Phospholipids of the Thiobacilli

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Phosphatidyl glycerol, disphosphatidyl glycerol, and phosphatidyl ethanolamine were found in all of the Thiobacillus species studied. T. thioparus possessed only these phospholipids. T. intermedius, T. neapolitanus, and T. thiooxidans contained phosphatidyl-N-monomethylethanolamine, and T. novellus lipids contained phosphatidyl-N-monomethylethanolamine, phosphatidyl-N,N-dimethylethanolamine, and phosphatidyl choline, in addition to the three phospholipids common to all of the thiobacilli. Methionine was found to act as a methyl donor in the biosynthesis of the methylated forms of phosphatidyl ethanolamine. Phosphatidyl inositol was not detected in any of the organisms. Changing the nutrient medium did not result in a qualitative change in the phospholipid spectrum of the cultures.

In 1963, Schaeffer and Umbreit (20) identified the sulfur "wetting agent" produced by Thiobacillus thiooxidans as phosphatidyl inositol (PI). Since that time, considerable interest has been directed toward the phospholipids of this organism (11, 21; D. S. Herson, Bacteriol. Proc., p. 108, 1967). Very recently (21), the phospholipids of T. thiooxidans were identified as phosphatidyl glycerol (PG), diphosphatidyl glycerol (DPG), phosphatidyl ethanolamine (PE), and phosphatidyl-N-monomethylethanolamine (PME). The phospholipid components of other thiobacilli, however, have not been determined. An analysis of the phospholipids of several Thiobacillus species was undertaken to provide comparative information regarding their physiology (e.g., the relationship of PI to sulfur utilization) and their phylogenetic relationships to other bacteria (10).

Materials and Methods

Cultures. The following Thiobacillus species were examined: T. intermedius (S. C. Rittenberg, University of California, Los Angeles), T. neapolitanus (W. Vishniac, University of Rochester, Rochester, N.Y.), T. novellus ATCC 8093 (R. Levin, State University of Iowa, Iowa City), T. thiooxidans ATCC (thiosulfate adapted culture of Barton and Shively (1)), and T. thioparus (R. L. Starkey, Rutgers, The State University, New Brunswick, N.J.).

Methods of cultivation. All of the cultures were grown autotrophically in the thiosulfate medium of Vishniac and Santer (23). T. novellus and T. intermedius were grown heterotrophically in nutrient broth and in the Vishniac-Santer medium supplemented with yeast extract (0.1 g per liter of medium), respectively. The sulfur medium was prepared by deleting the thiosulfate and adding 0.5 g of sterile sublimed flowers of sulfur to 50 ml of the Vishniac-Santer medium. The sulfur was sterilized separately by steaming 0.5-g quantities for 1 hr on 3 successive days. The media, except those for T. thiooxidans (pH 4.5 for thiosulfate and 4.0 for sulfur), were adjusted to pH 7.0.

The cultures were grown in 250-ml Erlenmeyer flasks containing 50 ml of the appropriate medium. Inoculations were made with 0.5-ml amounts of 48-hr thiosulfate (T. novellus, 6 day) and heterotrophic cultures and 5-day sulfur cultures. The flasks were incubated at 30°C on a rotary incubator shaker (model 27; New Brunswick Scientific Co., New Brunswick, N.J.) adjusted to 180 rev/min. The sulfur cultures were incubated for 3 days before shaking.

Preparation and analysis of the phospholipids. For 32P labeling, the concentrations of K2HPO4 and KH2PO4 in the Vishniac-Santer media were reduced to 0.05 g per liter. Higher phosphate levels were required for the growth of T. neapolitanus (0.2 g of each salt per liter) and T. thioparus (0.8 g of each salt per liter) when sulfur was supplied as the energy source. Even then, considerable difficulty was encountered because accumulated acid appeared to inhibit the organisms before much growth occurred. Adequate growth was obtained by readjusting the pH of the medium to 7.0 approximately every 12 hr.

