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Occurrence of Dialkyl Ether Phospholipids in *Stigmatella aurantiaca* DW4

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We investigated the lipid composition of vegetative cells of *Stigmatella aurantiaca*. Four phospholipids were isolated and identified: phosphatidylethanolamine as the main component, phosphatidylglycerol, lysophosphatidylethanolamine in an exceptionally large amount (17%), and phosphatidylinositol (18 to 25%), rare in procaryotic cells. This composition did not change significantly during growth. The fatty acids of total lipids were found to be rather similar to those of other strains of myxobacteria; the main fatty acids found were unsaturated and branched. We noted a different fatty acid pattern for each phospholipid. The presence of unusual alkyl ether linkages, established by chemical hydrolysis and infrared spectroscopy, was unexpected in these bacteria. Diacyl ester, dialkyl ether, and monoacyl-monoalkyl structures were shown in phosphatidylethanolamine and phosphatidylglycerol. Lysophosphatidylethanolamine was essentially a monoacyl form, whereas phosphatidylinositol was a unique dialkyl ether phospholipid.

Stigmatella aurantiaca, a gram-negative bacterium, exhibits a multicellular developmental process. Under appropriate conditions of starvation, cells aggregate by gliding on a solid surface to aggregation centers. Then, fruiting bodies are built up by the accumulation of a large part of the bacterial population; the resting cells differentiate into myxospores.

Envelope components may be involved in cellular interaction mechanisms (messenger receptors, cell-cell recognition, and adhesion). We have been interested in the function of lipids in these processes. In *Dictyostelium discoideum*, glycosphingolipids are directly implicated in cellular aggregation as cell surface receptors (20). It is generally held that the phospholipids, by their fatty acid content as well as by their polar head groups, affect the physical properties of membrane structure. In particular, during myxospore differentiation, the transformation of the rod-shaped cells to shorter and wider spores implies changes in the molecular packing of phospholipids or, perhaps, the segregation of lipid domains in the membranes. The function of the cell surface in the biochemical events of development is not yet known.

Consequently, in this preliminary work, we have studied the lipid composition of vegetative cells of *S. aurantiaca* DW4. The main features of the work were: (i) unexpected amounts of lysophosphatidylethanolamine and phosphatidylinositol in growing cells and (ii) unusual alkyl ether linkages in each of the phospholipids.

MATERIALS AND METHODS

Strain and culture. Strain DW4 of *S. aurantiaca* was from D. White. Cells were grown in liquid medium (12) on a rotary shaker at 30°C with vigorous aeration. Growth was followed turbidimetrically at 540 nm with a Pye-Unicam SP 600 spectrophotometer.

Extraction and identification of phospholipids. After the addition of 0.5 N perchloric acid, cells were harvested by centrifugation at 7,000 × *g*. Lipid pellets were extracted with a chloroform-methanol-water mixture by the Bligh and Dyer procedure (1). The final chloroform solution obtained by phase separation was used for the estimation of phospholipid content and for the analysis of fatty acid composition. Phospholipids were separated on silica gel plates (silica gel 60 precoated Merck thin-layer chromatographic plates) developed with solvent I (chloroform-methanol-water, 50:20:2.6 [vol/vol/vol]) or with solvent II (chloroform-methanol-ammonia-water, 50:20:0.6:0.8 [vol/vol/vol/vol]). Each molybdate-positive spot (6) was scraped off and eluted with methanol-chloroform (9:1 [vol/vol]). Phospholipids were labeled in the complex medium with [^{32}P]phosphate (Commissariat à l'Energie Atomique, Paris, France). After 2.5 generations, lipids were extracted as previously described.

Fatty acid analysis. Lipids were transesterified by heating with acidified anhydrous methanol (14) for 4 h at 75°C. Methyl esters were extracted into hexane and analyzed by isothermal gas chromatography at 190°C with a Hewlett-Packard 7620 A chromatograph on a nonpolar methyl silicone OV_1 column. Fatty acid

esters were identified after cochromatography with Analabs standards. The relative composition of each extract was calculated by the equation $\% Sn = h_n \times t_n / \Sigma(h \times t) \times 100$ in which h is the height of the peak and t is the retention time for the fatty acid S with n carbons.

