Trisporic Acid Synthesis in Homothallic and Heterothallic Mucorales

By BERTINE A. WERKMAN AND H. VAN DEN ENDE

*Department of Plant Physiology, University of Amsterdam, Amsterdam,
The Netherlands*

(Received 7 January 1974)

SUMMARY

The synthesis of trisporic acids, the sexual hormones, proceeds in a similar way in mated cultures of the heterothallic fungi *Mucor mucedo* and *Blakeslea trispora*. In both species precursors are secreted by both mating types and these can be converted to trisporic acids by the sexual partner. Precursor production is strongly stimulated by trisporic acids. In homothallic species the presence of biologically active substances can be demonstrated, which have characteristics similar to those found in heterothallic strains. Moreover, precursors of trisporic acids isolated from *Mucor mucedo* are readily converted to trisporic acids by homothallic species. These results suggest a close relationship between the hormonal systems of homothallic and heterothallic species.

INTRODUCTION

Trisporic acids, substances which induce the formation of sexual structures (zygophores) in *Mucor mucedo*, are produced by mated cultures of *M. mucedo*, *Blakeslea trispora* and *Phycomyces blakesleeanus* in the order Mucorales (Gooday, 1973). In *B. trispora* there is evidence that each mating type produces specific precursors which are converted to trisporic acids by the opposite mating type (Sutter, Capage, Harrison & Keen, 1973). Trisporic acids have a stimulatory effect on precursor production in both mating types (Werkman & van den Ende, 1973), which may help to explain the relatively large yields of trisporic acids in mated cultures of *B. trispora*. We wondered whether the same mechanism of trisporic acid formation operates in other heterothallic Mucors which produce much lower amounts of trisporic acids. Here *M. mucedo* has been studied as: (i) it has often been used in sexual studies in the Mucorales; (ii) it produces only small amounts of trisporic acids in mated cultures (e.g. Gooday, 1973); and (iii) it is not closely allied to *B. trispora* in a morphological sense.

We were also interested in the hormonal aspects of sexuality in related homothallic species. The occurrence of interspecific sexual reactions between homothallic and heterothallic strains suggests similar sexual mechanisms. If trisporic acids or related substances are involved in zygophore formation in homothallic species, one might suppose that in these species at least part of the biosynthetic pathway to trisporic acids would be operative. Therefore we investigated whether homothallic strains do produce biologically active substances similar to those isolated from heterothallic strains and whether they can convert precursor material derived from heterothallic strains to trisporic acids.

METHODS

Cultures. The strains used in the present investigation were: *Mucor mucedo* Bref. strain Saito (*minus*), CBS109.16; *Mucor mucedo* Bref. strain Burgeff (*plus*), CBS144.24; *Mucor genevensis* Lendner, NRRL1755; *Zygorhynchus moelleri* Vuill., CBS501.66; *Z. heterogamus* Vuill., CBS405.58; and *Syzygites megalocarpus* Ehrenb. ex Fr., CBS514.66. All strains were obtained from the Centraal Bureau voor Schimmelcultures, Baarn, The Netherlands, except *M. genevensis* which was kindly provided by Dr J. J. Ellis, U.S. Department of Agriculture, Peoria, Illinois, U.S.A.

All strains were cultivated at 20 °C in the dark in 30 % (v/v) beer wort adjusted to pH 8 before sterilization. Submerged cultures were grown for 4 days in 100 ml portions in 300 ml Erlenmeyer flasks on a reciprocal shaker; surface cultures were grown for an equal period of time in 30 ml portions in Petri dishes.

Extraction and incubation of biologically active substances was carried out as described previously (Werkman & van den Ende, 1973), except that the hexane extraction was omitted. One unit of zygothore-inducing material is defined as the smallest amount which induces the formation of zygothores in *Mucor mucedo* at 20 °C in the dark (van den Ende & Stegwee, 1971). The total amounts were estimated by serial dilutions (expressed in units). All other procedures were as described earlier (van den Ende, Werkman & van den Briel, 1972). Trisporic acids were derived from *Blakeslea trispora* and isolated as described earlier (van den Ende *et al.* 1972).