Cells were harvested for phospholipid analysis after 48 hr of incubation of the thiosulfate (T. novellus, 6 day) and heterotrophic cultures and after 5 days of incubation of the sulfur cultures. All of the cultures were in the maximal stationary phase of growth.

The phosphatides were labeled with 32P and 14C and were prepared for analysis as described by Shively and Benson (21). Samples of the concentrated washed lipids were spotted on activated (105°C, 1 hr) silicic acid-impregnated paper (Whatman SG-81) and were developed in disubutyl ketone-acetic acid-water (40:20:3, v/v) (14).

The products of the hydrolysis of the phosphatides by hydrochloric acid and methanolic potassium hydroxide.
hydride were separated by paper chromatography with the following solvent systems (21): phenol-water (100:38, v/v), n-butyl alcohol-propionic acid-water (142:71:100, v/v), and phenol-n-butyl alcohol-formic acid-water (5:5:3:1, w/v/v/v).

 Autoradiograms were prepared by exposure to non-screen X-ray film (Eastman Kodak Co., Rochester, N.Y.) and the individual spots (32P-lipids) were quantitated with a thin-window Geiger tube (Tracerlab, Inc., Waltham, Mass.).

 The phospholipids were identified as previously described (21). Phosphatidyl choline (PC), PE, and PI (Pierce Chemical Co., Rockford, Ill.) were used as chromatographic standards. In addition, the already identified phospholipids of T. thiocidans were useful reference compounds (21).

 Methionine as a methyl donor. After the initial discovery that several of the thiobacilli possessed methylated derivatives of PE, the species were examined for their ability to incorporate the methyl group of methionine into the phospholipids, as was found in other bacteria (5, 7, 12). The cultures were grown in 50 ml of Vishniac-Santer medium supplemented with 10 μc of 14C (methyl) l-methionine (Volk Radiochemical Co., Burbank, Calif.). After growth, the cells were harvested by centrifugation, and the lipids were extracted and washed (21). The lipids were hydrolyzed in 1.0 n HCl for 1 hr at 100 C, and the water-soluble products were chromatographed on Whatman no. 1 paper with phenol-n-butyl alcohol-formic acid-water (5:5:3:1, w/v/v/v). The products were identified by chromatography with reference compounds (2-aminoethanol, 2-methylaminoethanol, 2-dimethylaminoethanol, and choline chloride; Distillation Products Division of Eastman Kodak Co.).

 RESULTS

 PG, DPG, and PE were common to all of the Thiobacillus species studied (Table 1 and Fig. 1). T. thioparus possessed only these three phosphatides. T. intermedium, T. neapolitanus, and T. thiocidans contained PME, and T. novellus was able to synthesize PME, phosphatidyl-N-N-dimethylethanolamine (PDE), and PC, in addition to the other three phospholipids. Either PE or one of the methylated forms of PE was found to be the most abundant phospholipid in each of the species tested. PI was not detected in any of the cultures.

 The chromatography of the phospholipids on silicic acid-impregnated paper did not result in satisfactory separation of all the phosphatides (Fig. 1a). PE and PME always ran as one component. PDE did not separate from the other phospholipids. Chromatography of the phosphodiester gave the most satisfactory separations (Fig. 1b).

 Changing the nutrient medium did not result in the formation of any new phospholipids, although some quantitative differences in the phospholipid levels were noted when the nutrient was changed.

<table>
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<th>Table 1. Phospholipids of the thiobacilli</th>
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<td>Organism</td>
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<td>Thiobacillus intermedium</td>
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<td>T. neapolitanus</td>
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<td>T. novellus</td>
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<td>T. thiocidans</td>
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<td>T. thioparus</td>
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* Data calculated from 32P paper chromatograms of water-soluble decacylation products.
* Nutrient broth (NB); Vishniac-Santer medium (VS) with thiosulfate (S2O3) or elemental sulfur (S) provided as the energy source; yeast extract (YE).
* PG, phosphatidyl glycerol; DPG, dihydroxy fatty acid glyceride; PE, phosphatidyl ethanolamine; PME, phosphatidyl-N-N-dimethylethanolamine; PDE, phosphatidyl-N-N-dimethylethanolamine; PC, phosphatidyl choline.
* Trace.

