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Isolation and Characterization of an Ornithine-Containing Lipid from *Desulfovibrio gigas*

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The isolation and characterization of an ornithine-containing lipid obtained from *Desulfovibrio gigas* are reported. The general structure for this aminolipid is represented by NH_2-CH_2-$(CH)_2$-CHNH[CO-CH_2CH(O-COR^2)-R^1]-COOH, where R^1 represents 3-hydroxy palmitate linked through an amide bond to the α-amino group of ornithine, and R^2 represents a complex variety of fatty acids esterified to the hydroxyl group of 3-hydroxy palmitate. Fatty acids characterized were *n*-$C_{14:0}$ (21%), iso-$C_{14:0}$ (14%), anteiso-$C_{15:0}$ (43%), *n*-$C_{16:0}$ (2%), *n*-$C_{18:0}$ (8%), and *n*-$C_{18:1}$ (11%). The quantitative relationships between aminolipid and phospholipids showed the aminolipid to represent the major polar lipid. Isolation of the cytoplasmic and outer membranes of *D. gigas* showed the aminolipid to be evenly distributed between both membrane fractions, suggesting a compensatory role in phospholipid-deficient membranes.

A number of ornithine-containing lipids have been obtained from diverse microorganisms. An atypical mycobacterium was reported as yielding an ether-soluble ornithine derivative after saponification (10). *Rhodopseudomonas spheroides* was shown to contain a non-saponifiable, ornithine-containing lipid (3), and the occurrence of alkali-stable, ornithine-containing lipids has been reported for *Rhodomicrobium vannielii* (19), *Rhodospirillum rubrum* (2), *Pseudomonas rubescens* (26), and several other *Pseudomonas* species (27). *Streptomyces sioyaensis* was also described as containing both a lysine- and ornithine-containing lipid (7, 8). An ornithine-containing lipid has been characterized from two species of *Brucella* and from *Bordetella pertussis* (24, 25). Shively and Knoche have reported on the isolation and characterization of an ornithine-containing lipid from *Thiobacillus thiooxidans* (9, 23). This report describes the novel isolation and characterization of an ornithine-containing lipid from *Desulfovibrio gigas*.

MATERIALS AND METHODS

Organism. *D. gigas* was grown by the method of LeGall et al. (11). Cultures were harvested by centrifugation and washed in 50 mM phosphate buffer, pH 7.5.

Extraction of lipids. The extraction of total cellular lipid was described previously, including the procedures used for thin-layer (TLC) and column chromatography (14). TLC plates were developed in the following solvent systems unless otherwise specified: (i) solvent A, chloroform-methanol-water (65:25:4, vol/vol/vol); and (ii) solvent B, chloroform-methanol-concentrated NH_4OH (65:30:2, vol/vol/vol).

Analysis of fatty acids. Fatty acid methyl esters were identified with a Packard gas chromatograph, series 7500, consisting of a dual-column oven with coiled glass columns (4-mm inside diameter, 1.83 m long). The detection system was an argon ionization detector with column support systems consisting of 10% Apiezon L or 20% diethyleneglycol succinate (DEGS) on 70-80 AnaKrom A (AnaLabs, Inc.). Operating conditions were: column temperature, 150 or 180 C; detector temperature, 190 C; injection temperature, 180 C; outlet temperature, 205 C; argon flow rate, 50 ml/min; chart speed, 2.5 min/inch (ca. 2.54 cm). Fatty acids were methylated as described previously (13), and peak areas were determined by triangulation. All fatty acid identifications were substantiated by 10% Apiezon and 20% DEGS.

A Dupont mass spectrometer, model 21490, coupled to a Varian gas chromatograph, series 1200, was used to obtain the mass spectra. The column contained 20% DEGS (2-mm diameter, 3.7 m long).

Mild alkaline hydrolysis. The aminolipid was treated with 0.2 M KOH in methanol for 2.5 h at 37 C. The procedure of Gorchein (4) was modified by lowering the reaction mixture to pH 5.0 with 1 N HCl just before reducing the methanol to dryness. The lipid residue was partitioned with 1 volume of the upper phase and 2 volumes of the lower phase of water-isobutanol-chloroform (9:6:12, vol/vol/vol). The chloroform-soluble lipid was reduced to dryness, dissolved in chloroform, and applied to a silicic acid column. The fatty acids were recovered from the column by elution with 2 volumes of 5% methanol in chloroform. The deacylated aminolipid (lysoaminolipid) was recovered from the column by elution with 2 volumes of chloroform-methanol (1:1, vol/vol) followed by 2 volumes of methanol.