Deacylation by methanolysis of phospholipids. Lipids dried by vacuum evaporation were incubated in freshly prepared methanolic 0.1 N KOH for 75 min at 0°C (3); then, the pH was adjusted to 7.0 with 0.2 N perchloric acid (10). Under these conditions, deacylation was complete. Phase separation was achieved by the addition of adequate volumes of chloroform and water. Phospholipids and lysophospholipids were in chloroform solution; polar products in the aqueous phase were identified on Whatman paper no. 1 with solvent III (phenol-ethanol-acetic acid-water, 50:3:3:22 [vol/vol/vol/vol]).

Infrared spectra. The spectrum of each phospholipid, deacylated or not, was determined in KBr cells on a Beckman spectrophotometer. Diacyl ester phosphatidylethanolamine (Sigma Chemical Co., St. Louis, Mo.) and dialkyl phosphatidylethanolamine (Medmark) were used as standards.

RESULTS AND DISCUSSION

Phospholipid composition and identification. The phospholipid content of *S. aurantiaca* grown in the complex medium was about 4% on a cell weight basis. This amount was estimated by the incorporation of [^{32}P]phosphate (60 nmol/mg of dry weight). This value (4×10^{-11} μmol/cell) was lower than the result obtained for *Myxococcus xanthus* (9×10^{-11} μmol/cell) (11) but in the same order of magnitude. Thin-layer chromatography of the polar lipids revealed the presence of four components.

(i) PE. In solvents I and II, phosphatidylethanolamine (PE), the main compound, exhibited chromatographic properties identical to those of the PE of *Bacillus subtilis* previously identified (13). Alkaline hydrolysis of this ninhydrin-positive lipid yielded glyceryl phosphorylethanolamine (R_f, 0.66 with solvent III).

(ii) PG. Phosphatidylglycerol (PG) reacted with I_2 vapors, molybdate stain, and periodate-Schiff reagent (7). It had the same mobility as the PG of *B. subtilis* and yielded glyceryl phosphorylglycerol upon alkaline hydrolysis (R_f, 0.49 with solvent III).

(iii) Lyso PE. Lysophosphatidylethanolamine (lyso PE) reacted with ninhydrin and molybdate reagents. Upon alkaline hydrolysis, a product comigrating with GPE was obtained. Mild acid hydrolysis with 1 N HCl (100°C over 4 h) yielded a ninhydrin-positive compound which exhibited an R_f value similar to that of standard ethanolamine. Phospholipase A_2, under conditions described by Brockerhoff (4), was without effect on this lipid, whereas alkaline hydrolysis completely deacylated it. Our data were consistent with the structure acyl-1 PE, although such a high proportion (17%) of the total phospholipids was unexpected.

(iv) Phospholipid 4. Phospholipid 4 (with a very low R_f in solvent systems I and II) reacted with the periodate-Schiff reagent and migrated like standard phosphatidylinositol (PI). It was neither a sphingophospholipid nor a lyso PG. Strong acid hydrolysis (2 N HCl) yielded a compound which migrated as standard inositol. This phospholipid was tentatively identified as PI.

PE was quantitatively the main phospholipid (about 50%) in *S. aurantiaca* (Table 1) as in other gram-negative bacteria, whereas the proportion of PG was very low and decreased in stationary phase. These two phospholipids are also found in the other myxobacteria previously investigated (9, 11), but the proportion of PE (about 50%) was lower than the values observed in *M. xanthus* (76%) and in *Myxococcus fulvus* (72%). Large amounts of lyso PE (17%) have been found at different growth stages: the presence of this lipid has been reported in *M. fulvus* (9) and in *E. coli* (17); in this latter case, it was interpreted as an artifact of extraction (17). The activation of phospholipase could also explain the lyso PE formation although its proportion was high, constant, and independent of the PE amount during growth. A considerable content of PI was present in this strain, and its relative proportions changed from 18% at the end of log phase to 25% in stationary phase. The presence of PI is rare in procaryotic cells; it has also been found in another myxobacterium, *M. fulvus* (9), but only in small amounts.

