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Determination of the gram-positive bacterial content of soils and sediments by analysis of teichoic acid components

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Summary

Many gram-positive bacteria form substituted polymers of glycerol and ribitol phosphate esters known as teichoic acids. Utilizing the relative specificity of cold concentrated hydrofluoric acid in the hydrolysis of polyphosphate esters it proved possible to quantitatively assay the teichoic acid-derived glycerol and ribitol from gram-positive bacteria added to various soils and sediments. The lipids are first removed from the soils or sediments with a one phase chloroform-methanol extraction and the lipid extracted residue is hydrolyzed with cold concentrated hydrofluoric acid. To achieve maximum recovery of the teichoic acid ribitol, a second acid hydrolysis of the aqueous extract is required. The glycerol and ribitol are then acetylated after neutralization and analyzed by capillary gas-liquid chromatography. This technique together with measures of the total phospholipid, the phospholipid fatty acid, the muramic acid and the hydroxy fatty acids of the lipopolysaccharide lipid A of the gram-negative bacteria makes it possible to describe the community structure of environmental samples. The proportion of gram-positive bacteria measured as the teichoic acid glycerol and ribitol is higher in soils than in sediments and increases with depth in both.

Key words: *Community structure – Gram-positive bacteria – Sediments – Soils – Teichoic acid*

Introduction

One of the most effective measures of the bacterial content of environmental samples is the muramic acid content [1–5]. This amino sugar with its unusual lactoyl ether is uniquely found in the prokaryotic muramyl peptide cell wall. The determination of the bacterial numbers by muramic acid content is difficult as the

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gram-negative and gram-positive bacteria have different amounts of muramic acid per cell [1–3]. Moriarty [4, 5] has estimated the numbers of bacteria from the muramic acid by staining and counting transmission electron micrographs of organisms in sediments. In these preparations the distinctive morphology of the gram-positive bacteria enabled estimation of the ratio between the two major classes of bacteria. Examining transmission electron micrographs that are necessary to distinguish the cell wall morphology is tedious and expensive as only a tiny field can be viewed with each examination. This paper reports the development of a chemical methodology for detecting gram-positive bacteria based on their content of teichoic acid. When combined with the quantitative assay for the gram-negative bacteria based on the amounts and distribution of the hydroxy fatty acids covalently linked in the lipopolysaccharide lipid A [6], a sensitive chemical methodology for measuring the ratio between these two major classes of bacteria that is not subjected to the limitations of in situ transmission electron microscopy.

Teichoic acids are polymers with glycerol or ribitol joined together through phosphodiester linkages. Many of these polymers have glucosyl or D-alanyl residues and are located exclusively in the walls, capsules or membranes of gram-positive bacteria [7–9]. The teichoic acids may be divided into two groups by their cellular localization – the membrane teichoic acids or lipoteichoic acids linked covalently to lipids, and the wall teichoic acids linked covalently to the peptidoglycan. Wall teichoic acids may be composed of glycerol phosphate, ribitol phosphate and sugar-l-phosphate residues. Most of the ribitol containing teichoic acids also contain D-alanine residues. Wall glycerol containing teichoic acids may contain glycosidically linked sugars as part of the chain or as its substituents. Membrane teichoic acids can be extracted from the bacteria with 80% aqueous phenol and are exclusively of the glycerol phosphodiester type. Analyses of purified lipoteichoic acids show they contain hydrophilic carbohydrate residues and are not extracted with the classical lipid extraction procedures.

Ribitol, a wall teichoic acid component, has a very restricted distribution in nature. In addition to the wall teichoic acids ribitol is found in the riboflavin nucleotides. Ribitol is thus a valuable 'signature' for the wall teichoic acids.