Membrane preparation. Cells were suspended in 50 mM phosphate buffer, pH 7.5, and passed through a French pressure cell twice at 10,000 lb/in². This cell extract was centrifuged at 10,000 × *g* for 30 min, and the supernatant fluid was decanted and treated with deoxyribonuclease and ribonuclease at 1 mg/10 g of original wet cell weight for 30 min at 0 C. This nuclease-treated supernatant fluid was centrifuged at 105,000 × *g* for 3 h, and the resulting membrane pellet was suspended in phosphate buffer. A discontinuous density gradient was prepared consisting of: 55% sucrose (wt/wt), 8 ml; 41.7% sucrose (wt/wt), 15 ml; and 24% sucrose (wt/wt), 7 ml. The membrane suspension was layered on the 24% sucrose layer and centrifuged at 55,000 × *g* for 18 h in an SW25.1 Spinco rotor.

Preparation of dinitrophenyl-ornithine. The 2,4-dinitrophenyl (DNP) derivative of the intact aminolipid was made by the method of Gorchein (4). The DNP-lipid was hydrolyzed with 6 N HCl for 18 h at 105 C, and the solution was extracted with ether. The aqueous layer was reduced to dryness in vacuo, and the residue was dissolved in *n*-butanol-ethyl acetate (1:1, vol/vol). The DNP-ornithine was chromatographed on Silica Gel G plates in two solvent systems: (i) solvent C, *n*-propanol–ammonia (7:3, vol/vol); and (ii) solvent D, the upper phase of *n*-butanol-acetic acid-water (4:1:5, vol/vol/vol).

Aminolipid quantitation. Lipid extracts obtained from either whole cells or membranes were treated with 0.2 M KOH in methanol for 1 h at 37 C to hydrolyze all ester bonds. The lyso-aminolipid was recovered as described for mild alkaline hydrolysis. The resulting lyso-aminolipid was hydrolyzed with 6 N HCl for 18 h at 105 C, and the resulting ornithine-hydrochloride was measured by the ninhydrin colorimetric assay according to Rosen (21), with ornithine-hydrochloride as standard.

Analytical methods. Lipid phosphorus and cell nitrogen were determined as described previously (14). Protein was estimated by the method of Lowry et al. (12), with bovine serum albumin as a standard. Infrared spectra were obtained with a Beckman infrared spectrometer, model 33. Vicinal glycol was determined as specified in a previous report (15), with ethane-1,2-diol and propane-1,2-diol as standards.

RESULTS

A ninhydrin-positive, phosphate-negative lipid was observed in lipid extracts obtained from *D. gigas*. Exhaustive analyses of *D. desulfuricans* and *D. vulgaris* failed to reveal this specific aminolipid (18). The aminolipid was separated on diethylaminoethyl cellulose columns with the basic phospholipid fraction. Attempts to further purify this amino-lipid from phosphatidyletholamine by diethylaminoethyl-cellulose column chromatography proved unsuccessful by batch elution with increasing concentrations of methanol in $CHCl_3$ (i.e., chloroform-methanol, 9:1, 8:2, 7:3, 1:1, vol/vol). Silicic acid column chromatography with discontinuous gradients of chloroform-methanol (90:10, 85:15, 80:20, 70:30, vol/vol) did not improve the separation of this aminolipid from phosphatidylethanolamine. TLC with solvent systems A and B yielded separation of this aminolipid from phosphatidylethanolamine but remained generally inadequate for relatively large-scale purification.

The observation was made that a precipitate formed when a choloroform-methanol (2:1, vol/vol) solution of the basic diethylaminoethyl-cellulose column fraction was cooled to −20 C. The precipitate was collected, dissolved in fresh chloroform-methanol (2:1, vol/vol), and precipitated at low temperature. This procedure was repeated four times, yielding a single component which was ninhydrin positive and phosphate negative in solvent systems A and B. This lipid cochromatographed in solvent systems A and B with the TLC-purified aminolipid obtained from crude lipid extracts of *D. gigas* and *T. thiooxidans* (23). The low-temperature precipitation procedure was applicable to large volumes of solvent containing the aminolipid, yielding a homogeneous, pure component in apparently semiquantitative yield. Analysis of the chloroform-methanol-soluble phosphatidylethanolamine fraction indicated almost complete removal of the aminolipid.

