Phosphoglycerides of *Trichophyton terrestre* and One Phenotype Selected from the Apollo 16 Microbial Ecology Evaluation Device

R. T. SAWYER, D. C. DESKINS, AND P. A. VOLZ*

Department of Biology, Eastern Michigan University, Ypsilanti, Michigan 48197

Received for publication 11 December 1974

Total lipid extracted from wild-type *Trichophyton terrestre* CDC-X285 was found to be 2.0% of the dry cell weight. The total lipid contained the following phospholipid components identified by silicic acid-impregnated thin-layer and paper chromatography: phosphatidyl inositol, phosphatidyl choline, phosphatidyl serine, and phosphatidic acid. The total lipid extracted from the phenotype *T. terrestre* 7048-1 isolated from the Apollo 16 Microbial Ecology Evaluation Device (MEED) was found to vary according to the time at which the phospholipids were extracted. The *Trichophyton* phenotype was selected from a cuvette housed in the MEED exposed to specific space parameters including ultraviolet light of known wavelengths and energy levels in deep space. The phospholipid components identified in the phenotype were phosphatidyl ethanolamine and cardiolipin. The major lipid fraction was composed of digalactosyldiglyceride and monogalactosyldiglyceride. An unusual lipid was detected in the phenotype, which appeared to be sterol glycoside.

Wild-type *Trichophyton terrestre* CDC-X285 conidia were housed in the Apollo 16 Microbial Ecology Evaluation Device (MEED). The conidia were exposed in the MEED at a 90° angle to the sun for 10 min + 7 s in space during the Transearth Extra Vehicular Activity of Apollo 16 (18). Test spaceflight parameters monitored by the flight hardware included 254-, 280-, and 300-nm ultraviolet (UV) light at various energy levels, full solar light, and no light (18). *T. terrestre* 7048-1, referred to hereafter as the phenotype, isolated from one of the MEED cuvettes containing conidia of wild-type *T. terrestre*, was used in this study and compared to the parent.

The 0.05-cm² cuvettes were constructed of lexan tinted black to prevent light scatter. The cuvette body had a 7° internal slope to prevent possible shadowing of the organisms and a 25-mm² quartz window to allow for exposure of the UV light components of solar irradiation. A fillport was provided on the side opposite the quartz window. Peak wavelengths entering the cuvette chamber were selected by the use of quartz bandpass interference filters and quartz neutral-density filters controlled by the total radiant energy reaching the exposed fungal cells.

The wild-type conidia received 254-nm UV light at 9.6 × 10³ ergs per cuvette for the 10-min space exposure, whereas the parent was housed in a ground control cuvette. The morphological variant 7048-1 was selected after the spaceflight cuvettes were returned to the Lunar Receiving Laboratory, National Aeronautics and Space Administration, Houston Tex. Space-exposed conidia were allowed to develop colonies on Sabouraud dextrose agar. Phenotype selection and survival counts were made immediately after colony development (19). Weightlessness and the stresses related to launch and splashdown produced no variation in viability as indicated in ground control, vibration control, and darkness or flight control. Survival rates did vary in relation to spaceflight parameter exposure levels.

Previous studies concerning the lipid and hydrocarbon content of filamentous fungi have been performed (2, 4, 8). This study concerns the variation of phospholipids in wild-type *T. terrestre* and the space-flown phenotype obtained from the wild type after UV exposure in space.

**MATERIALS AND METHODS**

Conidia of wild-type *T. terrestre* CDC-X285 were housed in cuvettes in the Apollo 16 MEED. The isolate *T. terrestre* 7048-1 is a morphological variation of the parent isolated after the Apollo 16 spaceflight (18). Stock cultures of the microorganisms were
maintained on Sabouraud dextrose agar (Difco) slants at 24 C.

Both the wild type and the phenotype were grown in 250-ml Erlenmeyer wide-mouth flasks with Morton closures and containing 50 ml of Sabouraud maltose broth (Difco). The inoculum was prepared from agar slant cultures blended in 20 ml of distilled water for 1 min at 4 C. Flasks inoculated in sets of four were placed on a reciprocal shaker rotated at 100 rpm, and incubated at 24 C for 5, 7, and 10 days.

To minimize the potential contamination by trace hydrocarbons, glassware was cleaned with a chromic acid solution, rinsed with double-distilled water and then with methanol, and autoclaved. Before organisms were harvested, they were checked for purity by colony and microscopic characteristics, including Gram staining for possible presence of bacteria.