Teichuronic acid polymers replace the wall teichoic acids when gram-positive bacteria are grown in chemostats with phosphate as the limiting nutrient [9–11]. It is of interest that organisms with various types of wall teichoic acids under normal growth conditions form a single type of teichuronic acid polymer with phosphate-limiting growth conditions [12]. The membrane teichoic acids have a wider distribution amongst the gram-positive bacteria, a much more uniform structure, and their concentration is much less dependent on the growth conditions.

The possibility of using teichoic acids as an assay for the gram-positive bacteria in environmental samples depends on the specificity of cold concentrated hydrofluoric acid (HF) for the phosphodiester bonds. This was first utilized by Glaser and Burger in 1964 [13] and has been widely utilized in the elegant structural studies by R.A. Archibald [11]. In this paper the specificity of the cold concentrated HF assay made possible the quantitative measure of the teichoic acid-containing organisms of environmental samples. This assay, when combined with others, can be utilized to define the community structure of the microbial assembly.

Materials and Methods

Materials

Glass distilled solvents were used as purchased (Burdick and Jackson, Muskegon, MI) or ChromAR grade solvents (Mallinckrodt, St. Louis, MO) were used. Chloroform was distilled just prior to use in extractions and derivatizations. Standards and derivatizing reagents were purchased from Sigma Chemical Co., St. Louis, MO; Aldrich Chemical Co., Inc., Milwaukee, WI. Hydrofluoric acid (HF) 48% AR grade was purchased from Mallinckrodt, Inc., St. Louis, MO.

Organisms

Lyophilized cells of *Staphylococcus aureus* strain Newman D_2C, *Bacillus subtilis* (ATCC 6633), and *Escherichia coli* strain B were purchased from the Sigma Chemical Co., St. Louis, MO.

Sediment and soil samples

Sediment samples were taken from a subtidal mudflat adjacent to the Florida State Marine Laboratory (salinity 25 mg/l, depth 1 m) (29° 54'N; 84° 27.5'W), in three replicate 2.5 cm diameter cores. The cores were immediately put on ice without disturbing the sediment layers, and transported to the laboratory. The 0–1 cm and 10–11 cm horizons were recovered, washed through a 500 μm sieve with 2.5% saline and the sediment extracted.

The ground water aquifer sediments were recovered from the margin of a floodplain of a small creek near Lula, OK (34° 47'N; 96° 45'W) from 2.6 to 5 meters below the surface. The aseptic coring and recovery procedures utilized to protect the ground water aquifer sediments from contamination from surface microbes have been described [14]. Samples of surface soils 3–4.5 cm deep were also taken from this site.

Samples of the Bucatunna Clay, a deep aqualude, were recovered with a Christensen coring device from a test well drilled in Escambia County, northwest Florida (30° 35.68'N; 87° 14.73'W) at a depth of 410 m.

In recovery experiments exposed clay was taken from the surface soils adjacent to the Nuclear Research Building at Florida State University.

Lipid Extraction

Soils, sediments and monocultures were extracted with 8–10-times the volume of the one-phase chloroform-methanol (1:2, v/v) extraction [15]. After at least 2 h one volume of chloroform and one volume of water were added, and the suspension was shaken vigorously. The suspension was centrifuged at 5000 × *g* for 20 min and the supernatant poured into a separatory funnel. After partitioning, the lower chloroform phase was recovered by filtration through a Whatman 2V filter into a round bottom flask and the solvent removed in vacuo on a rotary evaporator. The extracted residues were recovered and lyophilized.

Phospholipid Analysis

The lipid fraction was digested in 23.3% perchloric acid at 180°C for 2 h and the phosphate determined colorimetrically [15].