Relative changes in the levels of the different phospholipids were very low and seemed to be independent of the growth stage. However, the

TABLE 1. Phospholipid composition of *S. aurantiaca*[a]

Phospholipid	Phospholipid composition at:		
	Mid-log phase (A_{540} = 0.306)	The end of log phase (A_{540} = 0.540)	Stationary phase
PE	48	52	49
PG	12	13	9
Lyso PE	17	17	17
PI	23	18	25
Total phospholipid content	5.3	9	13.2

[a] Phospholipids were labeled with [^{32}P]phosphate during growth in a complex medium (10 μCi/ml of medium). The total phospholipid content is expressed as nanomoles per milliliter of culture. The values of individual phospholipids are expressed as a percentage of the total phospholipid content. A_{540}, Absorbance at 540 nm.

TABLE 2. Diacyl, dialkyl, and monoacyl-monoalkyl phospholipids of *S. aurantiaca*[a]

Phospholipid	Diacyl	Dialkyl	Monoacyl-monoalkyl
PE	28	34	38
PG	36	14	50
Lyso PE[b]	94 (mono)	6 (mono)	
PI	0	100	0

[a] Values are expressed as a percentage of radioactive incorporation in the different forms of phospholipids, extracted during the log phase.

[b] Lyso PE is a monoacyl or a monoalkyl form.

radioactivity incorporated in lipids increased in the stationary phase (Table 1), and we observed that the quantity of PI was doubled (from 1.6 nmol/ml of culture at an absorbance at 540 nm of 0.540 to 3.3 in the stationary phase) as soon as cellular division stopped. A localization of pulse-labeled phospholipids in the two membranes after exponential growth will be necessary to understand the role of these changes in the molecular properties of the envelope.

Phospholipid structure. (i) Fatty acid composition. Under our experimental conditions, branched (42%), unsaturated (52%), and linear (6%) chains from C_{14} to C_{18} were the fatty acids of the total lipids extracted during the log phase. Thus, the fatty acid composition of *S. aurantiaca* DW4 grown in a complex medium was similar to that of other myxobacteria (15, 18). However, the proportions of branched $C_{15:0}$ (24%) and $C_{16:1}$ (37%) were rather different from the amounts observed in another strain of *S. aurantiaca* (15).

Unsaturated and linear chains are characteristic of gram-negative bacteria, but branched chains are usually found in gram-positive bacteria. Therefore, the synthesis of these fatty acids by *S. aurantiaca* required the metabolic pathways described for both gram-negative and gram-positive bacteria.

The fatty acid analysis of each phospholipid isolated by chromatography showed that unsaturated chains ($C_{16:1}$, $C_{18:1}$) are mainly present in PE and branched chains are mainly present in lyso PE and PG (br $C_{15:0}$, br $C_{16:0}$, br $C_{18:0}$); it was unusual to observe differences in the fatty acid content between individual phospholipids, yet, the absence of unsaturated chains in 1-acyl PE could be explained since they are generally acylated in position 2 of glycerol.

The function of such a complex fatty acid composition in maintaining the so-called membrane fluidity is not understood. The significant proportion of unsaturated and anteiso forms suggests an adaptation of this strain to lower temperatures; this could explain the optimal temperature growth at 30°C. But the polar head groups and the phospholipid organization in the two membrane structures play a large part in this adaptation.