Phenotype and wild-type cultures of *T. terrestre* were harvested after 5, 7, and 10 days of incubation. Experiments were conducted in replicates of eight. The cultures were transferred to 200-ml glass centrifuge jars, centrifuged at 2,000 x g for 60 min to achieve uniform packing of mycelial pellets, and then washed three times in 20 ml of sterile 0.9% saline. Washed cells were transferred from the centrifuge jars to pre-weighted 50-ml beakers and placed in a vacuum desiccator containing silica 16-mesh gel and Ascarite, vacuum pumped to a constant weight, and weighed on a Mettler H 33 analytical balance. Cell weights were adjusted to account for 75.9% water per 1 g of dry packed cells. All solvents used in this study were purchased from the Mallinckrodt Chemical Company and were of nanograde quality.

The total free lipids were extracted from the cells by the procedure of Bligh and Dyer (3). Although the Bligh-Dyer method has the advantage of making it unnecessary to homogenize or disrupt the cells, we found that phosphatidyl serine was not recovered from *T. terrestre* unless the cell mass was disrupted. Prior to extraction, dry packed cells were transferred to a sterile monometal container assembly and homogenized, as above, in 40 ml of sterile double-distilled water. The homogenate was transferred to a round-bottom flask and to it was added 100 ml of methanol and 50 ml of chloroform, bringing the final ratio of MeOH-CHCl₃-water to 10:5:4 (vol/vol/vol). The mixture was shaken and allowed to stand 18 to 24 h. The mixture was centrifuged to remove cellular debris, and supernatant was transferred to a 500-ml separatory funnel fitted with a Teflon stopcock. Chloroform and water were added, bringing the final ratio of the solvents MeOH-CHCl₃-water to 10:10:9 (vol/vol/vol). The separatory funnel was agitated gently and allowed to stand overnight. The solution separated into an upper methanolic-water phase and a lower lipid-containing chloroform phase. The chloroform-soluble extract was collected in a round-bottom flask and concentrated to 5 to 8 ml by evaporation of the chloroform in a Buchler flash evaporator.

Lipids were fractionated on a silicic acid column (100 mesh; Coleman and Bell, Norwood, Ohio). The silicic acid had been heat activated for 12 h at 110 C (10). Aliphatic hydrocarbons were eluted from the column with n-hexane. Chloroform eluted the neutral lipids, whereas the remaining lipids were eluted with methanol (10, 11).

Dry weights of extracted lipid material were determined by transferring concentrated samples from round-bottom flasks to pre-weighted centrifuge tubes with a Pasteur pipette. Samples were blown to dryness under a stream of dry nitrogen gas in a water bath at 40 C. Tubes containing the dry lipid extract were placed in a vacuum desiccator and vacuum pumped at 35 atm for 15 min. The vacuum was released by flooding the desiccator with nitrogen to prevent peroxidation of lipids. Lipid-containing tubes were weighed on the analytical balance, and total lipid weight was determined from the differences in tube weights.

Total lipids were chromatographed on Kodak silica gel-impregnated chromatrams (Eastman Kodak Chromagram Sheet no. 13181 silica gel absorbent with fluorescent indicator). Spots were applied in 2- to 10-μl amounts by using chloroform-cleaned Drummond glass Microcaps 2 cm apart and 5 cm from the bottom of the thin-layer chromatography plates (20 by 20 cm). These plates were developed by ascending methods using solvent system A (benzene-diethyl ether-ethanol-acetic acid, 50:40:2:0.2, by volume), drying the plates in a hood for 45 min, and developing in solvent system B (n-hexane-diethyl ether, 96:4, by volume) (13). Plates were dipped in a solution of Rhodamine 6 G and visualized with UV light (11). Polar lipids were separated by developing the plates in ascending solvent system C (chloroform-acetone-methanol-acetic acid-water, 50:20:10:10:5, by volume) (15). Chromatograms were run against a standard containing authentic phospholipids (Sigma Chemical Co., St. Louis, Mo.).

Whatman no. 61812 silicic acid-impregnated papers (42 by 57 cm) were cut into strips (2.5 by 46 cm), and total lipids were applied to each strip in 1- to 10-μl amounts. Chromatograms were developed in a lined chromatography tank (Scientific Products, Romulus, Mich.) by descending solvent system DAW (diisobutyl ketone-acetic acid-water, 40:24:5, by volume) and in solvent system D (n-butanol-acetic acid-water, 40:24:5, by volume) (11).

