Phospholipid Composition of \emph{Desulfovibrio} Species

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The phospholipids of \emph{Desulfovibrio desulfuricans}, Norway strain, \emph{D. vulgaris}, and \emph{D. gigas} were examined in relationship to their qualitative and quantitative composition. \emph{D. desulfuricans} and \emph{D. vulgaris} exhibited an essentially identical phospholipid composition consisting of phosphatidylethanolamine, phosphatidylglycerol, cardiolipin, and lysophosphatidylserine. Phosphatidylserine (10.9\%) was present in \emph{D. desulfuricans} but was not detected in \emph{D. vulgaris}. \emph{D. gigas} was found to contain only two phospholipids, phosphatidylethanolamine (30\%) and phosphatidylglycerol (70\%). An ornithine-containing lipid was detected in \emph{D. gigas} which was not present in the other two \emph{Desulfovibrio} species.

Comparative studies into the complex lipids of microorganisms have largely ignored the genus, \emph{Desulfovibrio} (4, 7). The presence of aldehydogenic lipids in anaerobic bacteria has been generally documented, including \emph{Desulfovibrio} species (4, 8). The phospholipid fraction obtained from a \emph{Desulfovibrio} species exhibited a plasmalogen-aldehyde:phosphorus ratio of 0.09 (8). A naphthoquinone identified as menaquinone-6 has been isolated from \emph{Desulfovibrio vulgaris} and \emph{D. gigas} (13, 18). An analysis of the phospholipids of several \emph{Desulfovibrio} species was undertaken to provide further comparative information relative to the genus \emph{Desulfovibrio} and their phylogenetic relationships to other bacteria (7, 9, 10, 15).

MATERIALS AND METHODS

Organisms and culture conditions. \emph{D. vulgaris} was grown in the medium of Saunders et al. (17), \emph{D. gigas} according to LeGall et al. (11), and \emph{D. desulfuricans}, Norway strain, in a medium consisting of: sodium lactate, 9 ml; MgSO\(_4\)\(\cdot\)7H\(_2\)O, 1 g; Na\(_2\)SO\(_4\), 2 g; NH\(_4\)Cl, 1 g; K\(_2\)HPO\(_4\), 0.5 g; NaCl, 25 g; sodium thioglycollate, 0.2 g; Fe(NH\(_4\))\(_2\)SO\(_4\), 0.1 g; yeast extract, 1 g (Difco); CaCl\(_2\)\(\cdot\)2H\(_2\)O, 0.1 g dissolved in 1 liter of tap water and adjusted to pH 7.1. Cultures were grown at 37\(^\circ\)C under nitrogen for 24 h in 400-liter lots, harvested by centrifugation, and the cell pellets were washed with 50 mM phosphate buffer, pH 7.0.

Extraction of lipids. The washed cell pellets were suspended in 10 volumes of chloroform-methanol (2:1, vol/vol) and stirred under nitrogen at 3\(^\circ\)C for 18 h. The cell residue was removed by filtration and the chloroform-methanol solution was washed with 0.9\% NaCl twice to remove nonlipid contamination. Further extraction of the cell residue with chloroform-methanol-concentrated HCl (2:1:0.1, vol/vol) indicated 97\% of the phospholipid was removed by the first chloroform-methanol extraction.

Thin-layer chromatography (TLC). Glass plates prepared with silica gel G (0.25 mm thick) were activated at 110\(^\circ\)C and used within 30 min. Silica gel H (0.4 mm thick) impregnated with either 1 mM sodium tetraborate or 1 mM sodium carbonate were used as specified. Development of TLC plates was in the following solvent systems unless otherwise specified: (i) solvent A, chloroform-methanol-water (65:25:4, vol/vol); (ii) solvent B, chloroform-methanol-concentrated NH\(_4\)OH (65:30:2, vol/vol); (iii) solvent C, chloroform-methanol-acetic acid-water (50:50:8:4, vol/vol). Lipids were visualized with the following spray reagents in the sequence specified: (i) total lipid with either iodine vapor or Rhodamine 6 G (0.005\%), (ii) amino nitrogen with 0.2\% ninhydrin in acetone followed by heating at 110\(^\circ\)C for 5 min, and (iii) organic phosphorus by the phosphate spray reagent of Dittmer and Lester (2).

Column chromatography. Whatman DE 23 was used for diethylaminomethyl-cellulose (DEAE-cellulose) column chromatography of phospholipids according to the method of Rouser et al. (16). The volume of the resin bed was 2 by 20 cm and approximately 150 \(\mu\)mol of lipid phosphorus was applied to the column.

