



Rapid screening method for quantitation of bacterial cell lipids from whole cells

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Abstract

Although specific lipids in bacteria can be quantitated, there is still a need to quantitate the total lipid content of a bacterial sample. The sulfo-phospho-vanillin reaction for quantitation of bacterial lipids has significant advantages over traditional methods for screening of engineered mutant strains. In this report we show that this methodology can be used directly on whole cell or homogenized biological material, without any extraction step. The cell components, and most of the reagents used for cell extraction, that were tested did not interfere with the reaction. The screening is based on the observation of physiologic variations using ratios of relative amounts: lipid/DNA and lipid/protein. Our results show that significant differences in those ratios can be detected when there is a modification of the phospholipid content of the cell. The sample manipulation required is minimal and could be automated. Used as a primary screening and/or characterization of engineered mutant strain, the test may lead to further investigation of the nature and distribution of lipids in the cell.

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1. Introduction

Bacterial membranes are complex, involved in a wide variety of functions, and known to contain a wide variety of lipids. Details on the type and the composition of microbial lipids can be found in review articles by O'Leary (1974) and Langworthy

(1985). The nature of the different components of membranes can change as a function of physiological and environmental changes (Sajbidor, 1997; Veld et al., 1993). The lipids are involved in protein secretion, solute transport, protein folding and topology, DNA replication, cell division, energy production, cell integrity and defense, pathogenicity, and immunogenicity (Binenbaum et al., 1999; Bogdanov and Dowhan, 1998; Mileykovskaya et al., 1998; Niedhardt, 1996; Sonenshein et al., 1993; van Klompenburg et al., 1997).

Genomics, proteomics, and other techniques can provide significant volumes of data but the process of screening bacterial mutant strains and of testing po-

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tentially effective drugs can be laborious. While there are numerous methods by which to quantitate DNA and proteins in the sample, it is still relatively difficult to quantitate lipids from a large number of engineered mutants. Current methods for quantitation of lipids in bacterial cells require an extraction step. This step allows precise quantitative and qualitative analysis of extracted lipids (for examples, see Gerhardt et al., 1994). However, the extraction step may require a significant amount of starting biological material, is partially selective, and does not include lipoproteins and lipopolysaccharides. Moreover, the extraction step is time consuming and should be used for only a limited number of mutants.

The color reaction given by lipids with vanillin in a medium of sulfuric acid and phosphoric acid was reported by Drevon and Schmit (1964). The colorimetric method based on the sulfo-phospho-vanillin reaction has been used for the determination of total serum lipids in humans (Drevon and Schmit, 1964; Frings and Dunn, 1970; Tietz, 1982). The major advantage of this technique is to avoid a lipid separation step. The term “total lipids” usually refers to all lipid material that can be extracted readily from a specimen. In this paper we have adopted the sulfo-phospho-vanillin method to quantitate bacterial lipids in extracted and nonextracted samples.

The proposed method is for screening using quantitative and comparative analysis. This methodology uses a small amount of biological material, does not require a lot of sample manipulation, can be automated, is reproducible, easy to implement, and rapid. Applications, other than screening, may include the quantitation of total cell lipids, and the observation of physiologic variation by comparison of ratios of relative amount between lipid, DNA, and protein. It is a first screening and characterization test that may lead to further investigation of lipid nature and distribution in the cell by other investigational methods, including chromatography and fluorescence microscopy.

2. Materials and methods

2.1. Strains, reagents, culture and molecular methods

Escherichia coli AD93 (William Dowhan, Houston, TX) (DeChavigny et al., 1991), and its parent

strain, *E. coli* W3899 (*E. coli* Genetic Stock Center, Yale University, New Haven, CT), were cultivated in Luria-Bertani (LB) broth containing 50 mM MgCl₂, at 37 °C. The strain AD93 is derived from the strain W3899, using phage P1 mutagenesis to inactivate *pss* and *recA* genes (DeChavigny et al., 1991). *Treponema denticola* ATCC33520 was grown in New Oral Spirochete medium (NOS) with 10% heat-inactivated rabbit serum and 10 µg/ml cocarboxylase, at 36 °C, in an anaerobic chamber (Coy Laboratory Products, Grass Lake, MI) with an atmosphere of 85% nitrogen, 10% carbon dioxide, and 5% hydrogen.