Mild alkaline hydrolysis. The pure aminolipid was subjected to mild alkaline hydrolysis, and the resulting products were analyzed by TLC in solvent system A. Two chloroform-soluble lipids were detected: (i) a ninhydrin-positive lipid that exhibited an R_f value of 0.23; and (ii) a ninhydrin-negative lipid with an R_f value of 1.0. The intact aminolipid exhibited an R_f value of 0.53 in the same solvent system.

The ninhydrin-negative lipid was analyzed by TLC in petroleum ether-diethyl ether-glacial acetic acid (80:20:1, vol/vol/vol) and found to be comprised of fatty acid. Methylation of this fraction and analysis by gas chromatography demonstrated a complex mixture of fatty acids (Fig. 1). The major fatty acids were n-$C_{14:0}$ (21%), iso-$C_{14:0}$ (14%), anteiso-$C_{15:0}$ (43%), and lesser amounts of n-$C_{16:0}$ (2%), n-$C_{18:0}$ (8%), and n-$C_{18:1}$ (11%).

Acid hydrolysis. The ninhydrin-positive lipid obtained from the mild alkaline hydrolysis reaction was purified further by silicic acid column chromatography. The recovered lipid was hydrolyzed in 6 N HCl at 105 C for 18 h. The acid hydrolysate was extracted twice with diethyl ether, and the ether extracts were pooled, dried over sodium sulfate, and reduced to dryness. The analysis of this fraction by TLC indicated only free fatty acid which was methyl-

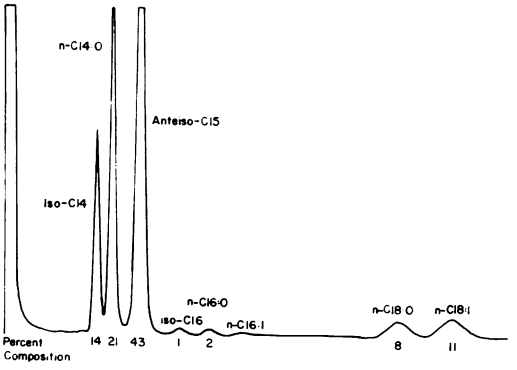

FIG. 1. *Ester-linked fatty acids derived from D. gigas aminolipid. Fatty acid methyl esters were analyzed on 20% DEGS (column temperature 150 C) and 10% Apiezon L (column temperature 180 C). Results shown were obtained by analysis on 20% DEGS.*

ated and analyzed by gas chromatography. Two fatty acids were identified by gas chromatographic analysis: (i) methyl hexadec-2-enoate and (ii) methyl 3-hydroxy hexadecanoate (Fig. 2). The mass spectrum of the methyl ester of the hydroxy fatty acid obtained from *D. gigas* aminolipid showed characteristic ions at *m/e* 286 (M^+), 268 ($M-H_2O$), 236, 194, 103, 71, and 43 (Fig. 3). Parallel analyses with the ornithine-containing lipid purified from lipid extracts of *T. thiooxidans* (9) and synthetic methyl 3-hydroxy hexadecanoate (16) served to provide known standards. The mass spectrum of methyl-hexadec-2-enoate showed ions at *m/e* 268 (molecular ion), 237 ($M-OCH_3$), 194, and 113, and an 87 base peak (Fig. 4).

The aqueous phase recovered from the acid hydrolysis reaction contained a ninhydrin-positive spot that remained at the origin after TLC on Silica Gel G in solvent system A. The water-soluble, ninhydrin-positive component was chromatographed on Silica Gel G in a solvent system consisting of phenol-water (75:25, wt/wt) containing 20 mg of NaCN per 100 g of solvent. The ninhydrin-positive component cochromatographed with authentic ornithine as a single component. Further analysis with a Beckman amino acid analyzer established its identity as ornithine.

Chemical linkages of the amino group. The DNP derivative of the intact aminolipid was prepared and subjected to acid hydrolysis. The resulting DNP-ornithine was found to cochromatograph with authentic δ-DNP-ornithine by TLC in solvent systems C and D. The mass spectrum of DNP-NH-CH-COOH (*m/e* = 241), which would result from the fragmentation of α-DNP-ornithine, was absent in the recorded mass spectrum. In addition, the mass spectrum obtained from standard δ-DNP-ornithine was identical to the DNP-ornithine derivative prepared from *D. gigas* aminolipid.