Hydrofluoric Acid Hydrolysis

Concentrated HF in 50 ml polypropylene centrifuge tubes was placed in the −70°C freezer. After the tubes were thoroughly cooled, lyophilized lipid-extracted residue was added to the tubes with a final ratio of 2 ml HF/g of residue. The tubes were capped and the mixture stirred for 72 h at 4°C with a teflon covered magnetic stirring bar. The sample was then centrifuged at 36 000 × G for 15 min at 4°C and the supernatant, containing the teichoic acid components, was decanted quantitatively into new tubes. The acid-treated residue was washed once with one volume of water and the water added to the supernatant. The HF was removed in a glass desiccator in vacuo over phosphorous pentoxide (P_2O_5) and KOH pellets at room temperature. The glass vessel superficially etches but maintains its strength. The strong vacuum that can be maintained in the desiccator speeds the removal of the HF. Generally the samples were dry after 24–48 h in the desiccator. The vacuum pump was protected by a 5 × 34 cm polyvinylchloride pipe containing sodium fluoride (NaF) in series with a glass trap cooled with dry ice-acetone. After over 100 h of pumping the glass trap showed no evidence of etching.

Hydrochloric acid hydrolysis

Hydrochloric acid, 6 N, was added to dried teichoic acid hydrolysate (0.5 ml/g) and heated to 60°C for 1 h. The hydrolysate was neutralized with 6 N KOH with a final adjustment to pH 7.8–8.0 with 1 N KOH with the formation of a precipitate. The mixture was centrifuged at 36 000 × *g* for 15 min at 4°C, and the supernatant containing the hydrolysate was quantitatively decanted into a glass test tube and reduced to dryness in vacuo at 50°C with the Haake-Buchler rotary evapo-mix solvent remover.

Peracetylation

The internal standard of 0.2 μmol 1,9-nonanediol dissolved in methanol was added to the test tubes and the solvent removed in a stream of nitrogen at 30°C. One ml of acetic anhydride:pyridine (1:1, v/v) was added, the solution mixed with a Vortex mixer for 5 min, and then heated for 2 h at 60°C. After cooling, 2 ml of chloroform was added and the solution partitioned against 2 ml of 20% tartaric acid with vortex mixing followed by centrifugation at 100 × *g*. The aqueous tartaric acid was removed and the chloroform washed twice more with tartaric acid. The chloroform was transferred to a clean test tube and the solvent removed in a stream of nitrogen. The derivatized material was then dissolved in a small volume of chloroform for gas-liquid chromatography (GLC).

Gas-liquid chromatography

The peracetylated materials were analyzed with a Varian 3700 gas chromatograph using a model 8000 autosampler. Data were fed into a programmable Hewlett-

Packard 3552 laboratory data system. One μl of sample was injected onto a 25 m vitreous silica capillary column, 0.2 mm internal diameter, coated with the polar 25% phenyl – 25% cyanopropyl silicone chemically bonded BP-15 phase (Scientific Glass Engineering, Austin, TX). The gas chromatograph was operated in the splitless mode with a 0.5 min venting time. The temperature program was initiated at 80°C and increased to 140°C at 5°C/min which was followed by a 2°C/min rise to 220°C and a 10 min isothermal period. The hydrogen carrier gas, supplied by an Elhygen electrolytic hydrogen generator (LDC/Milton Roy, Riviera Beach, FL) was at a flow rate of 1.5 ml/min at 0.92 kg/cm^2. The detection was by hydrogen flame. Under these conditions the molar response ratio of the peracetylated glycerol and ribitol to the 1,9-nonanediol was 2.0 and 1.5, respectively.

Mass spectrometry

Capillary gas-chromatography/mass-spectral fragmentography was performed with a Hewlett-Packard 5995A GC/MS using the same column and chromatographic conditions as utilized in GLC. The mass spectrometer was autotuned with decafluorotriphenylphosphine and utilized a 70 meV fragmentation energy. Spectra were recorded in the peakfinder scan mode at a scan speed of 380 AMU/s (4 samples/0.1 AMU) between 50 and 350 AMU. The threshold of detectability was set at 100 linear counts at an electron multiplier voltage of 1800 V.