RESULTS

Production of precursors and conversion to trisporic acids in *Mucor mucedo*. The following results demonstrate that the liquid from unmated *M. mucedo* cultures contains neutral substances which are converted to trisporic acids by the sexual partner. Radioactive *plus* mycelia (mycelia grown in culture media containing [U-¹⁴C]glucose) were incubated in a solution of glucose and trisporic acids to stimulate precursor production (see below). From the incubation liquid, neutral, ether-soluble materials were extracted and purified. Samples were assayed for biological activity towards *M. mucedo-minus*. Substances that gave a positive reaction were incubated with *M. mucedo-minus* mycelium in a liquid medium. After 2 days the incubation liquids were tested for the presence of trisporic acids by extraction and DEAE Sephadex chromatography (Werkman & van den Ende, 1973). By the same procedure radioactive precursor material was isolated from radioactive *M. mucedo-minus* cultures and incubated with *M. mucedo-plus* cultures, after which trisporic acids were isolated and purified by fractionation on columns of DEAE Sephadex. The radioactivity, biological activity and u.v.-absorption profiles are presented in Fig. 1 ai, bi. Part of the radioactive neutral compounds from both *plus* and *minus* (Fig. 1 ai and bi, respectively) had been converted by the opposite mating partner to radioactive compounds that were retained on DEAE Sephadex. These compounds were eluted at the same ionic strength as trisporic acids, were biologically active on both *plus* and *minus* *M. mucedo* and exhibited the same u.v.-absorption spectrum as trisporic acids; we therefore assume that they were trisporic acids.

When the radioactive neutral precursors were incubated alone or with non-radioactive cultures of the same mating type, they were unstable, as much of the radioactivity was lost within 2 days. Trisporic acids were not formed.

In a further experiment, ten Petri dishes, containing 20 ml culture medium with or without 54 µg trisporic acids, were inoculated with *plus* or *minus* *Mucor mucedo*. After four days

the media were collected, adjusted to pH 8 and extracted exhaustively with ether. The ether fraction was washed with 5 % (w/v) sodium bicarbonate and water, and the ether evaporated *in vacuo*. The residues were taken up in 5 ml water; with a series of dilutions (1:3) the dilution was determined which still induced zygothores in the standard assay with *M. mucedo* (0.5 ml per application) of opposite mating type. Trisporic acids stimulated up to tenfold the synthesis of biologically active precursors in both *plus* and *minus* *M. mucedo* (Table 1).

Isolation of zygothore-inducing substances from homothallic strains. The culture media from *Mucor genevensis*, *Zygorhynchus moelleri*, *Z. heterogamus* and *Syzygites megalocarpus* (fresh weights approx. 50 g) were extracted with ether, and the extracts subjected to DEAE Sephadex column chromatography as described above. Fractions were assayed for zygothore-inducing capacity towards *M. mucedo-plus* and *M. mucedo-minus*. In extracts of culture media from *Z. moelleri* and *Z. heterogamus* small amounts of acidic material were present with the same elution pattern on DEAE Sephadex as trisporic acids and with zygothore-inducing capacity towards both *plus* and *minus* mating type of *M. mucedo*. In extracts of *Z. moelleri* culture media (but not of *Z. heterogamus*) neutral material was present with the same chromatographic characteristics and biological activity as trisporic acid-precursor material from *M. mucedo-minus*. From the other homothallic strains no biologically active substances could be detected. These results suggest that at least in *Z. moelleri* and *Z. heterogamus* sexual morphogenesis is governed by similar low molecular weight substances as in heterothallic systems.

Conversion of precursors from Mucor mucedo to trisporic acids by homothallic strains. Radioactive trisporic acid-precursor materials isolated from *M. mucedo-plus* and *-minus* as described above were incubated with submerged cultures of *M. genevensis*, *Zygorhynchus moelleri*, *Z. heterogamus* and *Syzygites megalocarpus*. After 2 days the media were extracted after acidification with ether. The extracts were subjected to DEAE Sephadex column chromatography and the eluates were assayed for biological activity and radioactivity. All four strains appeared able to convert precursor material obtained from *M. mucedo-plus* to trisporic acids (Fig. 1 aii to v), since those fractions from the column that would contain authentic trisporic acids contained zygothore-inducing activity towards *both* mating types of *M. mucedo*. In controls of extracts of corresponding cultures incubated without precursor material, or the neutral precursor material incubated alone, no biological activity was found in the fractions in which trisporic acids might have been present. (This is not opposed to the previous isolation of zygothore-inducing substances from homothallic cultures, since in this experiment the total fresh weight of the cultures used was 14 times less.) In all cultures except *Z. heterogamus* radioactivity was associated with the biologically active fractions. Taken together with a measurable u.v.-absorption spectrum similar to that of trisporic acids, this suggests that in *M. genevensis*, *Z. moelleri* and *S. megalocarpus* the neutral precursor material of trisporic acids derived from *M. mucedo-plus* is as easily converted to trisporic acids as in *M. mucedo-minus*. In *Z. heterogamus* detectable trisporic acids were formed from added *plus* precursors, but considerably less efficiently than in other homothallic fungi. However, *Z. heterogamus* was superior to the other homothallic fungi in converting ^3H -*M. mucedo-minus* neutral precursors to trisporic acids, although not as efficient as *M. mucedo-plus* (Fig. 1 bii to v). The other homothallic strains, with the possible exception of *S. megalocarpus* from which some biological activity was recovered with the same retention as trisporic acids, showed no detectable conversion of *minus* neutral precursors to trisporic acids (Fig. 1 bii to v). After incubation of *M. genevensis* and *Z. moelleri* with the *plus* precursors, no starting material could be recovered, but from all the other incubations some unchanged starting material could be detected by bioassays.