Infrared analyses of the intact aminolipid exhibited absorption bands at 1,730 and 1,195 cm^{-1}, indicative of an ester function (Fig. 5A). These absorption bands were lost with the lyso-aminolipid (Fig. 5B). The amide bond function was indicated by absorption bands between 1,640 and 1,610 cm^{-1} and a broad absorption band in the region 3,300 to 3,030 cm^{-1}. Absorption throughout the region of 3,300 to 3,030 cm^{-1} may be due to the amide linkage, although hydroxyl absorption in the region of 3,400 cm^{-1} exhibited considerable overlap. These data indicate the α-amino group of ornithine is linked through an amide bond to the 3-hydroxy hexadecanoic acid, whereas the δ-amino group of ornithine is free.

Chemical analyses for diols were performed after base and acid hydrolysis, respectively, of aminolipid samples. The presence of formaldehyde was determined by the chromotropic acid reagent after periodic acid oxidation. All determinations were negative for diol. Ethane-1,2-diol was added as an internal standard at known concentrations to the purified aminolipid and the mixture was hydrolyzed. Quantitative recoveries of ethane-1,2-diol were obtained, indicating the absence of "connector" diols in the aminolipid. Additionally, the internal standard demonstrated that diols were not destroyed by the hydrolytic procedures employed.

Quantitative relationships between phospholipids and the aminolipid. A comparison of the amount of aminolipid to the amount of total phospholipid in whole cells is shown in Table 1. The aminolipid represented a major cellular

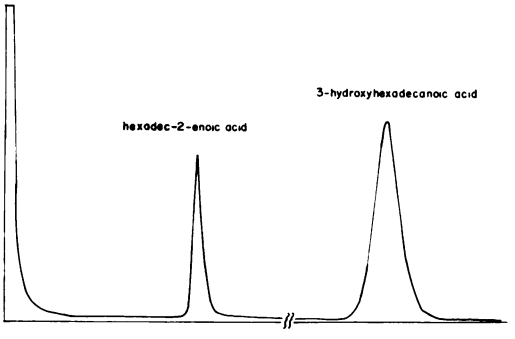

FIG. 2. *Fatty acids derived from acid-hydrolyzed lyso-aminolipid. Fatty acid methyl esters were analyzed on 20% DEGS (column temperature 150 C) and 10% Apiezon L (column temperature 180 C). Results shown were obtained by analysis on 20% DEGS.*