These differences, in all probability, were the result of the different ages of the cultures. It was previously noted that a shift from PG to DPG and from PE to PME occurred in T. thiocidans as the culture aged (21). Shifts of these types appeared to take place in the other thiobacilli. The use of 14C (methyl) l-methionine aided in the identification of PME, PDE, and PC in certain of the cultures (Fig. 2). As expected, no label was found in the water-soluble acid hydrolysis products of T. thioparus. Trace amounts of PDE were noted in cultures which were found to contain only PME by our other methods of analysis. The reason for this discrepancy has not been resolved at this time, but the labeled methionine may provide a more sensitive method of detection.

All of the cultures demonstrated an uptake of methionine. The transport of amino acids by the autotrophs has been recorded by other investigators (2, 22).

DISCUSSION

 PE is a common bacterial phospholipid, but the methylated forms of PE have been found in
only a few bacteria. PME has been shown to accumulate in *Clostridium butyricum*, *Proteus vulgaris*, and *T. thiooxidans* (5, 21). PC, a common constituent of higher plant and animal lipids, has been detected in *Agrobacterium*, *Hyphomicrobium*, *Rhodobacter*, *Rhodopseudomonas*, and *Rhodosporillum* (7, 10, 16). PC was found by Jones and Benson (11) in lipid extracts of *T. thiooxidans*, but we have been unable to duplicate this result.

PME, PDE, and PC are synthesized in bacteria by the stepwise methylation of PE, with S-adenosyl-methionine serving as the methyl donor (12). The same type of biosynthetic mechanism appears to be present in the *Thiobacillus* species capable of forming the methylated derivatives of PE. The cultures were not checked, however, for the presence of the cytidine diphosphate diglyceride pathway for the synthesis of these phosphatides.

The significance of the presence of PC in certain bacteria has not been elucidated. Extensive intracytoplasmic membranes and PC have been discovered in *Nitrocystis*, *Hyphomicrobium*, and the photosynthetic bacteria (3, 9, 17, 18). This lends support to the theory of Hagen, Goldfine, and Le B. Williams (7), who postulated a close relationship between the presence of PC and the occurrence of intracytoplasmic membranes. *T. novellus* has not been examined for the occurrence of these membranes, but *T. thiioxidans*, which can form PME, does not possess an extensive intracytoplasmic membrane system (13). Ikawa (10) pointed out that an efficient electron transport system, which would be required for photosynthesis and aerobic metabolism, is a common feature among the PC-containing organisms. He indicated, however, that PC is evidently not required because it is not found in all aerobes. It is interesting to note that PC does not appear to have an essential function in *Neurospora crassa* (8). PC-less mutants of *N. crassa*, which accumulated PME and PDE, were unable to carry out the final methylation step to form PC, but exhibited a normal vegetative and sexual life cycle. PME and PDE, however, may be able to fulfill the function(s) of PC.

The presence of the methylated forms of PE in the thiobacilli and photosynthetic bacteria and the ability of certain members of both groups to utilize reduced sulfur compounds (17, 19) supports the theory that these organisms are closely related. This theory is further supported by the recent finding that the growth of *T. neapolitanus* is stimulated by light (15). In addition, we have...
discovered the presence of non-phosphorus-containing ornithine lipid(s) in some of the thiobacilli. These lipid(s) appear to be very similar to the one(s) reported in the photosynthetic bacteria (4, 6). A complete characterization of the ornithine lipid(s) is now in progress.

PI, reported by Schaeffer and Umbreit (20) and D. S. Herson (Bacteriol. Proc., p. 108, 1967) to be the agent responsible for the "wetting" of sulfur by *T. thiooxidans*, was not detected in any of the thiobacilli studied. This was true regardless of the energy source (thiosulfate or elemental sulfur) supplied to the cells. PI, therefore, does not appear to be required for the oxidation of sulfur. Further testing will be necessary in order to establish the identity of the "wetting agent."

**ACKNOWLEDGMENT**

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**LITERATURE CITED**