Mild alkaline methanolysis. Phospholipids were deacylated by mild alkaline methanolysis at 0\(^\circ\)C (19). The water-soluble glycerylphosphoryl esters were separated on precoated cellulose thin-layer plates (Eastman 13255, Eastman Kodak Co., Rochester, N.Y.) in solvent system D (3.8 mM ethylenediaminetetraacetic acid-0.7 M ammonium bicarbonate in 90 mM ammonium hydroxide containing 67\% [vol/vol] ethyl alcohol) or solvent system E (ethyl alcohol-0.5 M ammonium acetate, pH 7.5 [7.3, vol/vol] [14]). The compounds were visualized with the phosphate spray of Hanes and Isherwood (5).

Analytical methods. Lipid phosphorus was measured by the method of Bartlett as quoted in (3). Cell nitrogen was determined by microKjeldahl (6).

Phospholipid quantitation. Approximately 100 to 150 nmol of lipid phosphorus were applied to a
borate-impregnated plate as four separate spots and developed in chloroform-methanol-water (95:35:5, vol/vol). One lane was sprayed with the phosphate spray reagent and the remainder of the plate was treated with I<sub>2</sub> vapor. After location of the phosphate-positive areas, the silica gel areas containing phospholipid were transferred to glass tubes. A blank area of the TLC plate corresponding in size to an average phospholipid spot was removed for use as the control blank. All recovered silica gel samples were analyzed in triplicate for lipid phosphorus by the Bartlett method. Recovery of the lipid phosphorus applied to the plate was 95 to 97%.

**Acid hydrolysis of lipids and paper chromatography of water-soluble products.** Ninhydrin-positive phospholipids were hydrolyzed overnight at 100°C in 6 N HCl. The water-soluble products were obtained by reducing the aqueous phase to dryness after ether extraction. Ascending chromatography on Whatman no. 1 filter paper was done in n-butanol-acetic acid-water (70:15:15, vol/vol). Ninhydrin was used to visualize the paper chromatogram.

**Materials.** L-[3H]serine was purchased from the New England Nuclear Corp. (specific activity, 3.38 Ci/mM). Silica gel G containing CaSO<sub>4</sub> binder and silica gel H were obtained from Brinkman Instruments Inc. All phospholipid standards were purchased from Servaly Research Laboratory, London, Ontario, Canada.

**RESULTS**

**Characterization of the phospholipids.** Initial TLC analyses on the crude lipid extracts obtained from *D. desulfuricans*, Norway strain, *D. vulgaris*, and *D. gigas* enabled a tentative identification of the phospholipids characterizing these microorganisms as well as the quantitative determination of the individual phospholipid species. Figure 1 shows the resolution of the polar lipids obtained from the three Desulfovibrio species. *D. gigas* exhibited a phosphate-negative, ninhydrin-positive lipid that was absent in the other two Desulfovibrio species. This lipid has been identified as an ornithine-containing lipid, whose isolation and characterization is in progress. Table 1 shows the qualitative and quantitative relationships of the polar lipid fraction obtained from these Desulfovibrio species. *D. desulfuricans*, Norway strain, and *D. vulgaris* exhibit a typical gram-negative phospholipid pattern with phosphatidylethanolamine (PE) comprising the major phospholipid species. *D. gigas* exhibited a phospholipid profile characteristic of many gram-positive microorganisms with phosphatidylglycerol (PG) as the major phospholipid. An additional difference was the presence of only two phospholipid species, PG and PE, in *D. gigas*. This compositional pattern, both qualitatively and quantitatively, is in significant contrast to those patterns characteristic of *D. desulfuricans*, Norway strain, and *D. vulgaris*. Further extraction of *D. gigas* with acidified chloroform-methanol did not release additional phospholipid. A further comparative contrast is evident with *D. gigas* in that the lipid phosphorus-cell nitrogen ratio was significantly lower than that obtained for the other two Desulfovibrio species. *D. desulfuricans*, Norway strain, contained 10.9% phosphatidylserine which was not detected in the other Desulfovibrio species.

Lipids obtained from the three Desulfovibrio species were fractionated by DEAE-cellulose column chromatography. The neutral lipid fraction was eluted with 10 column volumes of chloroform with no detectable lipid phosphorus being recovered in this fraction. Further elution with 10 column volumes of methanol removed PE. The acidic phospholipid fraction was eluted with 10 column volumes of a solvent system consisting of: chloroform-methanol-ammonium hydroxide-5 M ammonium acetate (400:100:10:5, vol/vol). The recovery of lipid phosphorus applied to the column was 100%. Since PE was the sole phospholipid obtained from the methanol column fraction, it served as an additional corroborative check on the TLC quantitation. The amount of PE recovered from the column was within 1% of that amount determined by quantitative TLC.

**PE.** PE was characterized further following purification from the DEAE-cellulose column. This fraction was pure as determined by TLC and co-chromatographed with authentic PE isolated from *Micrococcus cerificans* (12) in the following solvent systems: (i) solvent A, (ii) solvent B, and (iii) chloroform-methanol-water (95:35:5, vol/vol) with silica gel H containing 1 mM sodium tetraborate. PE was deacylated and the resulting water-soluble product co-chromatographed with authentic glycerylphosphorylethanolamine using cellulose TLC plates (Eastman 13255) in solvent systems D and E. The acid hydrolysis product was identified as ethanolamine by paper chromatography and the Beckman amino acid analyzer.