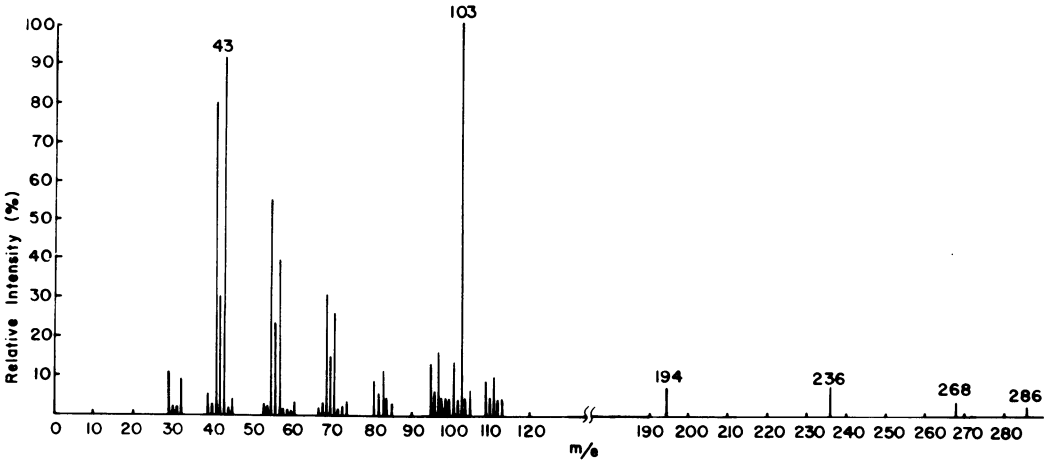

FIG. 3. *Mass spectrum of the methyl ester of 3-hydroxy palmitic acid.*

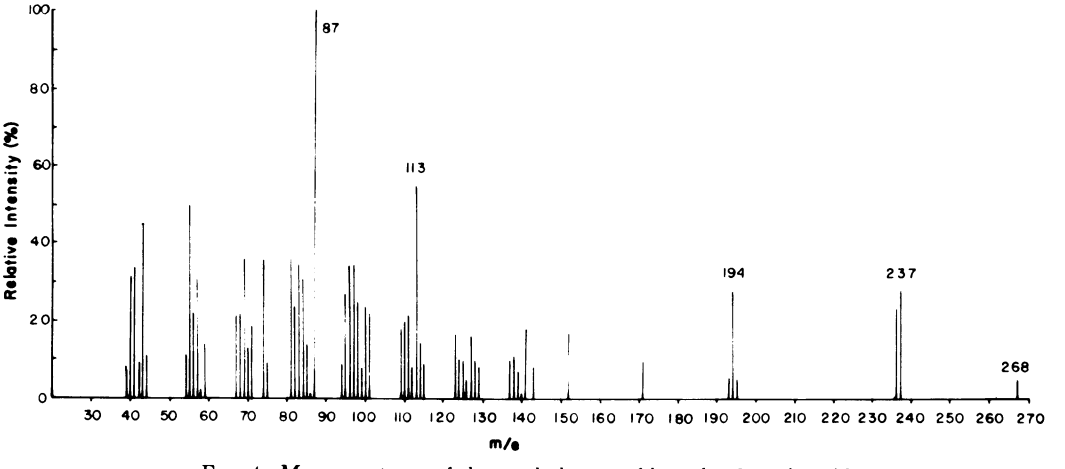

FIG. 4. *Mass spectrum of the methyl ester of hexadec-2-enoic acid.*

lipid constituent (637 nmol/mg of cell nitrogen). The contribution of the aminolipid to the total polar lipid fraction of *D. gigas* becomes compensatory to what appeared to be a polar lipid-deficient microorganism.

The membrane fraction was separated into two membrane bands by sucrose-density gradient centrifugation. A lighter membrane fraction banded at a density of 1.18 g/cm^3, characteristic of the cytoplasmic membrane reported for gram-negative bacteria (18, 22). A heavier membrane fraction banded at a density of 1.25 g/cm^3, similar to the outer membrane or wall fraction reported for gram-negative bacteria. The aminolipid represented a significantly large proportion of the total polar lipid content of the cytoplasmic membrane and the outer membrane of *D. gigas* (Table 1). The amount of aminolipid per milligram of membrane protein is also very similar for each membrane fraction, as contrasted to the phospholipids which decreased approximately 50% in the outer membrane fraction. Phosphatidylglycerol was the major phospholipid for both the cytoplasmic membrane and the outer membrane, comprising 67 and 75% of the total phospholipid composition, respectively.

A comparison of the esterified fatty acids of aminolipid with those of phosphatidylglycerol and phosphatidylethanolamine is shown in Table 2. A close qualitative similarity in fatty acid composition is evident although the proportion of n-$C_{14:0}$ is greater and anteiso $C_{15:0}$ is smaller in aminolipid as compared with phos-