Bovine serum albumin fraction V was from Pierce Chemical (Rockford, IL). Anhydrous ethanol was from Pharmco Products (Brookfield, CT). Hoechst 33258 (bisbenzimidazole) was from Molecular Probe (Eugene, OR). Tris and urea were from Bio-Rad Laboratories (Melville, NY). Phosphate-buffered saline was from GIBCO/BRL Life Technologies (Grand Island, NY). NaCl, MgCl₂, MgSO₄, ethylenediaminetetraacetic acid (EDTA disodium salt, dihydrate), dithiothreitol (DTT), anhydrous glycerol, methanol, phosphoric acid 85%, sulfuric acid, dextrose (alpha-D(+)-glucose), sodium phosphate monobasic, sodium phosphate dibasic, vanillin (4-hydroxy-3-methoxybenzaldehyde), lipopolysaccharide *E. coli* serotype 0127:B8, 3-*sn*-phosphatidylcholine 1,2-dilauroyl (1,2-didodecanoyl-*sn*-glycero-3-phosphocholine), L- α -phosphatidyl-DL-glycerol β -oleoyl- γ -palmitoyl (1-palmitoyl-2-oleoyl-*sn*-glycero-3-phospho-*rac*-(1-glycerol) ammonium salt), L- α -phosphatidylethanolamine type V from *E. coli* (1,2-diacyl-*sn*-glycero-3-phosphoethanolamine), cardiolipin sodium salt from bovine heart (diphosphatidylglycerol), tripalmitin, and triolein (1,2,3-tri[*cis*-9-octadecenoyl]glycerol) were from Sigma (St. Louis, MO). NaPi (0.5 M, pH 7.2) is a 0.5 M sodium phosphate monobasic solution, pH adjusted at 7.2 using a 0.5 M sodium phosphate dibasic solution.

Plasmid DNA (pTOPOCFPAERM) (Izard et al., 2001) was prepared by standard methodology (Maniatis et al., 1982). *T. denticola* ATCC33520 RNA was prepared following the RNaid Plus kit manufacturer protocol (Bio101, California).

For protein quantitation, the manufacturer's protocol of the Coomassie Protein Assay Reagent kit from Pierce Chemical was used. Bovine serum albumin was the protein assay standard. A Hoefer Scientific

Instruments (San Francisco, CA) TKO100 DNA fluorometer was used for DNA quantitation following the manufacturer's protocol for low range assay. Purified phage lambda DNA from New England Biolabs (Beverly, MA) was the DNA assay standard.

2.2. Dosage of lipids using phosphoric acid–vanillin reagent

To a stoppered glass tube, 100 µl of water (reference tube), 100 µl of the sample, or 100 µl of triolein standard dissolved in chloroform was added. The chloroform was evaporated using a nitrogen flux, and 100 µl of water were added. To each tube, two ml of sulfuric acid 18 M were added. The tubes were incubated in a boiling water bath for 10 min, and cooled for 5 min in a water bath at room temperature. Five milliliters of phosphoric acid–vanillin reagent were added to the tubes and incubated at 37 °C for 15 min. To prepare the phosphoric acid–vanillin reagent, 0.120 g of vanillin was added to 20 ml of water, and the volume adjusted to 100 ml with 85% phosphoric acid. The tubes were then cooled for 10 min in a water bath at room temperature. The optical density at 530 nm was read in a glass cuvette against a reference tube with 100 µl water as sample. The reference curve is composed of Triolein ranging from 10 to 100 µg with an increment of 10 µg.

2.3. Preparation of crude extract

Ten milliliters of a bacterial cell culture at an optical density of 0.5 at 660 nm was centrifuged at 13000 × g for 1 min. The cells were washed twice by resuspension of the pellet in the same volume of 10 mM sodium phosphate at pH 7.2 and centrifugation at 13000 × g for 1 min. The pellet was then resuspended in buffer at 1/20th of the starting volume for *T. denticola*, and at 1/60th of the starting volume for *E. coli*. A fraction was used directly to be tested (see above). The cell suspension was then sonicated for 1 min, using a W-385 sonicator from Heat Systems-Ultrasonics (Framingdale, NY). The final concentration of the sample is 1 optical density equivalent per 100 µl for *T. denticola*, and 3 optical density equivalent per 100 µl for *E. coli*. For quantitation of whole cell or crude cell extract,

0.3–3 optical density equivalent for *E. coli* and 0.25–2 optical density equivalent for *T. denticola*. A minimum of 3 points of different amounts is used for quantitation.