(ii) Ether linkages. ^{32}P-labeled PE and PG were not completely deacylated by mild alkaline hydrolysis under experimental conditions in which all acyl ester linkages were hydrolyzed (Table 2). Three fractions were obtained after the phase separation previously described: a dialkyl phospholipid, a monoalkyl phospholipid separated by chromatography, and a water-soluble product corresponding to the diacyl form. Lyso PE was almost entirely deacylated by this treatment, whereas PI was completely insensitive. Thus, diacyl derivatives of phospholipids, usually found in bacteria, existed in *S. aurantiaca*, but other structural conformations of phospholipids were also present. Alkenyl-acyl forms, previously reported in *M. fulvus* (9), were absent under our conditions since a specific hydrolysis of the vinyl ether linkages (19) was unsuccessful.

The infrared spectrum of PE (Fig. 1) exhibited an absorption peak at 1,730 cm^{-1} corresponding to the carbonyl group from the ester linkage

(—C—) and another strong band at 1,070 to 1,100 cm^{-1} characteristic of the ether linkage.

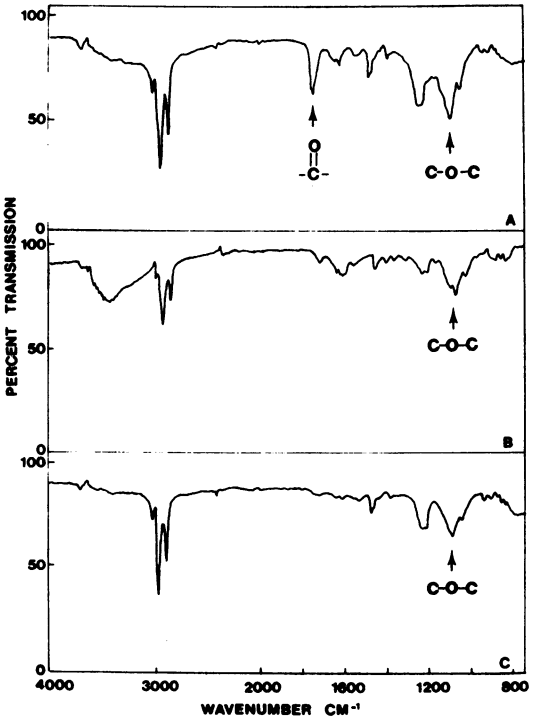

FIG. 1. Infrared spectra of PE (A), PE deacylated from *S. aurantiaca* DW4 (B), and of standard dialkyl ether PE (C).

The frequency of the C-O-C absorption at 1,070 to 1,100 cm^{-1} was confirmed with a standard dialkyl PE. After the deacylation of PE, the absorption at 1,730 cm^{-1} disappeared, whereas the 1,070 cm^{-1} band persisted; these data showed that alkyl ether linkages are present in the PE of *S. aurantiaca*. PE contained a large amount of the dialkyl form (34%) when lyso PE was mostly an acyl phospholipid (94%). The almost exclusive acyl form in lyso PE was indicated, since an ether linkage in position 1 of glycerol makes the ester bond in position 2 more resistant to the action of phospholipase A_2 (2). PG possessed a main acyl-alkyl form (50%), but dialkyl PG was present. PI was identified as a unique dialkyl ether phospholipid. The identification of alcohol chains is in progress.

The presence of alkyl ether bonds in each phospholipid of *S. aurantiaca* was unusual because, until now, ether-linked lipids have only been found in archaebacteria (5). Extremely halophilic bacteria contain exclusively the diphytanyl form of PG (8), but in *S. aurantiaca*, three forms coexisted: diether, diester, and monoether.

It has been established that many bacteria maintain, in response to environmental conditions, an optimal fluidity of their membrane lipid phase by adjustments of lipid composition (for example, homeoviscous adaptation [16]). The complexity of this composition suggests that many lipid structures are possible, but the function of the dialkyl or monoalkyl lipid forms for this adaptation is presently unknown. During the developmental cycle, most of the bacterial population aggregates to form fruiting bodies. Membrane structure may play an important role in cell-cell interactions, and phospholipid composition and localization are of particular interest in understanding the membrane function in this process.

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