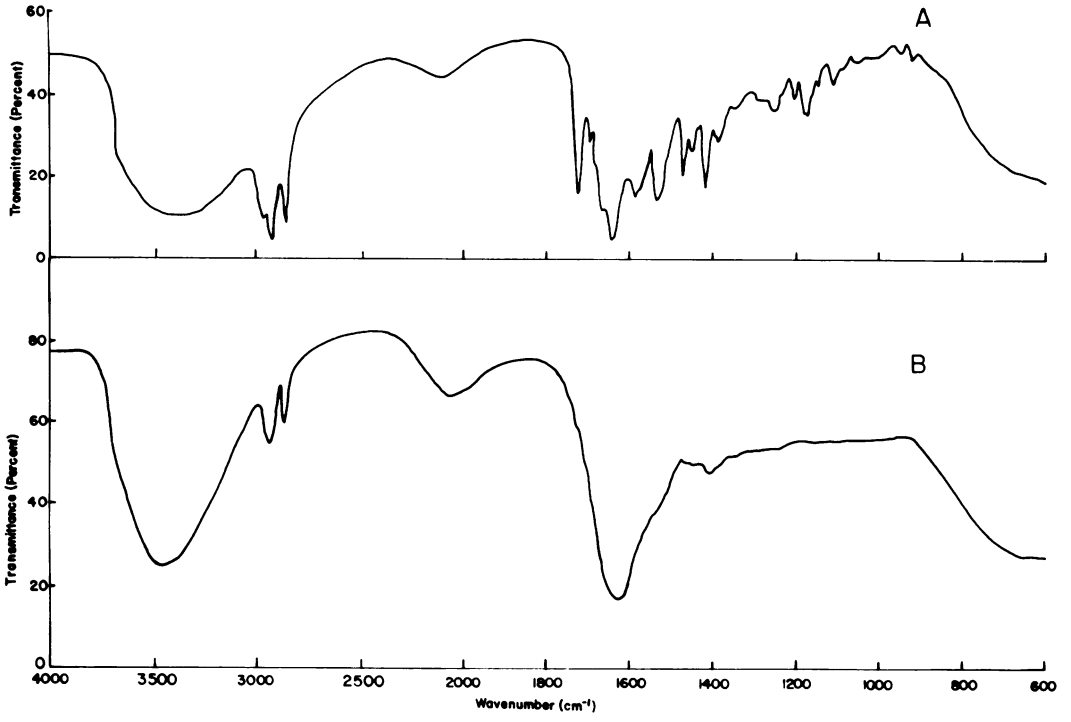

FIG. 5. *Infrared spectra of (A) aminolipid and (B) lyso-aminolipid.*

TABLE 1. *Quantitative relationships of aminolipid and phospholipid derived from D. gigas*

Cell fraction	Amino-lipid	Phosphatidylglycerol	Phosphatidyl-ethanolamine
Whole cells[a]	637	119	51
Cytoplasmic membrane[b]	385	92	45
Outer membrane[b]	350	54	18

[a] Nanomoles per milligram of cell nitrogen.
[b] Nanomoles per milligram of protein.

TABLE 2. *Esterified fatty acid composition of polar lipids*[a]

Fatty acid	Phosphatidyl-glycerol	Phosphatidyl-ethanolamine	Amino lipid
iso-$C_{14:0}$	9	8	14
n-$C_{14:0}$	3	3	21
Anteiso-$C_{15:0}$	84	58	43
iso-$C_{16:0}$	TR	TR	1
n-$C_{16:0}$	1	13	2
n-$C_{16:1}$	TR	TR	TR
n-$C_{18:0}$	2	13	8
n-$C_{18:1}$	TR	6	11

[a] Results are expressed as a percentage of the total fatty acids. The fatty acid methyl esters were analyzed on 20% DEGS and 10% Apiezon L columns. TR, Trace.

phatidylglycerol and phosphatidylethanolamine.

DISCUSSION

Ornithine-containing lipids that have been reported all contain a fatty acid linked at the α-amino position. The second fatty constituent is linked in one of three alternate ways. (i) A fatty alcohol is esterified to the carboxyl group of ornithine (4, 7). (ii) A fatty acid is esterified to the carboxyl-group of ornithine via a diol (24, 25). (iii) A fatty acid is esterified via the hydroxyl-group of a 3-hydroxy fatty acid which is linked at the α-amino position (9, 28).

The ornithine-containing lipid isolated from *D. gigas* represents an additional example of a fatty acid esterified to the hydroxyl group of a hydroxy fatty acid which, in turn, is linked to the α-amino group of a specific amino acid through an amide bond. The structure proposed for this aminolipid is shown in Fig. 6. The two alternate categories were eliminated for further consideration since fatty alcohols and diols were not detected in the hydrolysates of the amino-lipid. Whereas 3-hydroxy hexadecanoate is

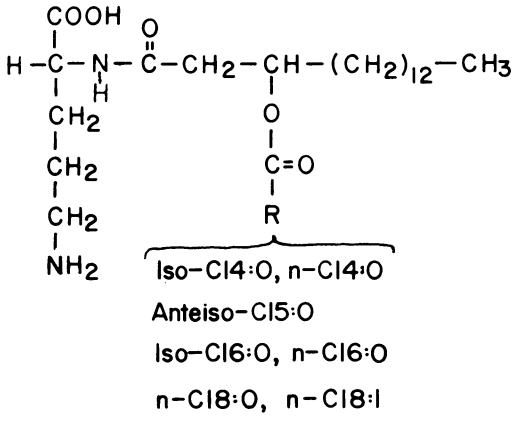

FIG. 6. *Proposed structure of the ornithine-containing lipid from D. gigas.*

linked to the α-amino group of ornithine, several fatty acids are linked through the hydroxyl group of 3-hydroxy hexadecanoate. The majority of the esterified fatty acids (57%) are branched chain and qualitatively similar to those fatty acids found in the phospholipid fatty acyl residues. No 3-hydroxy hexadecanoate was detected from fatty acids derived from the phospholipids.