2.4. Methanol–chloroform extraction

The methanol–chloroform extraction (Ames, 1968; Gerhardt et al., 1994) was performed after the sonication step. The methanol and chloroform were added to the sample with a ratio (2:1:0.8) (v/v). After mixing and a 10-min incubation at 4 °C, the lipids were separated from the water-soluble material by dilution of the extraction mixture with one volume of chloroform followed by one volume of water. The sample was then centrifuged for 15 min at 3000 × g. The chloroform layer was removed completely by gently inserting a pipette through the water–methanol phase and the material that collect

Table 1
Substances not affecting the sulfo-phospho-vanillin reaction

Substance tested	Compatible concentration/tested up to
DNA	tested up to 172 µg
dNTPs	< 1 mM
Protein (BSA) ^a	tested up to 200 µg
RNA	tested up to 10 µg
<i>Chelating agent</i>	
EDTA, pH 8.0	tested up to 0.5 M
<i>Reducing and thiol-containing agents</i>	
DTT	< 10 mM
Glucose	< 18 mg (< 0.1 mM)
2-Mercaptoethanol	tested up to 0.5 M
<i>Salts and buffers</i>	
MES, pH 6.0	tested up to 0.5 M
MgSO ₄	tested up to 1 M
NaCl	< 0.5 M
NaPi, pH 7.2 ^b	tested up to 0.5 M
PBS ^c	tested up to 1X
Tris–HCl, pH 7.2	tested up to 1 M
<i>Miscellaneous reagents and solvents</i>	
Ethanol, anhydrous	< 70%
Glycerol, anhydrous	< 3 µg
Methanol	tested up to 100%
Urea	< 8 M

^a Bovine serum albumin.

^b Phosphate buffer.

^c Phosphate-buffered saline.

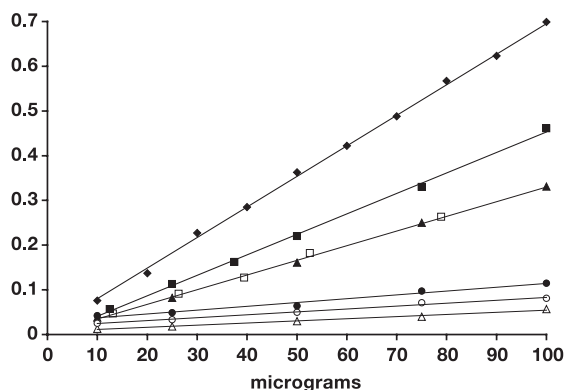


Fig. 1. Triolein reference curve and linearity of the sulfo-phospho-vanillin test for other substances. Triolein (◆), L- α -phosphatidyl-DL-glycerol β -oleoyl- γ -palmitoyl (■), L- α -phosphatidylethanolamine (▲), bovine heart cardiolipin (□), tripalmitin (●), lipopolysaccharide *E. coli* serotype 0127:B8 (○) and 3-*sn*-phosphatidylcholine 1,2-dilauroyl (△).

at the interphase. The chloroform extract was used immediately after removal.

3. Results

DNA, RNA, and proteins did not detectably react or interfere with the sulfo-phospho-vanillin reaction (Table 1). The reagents used for cell extraction as described in this report also did not affect the assay (Table 1). We noted that SDS, which is used in some bacterial lysis protocols, does react with the sulfo-phospho-vanillin reagent.

The sulfo-phospho-vanillin reaction detected microgram level of lipids (Fig. 1). The wavelength for reading the absorbance of the sample and of the reference was 530 nm. It was selected on the basis of the wavelength of maximal absorption of the tested synthetic lipids and purified lipids from biological material (data not shown).