Three different staining procedures were used to detect and partially identify the chromatographed phospholipids. In the first, chromatograms were dried in a hood for 15 to 30 min (less time if the lipids were susceptible to peroxidation), immersed in a 0.0012% solution of Rhodamine 6 G, rinsed in distilled water, and viewed immediately under UV light. Acidic phospholipids appeared blue or purple, whereas neutral phospholipids were yellow or orange.

In the second procedure, the dried chromatograms were sprayed with Ninspray (0.5% ninhydrin in butanol; Nutritional Biochemicals, Cleveland, Ohio). The papers were placed in an oven at 100 C on a clean glass plate and checked at 3-min intervals until mauve (blue-purple), positive spots appeared. Phospholipids containing amine groups such as phosphatidyl ethanolamine and phosphatidyl serine were visualized. The chromatograms were overstained with Rhodamine 6 G.

In the third method, the chromatograms were dried
for 15 min and stained according to the Periodate-
Schiff reaction (11). The chromatogram was dipped in
a solution of 0.25% sodium m-periodate and dried for
15 min in a hood. The chromatogram was dipped in a
1.0% solution of sodium m-bisulfite until colorless and
then dipped in a solution of 0.5% p-rosalaminewater-1.9% sodium metabisulfite (1:1:1, by volume).
This stain was specific for phosphatidyl glycerol,
phosphatidyl inositol, and glycolipids with vicinal hydroxyl groups.

RESULTS

Total lipid extracted from wild-type T. terrestre was found to be 1.8 to 2.0% on a dry
cell weight basis. A constant amount of total lipid was extracted from the wild type after 5, 7,
and 10 days of incubation at 24 C (Table 1). The phenotype harvested after 5 days of incubation
yielded low amounts of total lipid, 1% of the cellular dry weight. The phenotype total lipid
yield was 6 to 15% at 7 days of incubation and 31% at 10 days. No gross morphological differ-
ences were observed between each sample during incubation. Dry cell weights obtained from
various cultivations varied considerably.

It should be noted that neutral lipids were not separated from phospholipids for chromato-
graphic studies. Ames (1) noted that in Bligh-
Dyer extracts of Salmonella typhimurium the bright orange tint present was coenzyme Q.
Tornabene et al. (14) found that vitamin K also exhibits this property. A bright orange color was
apparent in our total lipid extracts of both T. terrestre wild type and phenotype. At this time
the orange tint in our samples is under investiga-
tion. There were no unsaponifiable materials
detected in any of our samples.

TABLE 1. Percent* total lipid extracted per gram (dry weight) of fungal cells harvested at 5, 7, and 10 days

| Organism         | Lipid extracted | Organism         | Lipid extracted |
|------------------|-----------------|------------------|-----------------|
|                  | % Total lipid   | % Dry mycelial   | % Total lipid   |
|                  | wt (g)          | wt (g)           | wt (g)          |
| 5                | 7               | 10               | 5               | 7               | 10               |
| T. terrestre     | 0.5             | 15.0             | 31.0            | 0.26            |
| phenotype 7048-1| 1.04            | 1.0             | 0.1            | 23.0            | 0.36            |
|                  | 1.2             | 1.03            | 0.6            | 19.0            | 0.35            |
|                  | 2.0             | 2.0             | 1.5            | 2.0             | 0.9             |

* Each average percentage represents extraction of
8 individual cultures.

When total lipid fractions from the wild type
were spotted on thin-layer chromatography plates and developed in polar lipid solvent
system C and in solvent systems A and B,
separation of eight phospholipid components
was observed. A tracing of this separation
appears in Fig. 1. The major phospholipids
observed were phosphatidyl inositol, phosphati-
dyl choline, phosphatidyl serine, phosphatidyl
ethanolamine, and phosphatidic acid. Also
present in parent extracts were digalactosyl
diglyceride and monogalactosyl diglyceride. A
small amount of neutral lipid was detected in
the wild type. Neither phosphatidyl glycerol nor
diphosphatidyl glycerol was found. In the total
lipid extract of the parent there appears to be a
high concentration of polar phospholipids.

