to low levels (30 to 75 units/ml in this case).

While a practical application of heterokaryosis to industrial fermentations may not appear to be too promising, its feasibility should not be discounted in view of the results obtained with balanced heterokaryons.

A practical application of the parasexual cycle was made by SERMONTI (1956, 1959) using *Penicillium chrysogenum*, by ISHITANI *et al.* (1956), and by IKEDA, NAKAMURA, UCHIDA and ISHITANI (1957), working with *Aspergillus oryzae* and *A. sojae*. The results reported by these investigators have been essentially comparable. While yields of the metabolic products involved have not on the whole been much greater than those of the parental strains, an occasional improvement in yields was achieved. A similar approach is being applied to the actinomycetes (Ann. N. Y. Acad. Sci. Vol. 81 art. 4, 1959).

Regarding the parasexual cycle in *E. salmosynnemata*, the results from fermentation studies of a diploid and its recombinants show an increase in antibacterial activity for the diploid and for some of the recombinants over the yields of the parent auxotrophs. Additional work with heterozygous diploids of *E. salmosynnemata* is needed before any definite trends can be formulated. The data shown, however, are essentially in agreement with the results reported by other investigators.

While only a low percentage of crossed cleistothecia have been recovered, the results indicate clearly that the possibility for recombination by sexual means does exist in this homothallic fungus. Crosses of a high and a low antibiotic producer gave recombinants with intermediate antibiotic yield, while prototrophic recombinants from low producers were generally good producers. Unfortunately, the introduction of genetic markers has usually resulted in low-producing strains, hampering from the start any attempt for a stepwise increase in antibiotic yields.

None of the recombinants recovered from sexual crosses of *E. salmosynnemata* produced antibiotic yields higher than those of the original wild-type strain (625 units/ml); on the other hand, the few prototrophic recombinants of *E. terricola* var. *glabra* showed an improvement of about 50 percent.

Since in all probability many genes are involved in the synthesis of the antibiotic substance, it may prove to be quite difficult to achieve, either sexually or parasexually, the proper combination of advantageous genetic factors which will produce the desired recombinant type. In any case, the investigation of the effects of heterokaryosis, diploidy, mitotic recombination, and meiotic recombination on yields of a metabolic product by a microorganism continues to be a stimulating and promising field of research.

The recovery of two parental genotypes and no recombinants from the same cleistothecium, in the cross between *E. terricola* (Colorado) and a mutant of *E. terricola* var. *glabra* is difficult to explain. Recombinant spores may well have been formed, but because of differences in the chromosomal makeup of the two varieties involved in the cross, they possibly were not viable. A more plausible suggestion, perhaps, would be the involvement of two separate ascogonia in the formation of a single cleistothecium, each ascogonium carrying only nuclei of one of the two species, respectively. This would produce a fruiting body with spores of two parental genotypes and no recombinant types, since cross-karyogamy has not occurred.

As previously mentioned, DURRELL (1959) indicates that in the genus Emericellopsis there are two distinguishable species, *mirabilis* and *terricola*. While it has not been the purpose of this investigation to pursue taxonomic relationships in the genus, some of the observations made in the course of this study tend to support DURRELL's view. Morphological changes in pigmentation, production or absence of synnemata, aerial or flat mycelium, were produced repeatedly by irradiation, and any attempt to assign these mutants to *E. salmosynnemata* or *E. terricola* var. *glabra* would have been difficult indeed. Also, some of the recombinant types of *E. salmosynnemata* recovered from sexual crosses were found to be very similar to some of the *E. terricola* var. *glabra* strains, and recombinants of the latter were morphologically more like the *E. terricola* Colorado and Indiana isolates of DURRELL. This intergradation of morphological characters observed in the few "species" covered by this study suggests that at least in these forms there exists a considerable degree of similarity, which lends support to DURRELL's view.

SUMMARY

Emericellopsis, an antibiotic-producing homothallic fungus with both an imperfect (Cephalosporium) and a sexual stage, was investigated genetically, including a study of the effect of genetic interactions on antibiotic activity. The process of heterokaryosis was found to be limited in frequency and extent. Antibiotic yields by heterokaryons synthesized from auxotrophs of low-yielding ability usually approached the activity of the original wild-type strain, but a decrease was observed upon dissociation of the heterokaryon. Heterokaryons from a low producer and a high producer generally gave intermediate degrees of activity. What appears to be a stable heterozygous diploid was observed in only one instance; antibiotic yields by the diploid and by some of the somatic recombinants were essentially comparable to those of the original wild-type strain and to the results shown by heterokaryons. Meiotic recombination between different parental biochemical mutants was found to occur rarely. Conclusive evidence for recombination, however, was obtained, and fermentation data for the recombinants show various degrees of antibacterial activity. Results from a few interspecific crosses are also reported, and a mutant with antifungal activity is described.

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