Each lipid was not detected with the same color intensity in function of their concentration (Table 2; Fig. 1). For the same amount by weight or by concentration, the reading varied as a function of the lipids' chemical characteristics (Table 2). The color reaction for all the substances detected was linear (Fig. 1).

There was no significant difference in the quantity of lipids detected between *T. denticola* cells resuspended in buffer or the same sample sonicated in the

same buffer (Fig. 2). However, there was a significant decrease in the amount of lipids detected in the case of the lipids extracted by a methanol–chloroform extraction, as compared to the amount of lipids detected in cells resuspended in buffer or sonicated in the same buffer (Fig. 2). The values obtained from three independent measurements, each for 1 unit of optical density (OD) at 660 nm of *T. denticola* cells were as follows: 46.10 ± 0.29 for the lipids extracted by a

Table 2

Amount in equivalent microgram of triolein of different substances detected by the sulfo-phospho-vanillin reaction, using the triolein reference curve

Substance	Assay value (μg) measured for 50 μg dry weight	Amount in μg (calculated for 100 nmol) ^a
Triolein	50	88.54
SDS	32.20	18.57
L- α -Phosphatidyl-DL-glycerol β -oleoyl- γ -palmitoyl	27.00	41.37
Cardiolipin from bovine heart	20.94	NA
L- α -Phosphatidylethanolamine type V from <i>E. coli</i>	19.96	NA
Tripalmitin	10.16	16.41
Lipopolysaccharide from <i>E. coli</i> serotype 0127:B8	7.13	NA
3- <i>sn</i> -Phosphatidylcholine 1,2-dilauroyl	3.86	4.80

^a This column reflects the relative molar color yield for each substance.

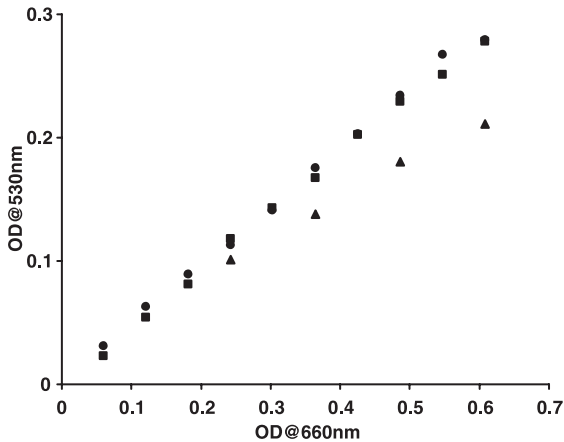


Fig. 2. Quantitation of lipids by the sulfo-phospho-vanillin method of *T. denticola* cells resuspended in buffer (●), sonicated in buffer (■), and cell lipids extracted by methanol–chloroform extraction (▲). The curve points have been obtained from the dilution of one sample prepared as described in Materials and methods. The axis scale represents the amount of sample converted as equivalent optical density (see Materials and methods).

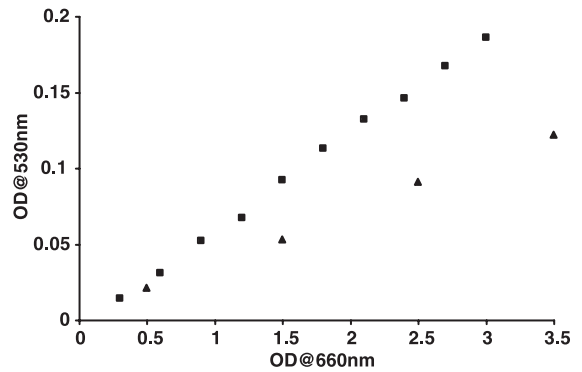


Fig. 3. Quantitation of lipids by the sulfo-phospho-vanillin method of *E. coli* cells sonicated in buffer (■), and cell lipids extracted by methanol–chloroform extraction (▲). The curve points have been obtained from the dilution of one sample prepared as described in Materials and methods. The axis scale represents the amount of sample converted as equivalent optical density (see Materials and methods).

methanol–chloroform extraction, 60.63 ± 1.15 for cells resuspended in buffer, and 59.91 ± 0.25 equivalent micrograms of triolein for the sonicated cells.