**Cardiolipin (CL).** CL was purified to homogeneity by TLC using solvent A. Chromatographic identity with authentic CL was established for CL purified from *D. desulfuricans*, Norway strain, and *D. vulgaris* in solvent systems A and B and with silica gel H impregnated with 1 mM sodium carbonate in a solvent system consisting of: chloroform-methanol-5 N NH<sub>4</sub>OH (60:30:5, vol/vol). CL was deacylated by mild alkaline methanolysis (MAM) and the water soluble product, glycerylphosphoryl-
glycerylphosphorylglycerol, was determined with cellulose containing TLC plates in solvent system E. Standard glycerylphosphorylglycerolphosphorylglycerol was derived by MAM from known CL. Chromatographic identity was established between authentic glycerylphosphorylglycerolphosphorylglycerol and the sample.

**PG.** PG was purified from the other acidic phospholipids by TLC in solvent system A. Chromatographic identity of PG was established with authentic PG in the three solvent systems specified for CL. MAM of known PG and PG purified from *D. desulfuricans*, Norway strain, *D. vulgaris*, and *D. gigas* yielded the water soluble product, glycerylphosphorylglycerol, as determined by cellulose TLC.

**Phosphatidylserine (PS).** PS was purified from the lipid extract obtained from *D. desulfuricans*, Norway strain, by fractionation on DEAE-cellulose and TLC in solvent system C. The ninhydrin-positive, phosphate-positive phospholipid was identified as PS on the basis of its chromatographic behavior with authentic PS by TLC in solvent systems A, B, and C. MAM of PS established the identity of the water-soluble product, glycerylphosphorylserine.

**Lysophosphatidylserine (LPS).** The lipids obtained from *D. desulfuricans*, Norway strain,
and *D. vulgaris* contained a ninhydrin-positive, phosphate-positive lipid that was not detected in *D. gigas*. This polar lipid was purified from the acidic lipid fraction obtained from the DEAE-cellulose column by TLC in solvent C. TLC in solvent systems A, B, and C established this polar lipid resolved below PS. MAM resulted in a water-soluble product which co-chromatographed with glycerylphosphorylseryl on cellulose TLC. Labeling of *D. desulfuricans*, Norway strain, with L-[3H]serine and purification of this component resulted in the incorporation of radioactive serine into this lipid. The distribution of [3H]serine radioactivity was 97, 2, and 1% for PE, PS, and LPS, respectively. On the basis of these results this lipid was identified as LPS.

**DISCUSSION**

A striking feature of the phospholipids obtained from these three *Desulfovibrio* species was the qualitative diisomeric pattern that served to characterize *D. gigas* apart from *D. desulfuricans* and *D. vulgaris*. The latter two *Desulfovibrio* species exhibited a compositional profile characteristic of many gram-negative bacteria with PE representing the major phospholipid. PS was observed to represent an unusually high percentage of the total phospholipids in *D. desulfuricans*. This phospholipid is normally found in trace amounts. The apparent absence of mono-, di-, and trimethylated derivatives of PE was characteristic of all three species although repeated efforts were made to detect these polar lipids as trace components.

The anomalous phospholipid composition of *D. gigas* is apparent. Its description as a gram-negative, nonspore-forming, sulfate-reducing bacterium (10) contrasts sharply with its phospholipid composition. Goldfine (4) has broadly classified the gram-positive bacteria into two groups on the basis of phospholipid composition. Asporogenous gram-positive bacteria generally contain phospholipids of the PG family with the absence of PE and its methylated derivatives. Sporogenous gram-positive bacteria are characterized by the presence of PE as well as PG and those lipids derived directly from PG. Gram-negative bacteria appear to uniformly exhibit a preponderance of PE and its methylated derivatives over the PG-related series of phospholipids. In comparison to these general relationships, *D. gigas* appears to be an atypical gram-negative bacterium.

A further anomalous relationship reported for *D. gigas* in the singular presence of a naphthoquinone. Analyses performed on a variety of microorganisms have indicated that gram-negative bacteria contain either ubiquinone and menaquinone or ubiquinone alone, whereas those gram-positive bacteria studied contained only menaquinone (1). Interestingly, *D. gigas* and *D. vulgaris* contain only menaquinone (13, 18).

These studies indicate the necessity for further comparative analyses into the lipids of diverse *Desulfovibrio* species as an aid in the phylogenetic classification of this ubiquitous group of microorganisms as well as for obtaining further insight into their basic physiological and biochemical properties.

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**LITERATURE CITED**

PHOSPHOLIPID COMPOSITION OF *DESULFOVIBRIO*