Fig. 1. For legend see facing page.

Table 1. Influence of trisporic acids on neutral precursor production in *plus* and *minus* mycelium of *M. mucedo*

The total amounts determined for *plus* and for *minus* precursor materials are not comparable. Values are averages of duplicates. Deviations from the average are indicated.

Mating type	Trisporic acids added	Neutral precursor material produced (units)
<i>plus</i>	No	69 ± 39
<i>plus</i>	Yes	891 ± 81
<i>minus</i>	No	207 ± 117
<i>minus</i>	Yes	1863 ± 1053

DISCUSSION

Blakeslea trispora was the fungus first shown to produce trisporic acids and the fungus used to investigate how hormone synthesis is initiated (Caglioti *et al.* 1964, Sutter *et al.* 1973). As Gooday (1973) remarked, however, it should be remembered that this species may not necessarily exhibit a situation representative for all heterothallic Mucorales, because it gives excessively high hormone yields. Therefore any scheme proposed for the sexual behaviour of *B. trispora* should be tested with *Mucor mucedo* and other species. Our results show that the hormonal situation in these two fungi may be very similar.

Part of this study was concerned with homothallic Mucorales, because the suggestion has repeatedly been made (e.g. Gooday, 1973) that in these species sexuality is also controlled by trisporic acids. This suggestion was based on the following observations: (i) inter-specific though incomplete sexual reactions between homo- and heterothallic species are very common (Burgeff, 1924; Satina & Blakeslee, 1930); (ii) zygospore formation in seemingly sterile homothallic strains can be induced by contact with other homo- or heterothallic species (Schipper, 1971); (iii) incubation of the *plus* strain of *B. trispora* or *M. mucedo*

Fig. 1. Radioactivities and biological activities after DEAE Sephadex column chromatography of extracts of cultures incubated with trisporic acid-precursor material. (ai) Twenty-eight submerged *plus M. mucedo* cultures were grown in the presence of 30 μ Ci [$U-^{14}C$]glucose (120 nmol). After harvesting, the mycelia (approx. 5 g fresh weight each) were incubated in 100 ml of a solution of 5 μ g trisporic acids (isolated as described previously, van den Ende *et al.* 1972) per ml and 1 % (w/v) glucose for 4 days (with one further addition of trisporic acid and glucose). The collected incubation liquids were extracted with diethyl ether after adjusting the pH to 8. After evaporating the ether from the extract *in vacuo*, the aqueous residue was put on a DEAE Sephadex column which was eluted with 0.01 ionic strength tris-HCl buffer, pH 8.5. The fractions with biological activity towards *M. mucedo-minus* were pooled and one-seventh added to *M. mucedo-minus* cultures (total fresh weight, 3.5 g; added radioactivity, 30 518 d.p.m.). After 48 h the culture liquids were acidified to pH 2 and extracted with ether to yield any trisporic acids. From the acid ether fraction, the solvent was evaporated *in vacuo*, and the aqueous residue subjected to DEAE Sephadex chromatography, with a discontinuous gradient of tris-HCl buffer pH 8.5, containing an increasing concentration of KCl (starting at total ionic strength of 0.01, van den Ende *et al.* 1972). Fractions (5 ml) were collected, in which radioactivities and biological activities towards both *plus* and *minus* strains of *M. mucedo* were determined. (bi) A slightly modified procedure was followed to obtain radioactive material from *M. mucedo-minus* cultures. Twenty-eight unlabelled *minus* mycelia were incubated in 100 ml of a solution containing 10 μ Ci [$U-^{14}C$]glucose (40 nmol), 5 μ g trisporic acids/ml and 2 % (w/v) glucose for 4 days. The procedure described above was then followed. The radioactive extract was incubated with *M. mucedo-plus* cultures (total fresh weight, 2.3 g; added radioactivity, 175 545 d.p.m.). (aii to v) Radioactive *M. mucedo-plus* material as in (ai) added to cultures of *M. genevensis*, *Z. moelleri*, *Z. heterogamus* and *S. megalocarpus*, respectively. (bii to v) Radioactive *M. mucedo-minus* material as in (bi) added to homothallic cultures, with the same quantities as in (bi). —, Total radioactivity per fraction; ----, u.v. absorbance of peak at 325 nm; |——| zygophore-inducing activity on both *plus* and *minus* mating types of *M. mucedo*.

with the homothallic *Zygorhynchus moelleri* gives measurable trisporic acid synthesis (van den Ende, 1968).