With *E. coli* cells, there was also a significant decrease in the amount of lipids detected, after methanol–chloroform extraction, as compared to the amount of lipids detected in cells resuspended in buffer (Fig. 3). The value obtained from three independent measurements for 1 OD at 660 nm for *E. coli* cell with the lipids extracted by a methanol–chloroform extraction was 4.41 ± 0.002 equivalent microgram of triolein, and for *E. coli* sonicated cells in buffer the value was 7.85 ± 0.01 equivalent microgram of triolein.

In the *E. coli* AD93 strain, the phosphatidylserine synthase gene is inactivated (DeChavigny et al., 1991). Consequently, there is over 99% decrease in phosphatidylethanolamine production (DeChavigny et al., 1991). The parent wild-type (W3899) and the mutant AD93 strains were tested using our methodology. Quantitation of total lipid, protein, and DNA was done as described in Materials and methods. To compare the data obtained, the ratio between the different components was analyzed (Fig. 4). The lipid/protein ratio was calculated using microgram

equivalent triolein per milligram equivalent of bovine serum albumin. The DNA/lipid ratio was calculated using nanogram equivalent lambda phage DNA per microgram equivalent triolein. The DNA/protein ratio was calculated using nanogram of equivalent lambda phage DNA per microgram equivalent bovine serum albumin. There was a significant difference between the wild-type and the mutant strains (Fig. 4). For the AD93 mutant, there was a decrease in the amount of lipids detected per milligram of protein in the cell. There was an increase in the amount of DNA measured per microgram of lipid detected in the mutant

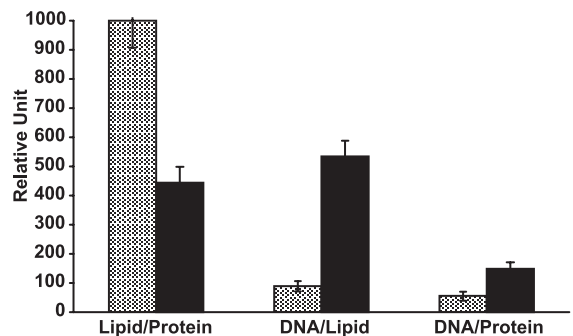


Fig. 4. Lipid, protein, and DNA ratios measured for *E. coli* W3899 wild-type (shaded column) and AD93 mutant (black column) strains.

cells. There was also an increase of the amount of DNA measured per microgram of protein in the cell.

4. Discussion

The screening method described in this report allows quick quantitation of lipids from bacterial samples without an extraction step. The sample containing lipids is heated with concentrated sulfuric acid, and then vanillin and phosphoric acid are added to yield a pink-colored product. The chemical reactions that form the basis of this method remain unknown. One assumption is that the unsaturated components of a lipid specimen first become oxidized to ketones, and then the ketones condense with vanillin or a derivative of the vanillin under the influence of acid catalysis. Following the assumed condensation reaction, dehydration of an aldol-type intermediate is further assumed to yield a more highly unsaturated product that absorbs visible light (Tietz, 1982).

The color yield among lipids varies. For example, the color yield from triolein is greater than that of tripalmitin (Table 2). Previous work has shown that the color yield from oleic acid is greater than the yield from linoleic acid, and both of those acids produce greater color yields than does cholesterol (Tietz, 1982). The variation of color yield limits the accuracy of the method. However, a modification of the proportion of each lipid will modify the amount of total lipid measured by reference to the triolein curve, and will be detectable. Changes in the constitution and amount of lipids per cell are detectable with this technique, as we have shown using a phosphatidylethanolamine-deficient mutant of *E. coli*. The detection of a change between two strains does not provide information on the type of modification. Consequently, further investigations should be required, those tests may include the isolation of classes and/or the identification of specific lipids. Those tests should provide the nature of the lipids, the relative amounts of extractable lipids, or the nature of the lipids complexed to macromolecules.

The typical extraction of lipids uses a two-phase separation that requires a significant amount of biological material as well as a significant amount of manipulation of the biological material preceding the quantitation (Gerhardt et al., 1994). The amount of

quantifiable extracted material by the sulfo-phosphovanillin reaction is lower after methanol–chloroform extraction than that in the whole cell, sonicated or not (Fig. 3). The significant difference observed could be related to the amount of lipids that are associated with lipoproteins, not extractable, or part of the lipopolysaccharide family. There was no significant difference between the amount of lipids quantitated in cells resuspended in buffer or sonicated in the same buffer.