Results

Identification

The peracetylated derivatives of glycerol and ribitol are readily eluted from a 25 m fused silica capillary gas-chromatographic column coated with chemically bonded polar silicon (Fig. 1). The multitude of unidentified components detected in the environmental sample illustrated in Fig. 1 do not contain amino groups as they are not retained on Dowex 50 cation exchange column (hydrogen form).

The putative triacetylglycerol coeluted with an authentic standard and showed major ions at M/z 103 (100%), 145 (76%), 116 (45%), 115 (32%), 86 (19%) and 73 (15%) of the authentic compound in mass spectral fragmentography. Pentaacetylribitol coeluted with authentic standards and showed major ions at M/z 60 (100%), 85 (83%), 98 (52%), 103 (47%), 115 (88%), 145 (45%), 187 (19%) and 217 (10%).

Sensitivity

Using the flame ionization detector operating at a sensitivity of 4×10^{-12} A/mV, 1×10^{-11} mol of triaetylglycerol or pentaacetylribitol gave a response 5-times the background noise level.

Hydrolysis

The relative specificity of cold concentrated HF for hydrolysis of polyglycerol or polyribitol phosphate esters formed the basis of this assay. Fig. 2A shows the optimum release of both glycerol and ribitol from the teichoic acids of 10 mg

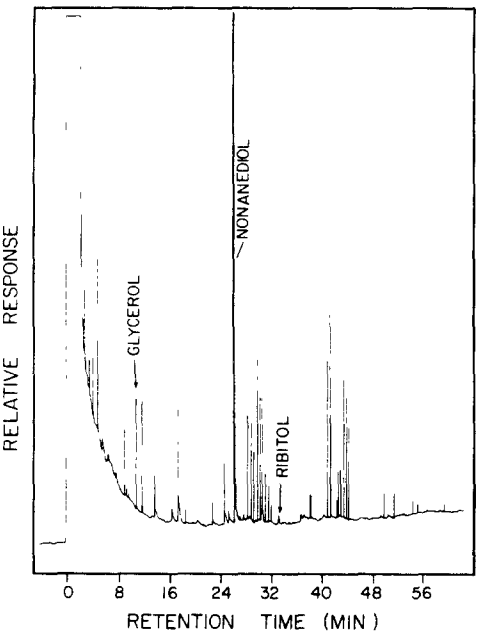

Fig. 1. Chromatogram of triacylglycerol (0.12 nmol), 1,9-nonanediacetyldiol (2.0 nmol), and pentaacetylribitol (0.0086 nmol) recovered from 23 mg dry wt subsurface aquifer clay