In interspecific conjugations between homo- and heterothallic species, some homothallic strains react predominantly with *plus*, others with *minus* strains. Therefore, Satina & Blakeslee (1930) attributed a *minus* 'tendency' to some homothallic strains, and a *plus* 'tendency' to others, although yet other homothallic species react with both *plus* and *minus* strains. *Zygorhynchus moelleri* is a homothallic strain with a *minus* tendency. We have found that this fungus converts precursor material derived from *Mucor mucedo-plus* about as efficiently as *M. mucedo-minus*, and also produces small amounts of precursor material very similar to that isolated from *M. mucedo-minus*. This explains the production of trisporic acids in a mated culture of *Z. moelleri* and a *plus* strain of *M. mucedo* or *Blakeslea trispora*, and the more abundant production of zygosporophores in *M. mucedo-plus* than in *M. mucedo-minus* when mated with *Z. moelleri*. *Z. heterogamus*, on the other hand, exhibits a *plus* tendency. Although no '*plus-like*' trisporic acid-precursor material has been detected in its culture media, this fungus may convert *minus* precursors to trisporic acids. The weak sexual responses of this strain seem to be reflected by its rather low efficiency of precursor conversion.

Satina & Blakeslee (1930) found that *Mucor genevensis* reacted with *plus* and *minus* strains. This character was not reflected in our experiments: our strain effectively converted *plus* precursors but not *minus* precursors to trisporic acids.

Syzygites megalocarpus has been described as sexually indifferent to heterothallic species (Satina & Blakeslee, 1930). However, our strain transformed both *plus* and *minus* precursors to trisporic acids. We observed that this species was unable to produce zygosporophores under standard conditions (described above) but did so abundantly when trisporic acids or its precursors isolated from *Mucor mucedo* were applied.

Although the study of hormonal aspects of sexuality in homothallic species has just begun, the conclusion that the hormonal system in homo- and heterothallic species of the Mucorales is closely related seems to be justified.

REFERENCES

- BURGEFF, H. (1924). Untersuchungen über Sexualität und Parasitismus bei *Mucorineen*. I. *Botanische Abhandlungen* **4**, 1-135.
- CAGLIOTI, L., CAINELLI, G., CAMERINO, B., MANDELLI, R., PRIETO, A., SALVATORI, T. & SELVA, A. (1964). Sulla costituzione degli acidi trisporici. *Chimica e l'industria (Milano)* **46**, 961-966.
- VAN DEN ENDE, H. (1968). Relationship between sexuality and carotene synthesis in *Blakeslea trispora*. *Journal of Bacteriology* **96**, 1298-1303.
- VAN DEN ENDE, H. & STEGWEE, D. (1971). Physiology of sex in *Mucorales*. *Botanical Review* **37**, 22-36.
- VAN DEN ENDE, H., WERKMAN, B. A. & VAN DEN BRIEL, M. L. (1972). Trisporic acid synthesis in mated cultures of the fungus *Blakeslea trispora*. *Archiv für Mikrobiologie* **86**, 175-184.
- GOODAY, G. W. (1973). Differentiation in the *Mucorales*. *Symposia of the Society for General Microbiology* **23**, 269-294.
- SATINA, S. & BLAKESLEE, A. F. (1930). Imperfect sexual reactions in homothallic and heterothallic *Mucors*. *Botanical Gazette* **90**, 299-311.
- SCHIPPER, M. A. A. (1971). Induction of zygosporophore production in *Mucor saximontensis*, an agamic strain of *Zygorhynchus moelleri*. *Transactions of the British Mycological Society* **56**, 157-158.
- SUTTER, R. P., CAPAGE, D. A., HARRISON, T. L. & KEEN, W. A. (1973). Trisporic acid biosynthesis in separate *plus* and *minus* cultures of *Blakeslea trispora*: identification by *Mucor* assay of two mating type specific components. *Journal of Bacteriology* **114**, 1074-1090.
- WERKMAN, B. A. & VAN DEN ENDE, H. (1973). Trisporic acid synthesis in *Blakeslea trispora*. *Archiv für Mikrobiologie* **90**, 365-374.