The choice of triolein as a standard was arbitrary. Triolein is an unsaturated lipid that reacts with a good yield. It is easy to manipulate, inexpensive, and can be purchased already quantitated. It requires a minimum of manipulation to prepare a reference standard curve, and the experiment is adaptable to a laboratory not specialized in lipid chemistry. The small volume of sample required (0.1 ml), in addition to the simplicity, speed, and reliability of the proposed method, makes it suitable for small- and large-scale analyses. No extraction is necessary and the method can be automated. The testing may be integrated in a high-throughput screening process.

The OD measurement was used to monitor the growth of pure culture. For each specific bacterial strain, correlation was obtained between the absorbance and the amount of organisms per milliliter of culture. It is possible to represent the data as OD_{530 nm} (quantity of lipids measured) vs. OD_{660 nm} as well as OD_{530 nm} vs. number of cells, since for a given organism there is a relation between the absorbance and the number of organism. The number of cells could also be substituted by the amount of total protein or other parameter used in the high-throughput screening. Different absorbances can be observed for the same amount of organism per milliliter as a function of the size and shape of bacterial cells. Therefore, the different absorbances found per particle (cell) may be reflected in the different amount of total lipid quantitated for different organisms. The long wave-shaped *T. denticola* cell differs significantly from the rod-shaped *E. coli* cell, and it does not have the same absorbance. Thus, the amount of starting material required for total lipid measurement of *E. coli* differed from *T. denticola*. The equivalent microgram of triolein per OD unit is higher for *T. denticola* than for *E. coli*.

Phosphatidylethanolamine is the only zwitterionic phospholipid in *E. coli*. It accounts for 70–80% of the

total glycerophospholipids of this organism (DeChavigny et al., 1991). In strain AD93, the inactivation of the phosphatidylserine synthase, which catalyzes the committed step to the synthesis of phosphatidylethanolamine, results in a mutant with traces of phosphatidylethanolamine (DeChavigny et al., 1991). There is a small decrease in the total phospholipid content in the mutant cell. The absence of zwitterionic phospholipid is compensated by an increase in phosphatidylglycerol and cardiolipin to maintain the normal phospholipid to protein ratio in the cell membrane (DeChavigny et al., 1991). There is a reorganization of the lipid content in the membranes. Our results show a decrease of over twofold in the ratio of lipid/protein between the wild-type and the mutant strain. We were able to detect a modification of the amount of the lipid detected, for a fixed amount of total protein measured. Moreover, we were able to show a significant increase of the DNA/lipid ratio between the wild-type and the mutant strain. Previous experiments have shown that the mutant is filamentous and shows some cell division defects (Mileykovskaya et al., 1998). It has been proposed that the cell division defects are due to the loss of phosphatidylethanolamine, or to the drastic modification of the ratio of phosphatidylethanolamine to anionic phospholipids (Mileykovskaya et al., 1998). The sulfo-phosphovanillin method detects each lipid with a different yield, and detects all lipids present in a sample. Consequently, if the proportion of each lipid differs between a mutant strain and the wild-type grown in the same conditions, our methods should detect the change in lipid composition. It would be the first screening step to warrant further investigations.

One direct application of this method is to screen randomly mutated strains obtained by different techniques including transposition (Akerley et al., 1998), by analyzing biochemical phenotypic characteristics in a defined medium. The sulfo-phosphovanillin method can be used in conjunction with the quantitation of other cell components, like DNA and proteins, using the same sample. This analysis of the different ratios can be used as an indicator of significant physiological conditions altering lipid, protein, or DNA synthesis in the mutant strain when compared to the wild-type strain. The detectable phenotypes are associated with lipid synthesis alteration, or alteration of the cell regulation, or the cell division process. The same

techniques can be used for analysis of the biochemical effects of chemical products blocking major pathways, or during the screening process, or the evaluation of a drug. This technique will provide a biochemical overview of the changes in expression caused by a compound, stress, or medium, and it is complementary to other proteomic techniques.

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