A second fatty acid, hexadec-2-enoate, has been shown to be a degradation product resulting from the acid hydrolysis of 3-hydroxy hexadecanoate (9). When the time of acid hydrolysis was reduced from 18 to 4 h, no significant amounts of hexadec-2-enoate were found with the 3-hydroxy hexadecanoate derived from *D. gigas* aminolipid.

The ornithine-containing lipid obtained from *D. gigas* represented a major polar lipid constituent of whole cells. Furthermore, it appears to be quantitatively distributed between the component membranes of *D. gigas* in equal proportions, suggesting a possible structural role for membrane integrity. This relationship is supported further by the apparent quantitative phospholipid-deficient composition of *D. gigas* whole cells as compared with *D. vulgaris* or *D. desulfuricans* (14).

A diversity of structural variations has been determined for ornithine-containing lipids obtained from different microorganisms. The general structure $NH_2\text{-}CH_2\text{-}(CH_2)_2\text{-}CHNH[CO\text{-}CH_2CH(O\text{-}COR^2)\text{-}R^1]\text{-}COOH$ has been determined for *T. thiooxidans* (9) and *P. rubescens* (28), where R^1 represents 3-hydroxy fatty acid and R^2 represents normal, branched, cyclopropane-, or unsaturated fatty acids esterified to the 3-hydroxy fatty acid. The general structure $H_2N\text{-}CH_2(CH_2)_2\text{-}CH\text{-}NH(\text{-}COR^2)\text{-}CO\text{-}O\text{-}CH_2CH_2\text{-}O\text{-}CO\text{-}R^1$ has been described for *Mycobacterium bovis* (20), *Brucella melitensis* (24), *B. abortus* (24), and *Bordetella pertussis* (24). In *M. bovis*, tuberculostearic acid (R^1) is esterified and 3-hydroxy stearic acid (R^2) is bound in an amide linkage. The aminolipids characterized from *B. abortus* and *B. melitensis* are characterized by predominantly esterified lactobacillic acid (R^1) with palmitic and stearic acid in the amide linkage (R^2). A strain of *B. melitensis* differs by having propane-1,2-diol as the polyhydric ligand and instead of ethane-1,2-diol (20). The structural variation $NH_2\text{-}CH_2\text{-}(CH_2)_2\text{-}CH\text{-}NH(COR^2)\text{-}CO\text{-}O\text{-}R^1$ has been described for aminolipid derived from *Rhodopseudomonas spheriodes* (4) and *S. sioyaensis* (6), where R^1 represents a fatty alcohol and R^2 represents a 3-hydroxy fatty acid. An aminolipid has been obtained from *Actinomyces* strain 660-15 and represents a further structural modification (1). The general structure $NH_2\text{-}CH_2\text{-}(CH_2)_2\text{-}CH\text{-}NH(\text{-}CO\text{-}CH_2\text{-}CH(OH)\text{-}R^2)\text{-}CO\text{-}O\text{-}CH(CO_2H)R^1$ has been proposed, where R^1 represents a 2-hydroxy fatty acid esterified to the carboxyl-group of ornithine, and R^2 represents a 3-hydroxy fatty acid linked by an amide bond to the α-amino group of ornithine.

The aminolipid described from *P. rubescens* has been reported as representing a major polar lipid constituent from nutrient agar-grown cells but was undetectable in lipid extracts derived from nutrient broth-grown cells (28). Recently, Minnikin and Abdolrahimzadeh (17) demonstrated the absence of aminolipid in magnesium-limited cultures of *P. fluorescens*. However, under conditions of phosphate limitation, the aminolipid becomes the sole polar lipid. The aminolipid characterized from *R. spheroides* was shown to be metabolically stable under conditions of aerobic growth followed by adaption to photosynthetic growth (5). The suggestion was made that aminolipid serves the role of a structural component in the membrane.

Ornithine-containing lipids appear to be widely distributed among bacterial species and are characterized by a high degree of structural heterogeneity. The biological significance of these lipids remains obscure, although the lipid exhibits those amphipathic properties characteristic of membrane polar lipids.

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