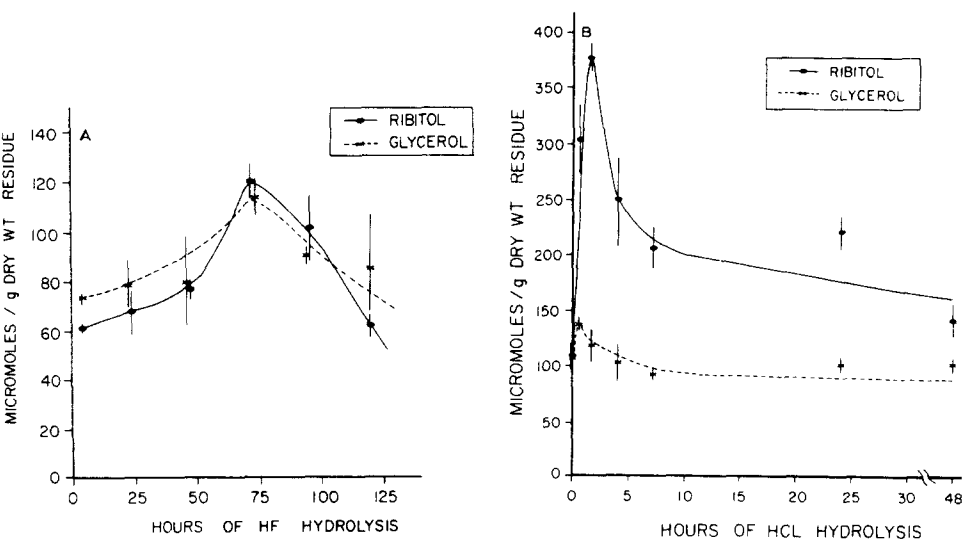

Fig. 2 A. Yield of glycerol (x) and ribitol (•) with exposure of the lipid-extracted residue of *S. aureus* to concentrated HF at 4°C. Mean and standard deviation are shown for each determination, $n = 3$. B. Yield of glycerol (x) and ribitol (•) from lipid extracted residue of *S aureus* exposed to 6 N HCl at 60°C after 72 h exposure to HF.

samples of *S. aureus* from which the lipid had previously been extracted. The optimum recovery occurred after a hydrolysis period of 72 h. The wall teichoic acid contained components covalently linked to the polyglycerol and polyribitol backbones. Continued hydrolysis in 6 N HCl for 1 h yielded further increases in polyol release (Fig. 2B). Treatment with HF for 72 h released 115 ± 8 μmol/g dry wt glycerol and 120 ± 8 μmol ribitol/g dry wt lipid-extracted residue of *S. aureus*. An additional 1 h of HCl hydrolysis increased the yields to 140 ± 14 μmol glycerol and 370 ± 52 μmol ribitol/g dry wt lipid-extracted residue of *S. aureus*. Similar experiments showed that *Bacillus subtilis* contained 22.9 ± 2.5 μmol teichoic acid glycerol/g dry wt.

Recovery from soil

Lyophilized 7–8 mg samples of lipid-extracted residue of *S. aureus* were thoroughly mixed with 2 g of surface clay taken from a fill site adjacent to the laboratory. The recovery of teichoic acid glycerol and ribitol after HF and HCl hydrolysis from the mixture was 114 ± 11% and 123 ± 47% of that expected (Table 1). Despite the formation of silicon tetrafluoride with the exposure of sand to HF, it was possible to recover 86 ± 10% of the glycerol and 81 ± 14% of the ribitol of added *S. aureus* from 10 g of Mallinckrodt washed and ignited sand.

Teichoic acid-containing bacteria in environmental samples

The proportion of the total microbes that form teichoic acids increases with increasing depth in both sediments and soils (Table 2). The total microbial biomass which includes both prokaryotes and microeukaryotes was measured by the total phospholipid [15], and the ratio of glycerol from teichoic acid to phospholipid increased with depth. Soils contain a higher proportion of organisms forming teichoic acids than marine sediments (Table 2).

Not only do the teichoic acid-containing bacteria increase with depth but the ratio of glycerol to ribitol teichoic acids increases markedly in bacteria from deeper horizons in the soil. A surface clay sample from near the laboratory contained 15 ± 2 nmol teichoic acid glycerol and 209 ± 76 nmol teichoic acid ribitol/g dry wt.

TABLE 1

RECOVERY OF ADDED *S. AUREUS* TEICHOIC ACID GLYCEROL AND RIBITOL FROM CLAY

Sample	Teichoic acid glycerol (μmol/g dry wt)	Teichoic acid ribitol (μmol/g dry wt)
S. aureus residue	0.91 ± 0.29[a]	3.12 ± 0.98
Clay	0.03 ± 0.003	0.42 ± 0.15
Expected recovery	0.95 ± 0.26	3.6 ± 1.04
Measured recovery[b]	1.02 ± 0.22	3.57 ± 1.11

[a] Data expressed as mean ± standard deviation, $n = 3$.

[b] Measured from a mixture of 8 mg lyophilized residue of lipid-extracted *S. aureus* plus 2 g dry wt subsurface clay.