Thin-layer chromatography separation of
total lipid extracts of the space-flown pheno-
type is presented in Fig. 1. The major phos-
pholipids present in the phenotype were phosphati-
dyl ethanolamine, which is present in large
amounts relative to other lipids, and diphos-
phatidyl glycerol (cardiolipin). Neutral lipids,
digalactosyl diglyceride, and monogalactosyl
diglyceride were detected. A lipid with staining
properties and Rf values of sterol glycoside (15)
was also observed. Phosphatidyl inositol, phos-
phatidyl serine, phosphatidyl glycerol,
phosphatidyl choline, and phosphatidic acid were
not found. The largest concentration of lipid material in the phenotype was present in non-

polar lipids.

The phospholipids observed by paper chromatography were identical to those seen by thin-layer chromatography of both the wild type and phenotype. A tracing of paper chromatography and staining reactions appears in Fig. 2. The phenotype total lipid contained an unusual component, not developed by thin-

layer chromatography, tentatively identified as sterol glycoside.

**DISCUSSION**

Phospholipids found in *T. terrestre* 7048-1, a phenotype isolated from the Apollo 16 MEED, varied from those of the wild type. Increasing percentages of total lipid were extracted from the phenotype with extended incubation. This increase in total lipid was not accompanied by morphological variation in growth of the phenotype. In a previous study another group of phenotypes selected from the Apollo 16 MEED isolates indicated that both *T. terrestre* and *Chaetomium globosum* phenotypes had similar logarithmic growth rates for each species (18).

Under normal growth conditions a stable membrane configuration, as represented by a low turnover of phospholipids, is seen at the end of logarithmic growth and the beginning of stationary growth phases of a normal growth curve. This appears to be true of the wild type. The phenotype total lipid content was unstable during stationary growth. The phospholipid contents of the phenotype harvested at different times did not vary.

Many phenotypic changes of the MEED-flown strains of *T. terrestre* and *C. globosum* have been recorded. Noted were changes in growth dynamics at the microscopic level including: hyphal branches, irregular hyphal walls, wavy hyphae, and blunt hyphal apices; variation in analysis of deoxyribonucleic acid composition; changes in colony morphology and growth characteristics; and variation in the ability of *T. terrestre* phenotypes to degrade human hair (18). Other changes are currently under investigation with various phenotypes. The phenotypes were selected from the Apollo 16 MEED mycology cuvettes after exposure to specific spaceflight parameters. Except for survival variation to specific UV irradiations, changes appeared random at the individual cell level. The space-flown phenotype of *T. terrestre* differed from the parent in phospholipid content.

Variations of the phosphoglyceride contents among different species of filamentous fungi have been noted. Bruszewski et al. (4) found that six thermophilic fungi had total lipid extracts varying as much as 20% and phospholipid contents varying up to 30% of the total lipid. The lack of numerous phospholipids among species of *Actinomycetales* is well documented. *Mycobacterium tuberculosis* may contain phosphatidyl inositol as its only phospholipid (9). The presence of unusual lipid material such as sphingolipids has been demonstrated in the *Phycomycetes* and the *Basidiomycetes* (21). Weete has extensively reviewed the literature concerning distribution of phospholipids among the fungi (20). Our results indicate that as the age of the culture increases the total phospholipid content increases and the average dry cell weight decreases. This suggests that a gross variation in total lipid between parent and phenotype *T. terrestre*, although not an artifact, may not be unprecedented.

Das and Banerjee (5) reported the presence of phosphatidylinositol, phosphatidyl serine, phosphatidyl choline, phosphatidyl ethanolamine, and phosphatidic acid in *Trichophyton rubrum*, all of which were detected in the wild-type *T. terrestre* in our studies. They also indicated the presence of phosphatidyl glycerol in *T. rubrum*, whereas our studies indicate that monogalactosyl diglyceride was present in the wild-type *T. terrestre*. The phenotype *T. terrestre* contained phospholipids common to both *T. rubrum* and the wild-type *T. terrestre*.
The phenotype contained diphosphatidyl glycerol not found in either *T. terrestris* wild type or *T. rubrum*. Although total phospholipids in *T. rubrum* appeared to remain constant throughout incubation, variation among individual components was found. This variation with age could account for the increase in total phospholipid in the phenotype *T. terrestris*.

In a recent review of space microbiology (12), it was stated that “Most of the space microbiology studies conducted over the last forty years have failed to establish any demonstrable changes in microbes returned to earth, although there were some positive findings.” We have concluded from our studies that exposure of wild-type *T. terrestris* resulted in a phenotype whose whole-cell phospholipid contents varied from that of the wild type. Other studies support the fact that exposure of microbes to space environment results in changes detected upon return to earth (6, 7, 16).