TABLE 2

PROPORTION OF BACTERIA CONTAINING GLYCEROL TEICHOIC ACID IN SURFACE AND SUBSURFACE SEDIMENTS AND SOILS

Sample	Teichoic acid glycerol (nmol/g dry wt)	Total phospholipid (nmol/g dry wt)	Teichoic glycerol/ phospholipid (nmol/g dry wt)
Marine sediment			
Surface	4 6 ± 1 0[a]	45 ± 6	0.10 ± 0 04
Subsurface (10–11 cm)	3.4 ± 0 4	14 ± 1	0 24 ± 0 03
Soil·			
Surface clay	37 ± 16	2 1 ± 0 16	18 ± 7
Subsurface (2–3 m)	6 ± 3	0.07 ± 0.02	85 ± 24
Subsurface (410 m)	29 ± 1	0 5 ± 0.1	55 ± 3

[a] Data expressed as mean ± standard deviation, $n = 3$

The Bucatunna clay recovered from 410 m below the surface contained 29 ± 1 nmol teichoic acid glycerol and 2.8 ± 0.7 nmol teichoic acid ribitol/g dry wt. This represents an increase in the ratio of glycerol to ribitol teichoic acids of 500-fold.

Measurements of bacterial biomass

Estimating the bacterial biomass by measuring the muramic acid for the total bacteria [3, 16], the hydroxy fatty acids from the LPS-lipid A for the gram-negative

TABLE 3

ESTIMATES OF THE MICROBIAL BIOMASS OF SEDIMENTS AND SUBSURFACE SOILS

Analysis	Estuarine sediment	2–6 m Subsurface soil (nmol/g dry wt)	410 m Subsurface soil (nmol/g dry wt)
Muramic acid	22 ± 0.3[a]	2.1 ± 1.24	0.62 ± 0.06
Glycerol teichoic acid	3.2 ± 1.0	4.3 ± 0.8	29 ± 0 1
LPS-Lipid A OHFAME	2.3 ± 0.3	0.15 ± 0.07	0 09 ± 0.04
Phospholipid	26 ± 7	1.0 ± 0 2	0.52 ± 0 1
Phospholipid FAME	77.6 ± 6.4	5.33 ± 4 4	1 07 ± 0.18
Estimated number of bacteria at 10^{12}/g dry wt			
Muramic acid[b]	2.5–7.5 × 10^8	2.5–7.1 × 10^7	0.7–2 1 × 10^7
Glycerol teichoic acid[c]	2.2 × 10^7	3.1 × 10^7	2 1 × 10^8
LPS-Lipid A OHFAME[d]	1 5 × 10^8	1.0 × 10^7	5 × 10^6
Phospholipid[e]	5.2 × 10^8	2 × 10^7	1 4 × 10^7
Phospholipid FAME[f]	7.7 × 10^8	5.3 × 10^7	1 1 × 10^7

[a] Values expressed as $X \pm$ S.D., $n = 4$.

[b] Estimated as 29 μmol muramic acid/g dry wt (gram-negative), 88 μmol muramic acid/g dry wt (gram-positive) [3].

[c] 140 μmol glycerol teichoic acid/g dry wt.

[d] 15 μmol hydroxy fatty acid methyl esters (OHFAME)/g dry wt [6].

[e] 50 μmol phospholipid/g dry wt [15].

[f] 100 μmol phospholipid fatty acid methyl esters (FAME)/g dry wt [17]

bacteria [6], the teichoic acid glycerol and ribitol for the gram-positive bacteria [this paper], the phospholipids [15] and ester-linked fatty acids [17] provides quantitative description of the microbial community (Table 3). The factors for the conversion of these measurements into actual numbers of bacteria are based on measurements of bacterial monocultures. The estimates of the bacterial biomass using each component are consistent. The ratio between the organisms estimated as gram-positive (teichoic acids) to gram-negative (LPS-lipid A hydroxy fatty acids) increases with increasing depth of soil. The ratios of gram-positive to gram-negative bacteria as estimated from the data of Table 3 are 0.15 for the surface estuary, 3.1 for the shallower subsurface soil, and 4.1 for the deep subsurface soil.

Single analysis for gram-negative and gram-positive bacteria

Sequentially treating washed and ignited sand, in which lyophilized and lipid-extracted *S. aureus* and *E. coli* had been thoroughly mixed, with cold concentrated HF followed by a mild acid hydrolysis and lipid extraction for the LPS lipid A hydroxy fatty acids (gram-negative bacteria), resulted in a loss of the 3-hydroxy myristic acid from the *E. coli*. Reversing the procedure, with a mild acid hydrolysis followed by lipid extraction for the lipid A hydroxy fatty acids, and then with the cold concentrated HF hydrolysis, resulted in quantitative recovery of the teichoic acid glycerol but a loss of 80 ± 16% of the teichoic acid ribitol of the *S. aureus*. Adding a second stronger acid hydrolysis of the water extract after the HF hydrolysis did not increase the yield of ribitol. Recoveries of added glycerol or ribitol indicated there were no significant losses in the procedures.

Discussion

The specificity of the cold concentrated HF hydrolysis for the phosphodiester bonds of the teichoic acid makes possible the recovery of glycerol and ribitol from the lipid-extracted residue of soils and sediments. Because of the restricted localization of ribitol in nature it becomes an especially valuable 'signature' for a portion of the gram-positive microbial community. The removal of the lipids with their content of glycerol [14] makes the HF-releasable glycerol an adequate marker. The relative immunity of the glycerol from the lipoteichoic acids to the chemical pleomorphism induced by growth with phosphate limitation [8, 9] increases the value of the HF-releasable glycerol as an indicator of gram-positive bacteria. With detection limits of 10 pmol it is possible, with the assay described here, to detect the presence of 10^4 bacteria with the teichoic acid content of *S. aureus*. The rapid turnover of the teichoic acids in monocultures [9, 18] indicates that the HF-releasable glycerol and ribitol are measures of the 'active' biomass much like the muramic acid. Both isolated cell walls and inactive whole bacteria are rapidly degraded in nature [19, 20].

With this method in hand it was possible to show that the proportion of gram-positive cells increases as the depth of the soil or sediment increases (Table 2). Soils have a higher content of gram-positive bacteria than sediments. Initial studies indicate that contamination of the ground water aquifer sediments with phenols decreases the proportion of gram-positive bacteria.