Although preflight studies using simulated exposure to solar parameters indicated that phenotypic variation did occur, no correlation between these experiments and ours has been made (17). It would be difficult to trace the variation in phospholipids in the space-flown phenotype to a primary mutation event. However, since exposure to high levels of UV irradiation does induce mutation, this cannot be ruled out as a factor in varying phospholipid content.

ACKNOWLEDGMENTS

We wish to thank Thomas G. Tornabene of Colorado State University for suggestions and review of this study.

Appreciation is extended to the National Research Council, the American Association for Engineering Education, and the National Aeronautics and Space Administration for their support on the Apollo 16 MEED project.

LITERATURE CITED

1. Ames, G. F. 1968. Lipids of Salmonella typhimurium and *Escherichia coli*: structure and metabolism. J. Bacteriol. 95:833-843.
2. Bartinicki-Garcia, S. 1968. Cell wall chemistry morphogenesis and taxonomy of fungi. Annu. Rev. Microbiol. 23:87-108.
3. Bligh, E. G., and W. J. Dyer. 1959. A rapid method of total lipid extraction and purification. Can. J. Biochem. Physiol. 37:911-917.
4. Bruszewski, T. E., C. L. Fergus, and R. O. Mumma. 1972. Thermophilic fungi. IV. Lipid composition of six species. Lipids 7:659-668.
5. Das, S. K., and A. B. Banerjee. 1974. Phospholipids of *Trichophyton rubrum*. Sabouraudia 12:281-286.
6. Dublin, M., and P. A. Volz. 1973. Space-related research in mycology concurrent with the first decade of manned space exploration. Space Life Sci. 4:225-230.
7. Dublin, M., P. A. Volz, and G. S. Bulmer. 1974. The antifungal activity of host compromised saliva on spaceflight fungal phenotypes. Mycopathol. Mycol. Appl. 54:499-516.
8. Erwin, J. A. 1973. Lipids and biomembranes of euukaryotic microorganisms. Academic Press Inc., New York.
9. Ikawa, M. 1967. Bacterial phospholipids and natural relationships. Bacteriol. Rev. 31:54-64.
10. Kates, M. 1964. Bacterial lipids, p. 17-90. In D. Kriche (ed.), Advances in lipid research, vol. 2. Academic Press Inc., New York.
11. Marinetti, G. V. 1967. Lipid chromatographic analysis. Marcel Dekker, Inc., New York.
12. Taylor, G. R. 1974. Space microbiology, p. 121-137. In M. P. Starr, J. L. Ingraham, and S. Raffel (ed.), Annual review of microbiology, vol. 28. Annual Reviews, Palo Alto, Calif.
13. Tornabene, T. G. 1973. Lipid composition of selected strains of *Yersinia pestis* and *Yersinia pseudotuberculosis*. Biochem. Biophys. Acta 306:173-185.
14. Tornabene, T. G., M. Kates, E. Gelpi, and J. Oro. 1969. Occurrence of squalene, di- and tetracyhdroxylanes and vitamin MK* in an extremely halophilic bacterium, *Halobacterium cutirubrum*. J. Lipid Res. 10:294-303.
15. Tornabene, T. G., and J. E. Ogg. 1971. Chromatographic studies of the lipid components of *Vibrio fetus*. Biochem. Biophys. Acta 229:133-141.
16. Volz, P. A. 1974. The Apollo 16 MEED Mycology. BioScience 24:486.
17. Volz, P. A. 1974. The Apollo 16 Microbial Ecology Evaluation Device Mycology Studies 1971-1974. National Aeronautics and Space Administration NAS 9-11562. NASA Johnson Space Center, Houston.
18. Volz, P. A., and M. Dublin. 1973. Filamentous fungi exposed to spaceflight stresses including known levels of ultraviolet irradiation. Space Life Sci. 4:402-414.
19. Volz, P. A., Y. C. Hsu, J. L. Hiser, and D. E. Jerger. 1974. The Microbial Ecology Evaluation Device mycology spaceflight studies of Apollo 16. Mycopathol. Mycol. Appl. 54:221-233.
20. Weete, J. D. 1974. Fungal lipid biochemistry. Plenum Press, New York.
21. Weiss, B., R. L. Stiller, and R. C. M. Jack. 1973. Sphingolipids of the fungi *Phycomyces blakesleeanus* and *Fusarium lini*. Lipids 8:25-30.