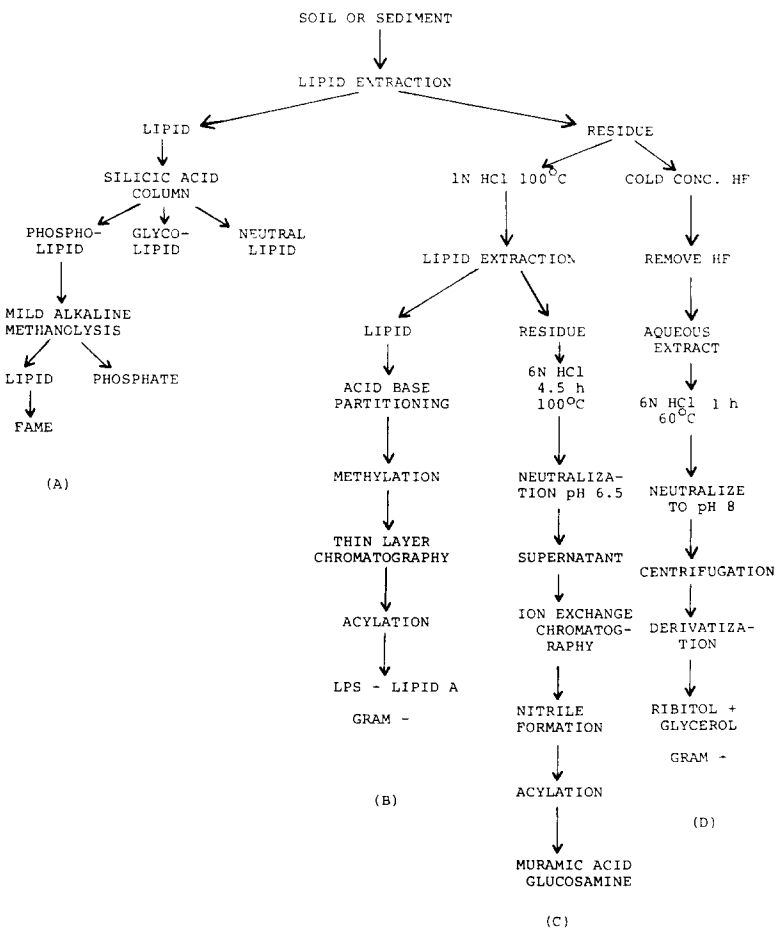

Fig. 3. Scheme for the analysis of phospholipid (A) from the lipid extraction [15]; LPS-lipid A hydroxy fatty acids (B) after mild acid hydrolysis and extraction [9]; muramic acid and glucosamine (C) after strong acid hydrolysis, neutralization, and column chromatography [6], and glycerol and ribitol from the teichoic acids (D) after HF and HCl hydrolysis [this paper].

The analysis for teichoic acid can be combined with other measures of microbial biomass to provide a comprehensive measure of the gram-negative bacteria, the gram-positive bacteria, the total bacteria, and the total microbial (bacterial plus microeukaryotic) biomass as illustrated in Fig. 3. The sediment or soil is lyophilized and extracted with the one phase chloroform-methanol extraction procedure as described above. The lipid is recovered from the organic phase and fractionated by column chromatography on silicic acid into neutral lipids, glycolipids and phospholipids, by successive elutions with chloroform, acetone and methanol as described. The glycerol and glycerol phosphate released by acid hydrolysis can then be determined [16].

The residue of the soil or sediment remaining after the lipid extraction is divided

in half. The first portion is subjected to mild acid hydrolysis (1 N HCl for 4.5 h at 100°C) and extracted again with the one phase chloroform-methanol solvent. The lipid fraction is dissolved in petroleum ether, the fatty acids extracted with aqueous base, and then recovered in chloroform after titration of the aqueous phase to acid pH. Methyl esters are then formed and the components purified by thin layer chromatography. The band corresponding to the monohydroxy fatty acid methyl esters is then recovered, acylated with heptafluorobutyric anhydride and analyzed by GLC for the lipopolysaccharide-lipid A hydroxy fatty acids as described by Parker et al. [6]. This is utilized as a measure of the gram-negative bacteria.

The residue left from the hydroxy fatty acid analysis for gram-negative bacteria was then hydrolyzed in strong acid (6 N HCl for 4.5 h at 100°C), the hydrolysate neutralized, and centrifuged. The supernatant after purification by ion exchange chromatography was derivatized and analyzed for its muramic acid and glucosamine content as described by Findlay et al. [3].

The second portion of the lipid-extracted residue was hydrolyzed in concentrated HF for 72 h at < 4°C, the HF removed, the suspension neutralized, centrifuged, and the supernatant fractionated with a cation exchange column. The neutral (uncharged) components recovered from the ion exchange column were acetylated and analyzed by GLC as described above.

The hydrolysis conditions necessary for the release of the lipopolysaccharide lipid A hydroxy fatty acids (a measure of gram-negative bacteria) and the HF and secondary acid hydrolysis of the lipid-extracted residue for the teichoic acids for the gram-positive bacteria are mutually exclusive. By splitting the sample and combining the analysis of the lipids as illustrated in Fig. 3, it is possible to define the community structure of a sediment (Table 3). With the addition of GLC analysis of the lipid components it proved possible to show the presence of an unusual bacterial flora of a ground water aqualude clay that was recovered from 410 m below the surface of the soil [21].

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