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Phosphatidylglyceroylalkylamine, a Novel Phosphoglycolipid Precursor in *Deinococcus radiodurans*

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We report here the structure of a previously uncharacterized phospholipid in the radiation-resistant bacterium *Deinococcus radiodurans*. This phospholipid, designated lipid 4, was shown by chemical analysis, HF hydrolysis, and nuclear magnetic resonance spectroscopy to be phosphatidylglyceroylalkylamine. Lipid 4 thus contains the unusual lipid constituents glyceric acid and alkylamines, which have previously been identified in two complex phosphoglycolipids from this organism. By [^{32}P]phosphate pulse-chase labeling techniques, lipid 4 was shown to be the precursor of the complex phosphoglycolipids α-galactosyl- and α-*N*-acetylglucosaminyl-phosphatidylglyceroylalkylamine. While phosphatidylglyceroylalkylamine is rapidly biosynthesized from P_i, its subsequent glycosylation occurs much more slowly. Therefore, we conclude that the final glycosylation step is the rate-limiting event in the biosynthesis of the complex phosphoglycolipids α-galactosyl- and α-*N*-acetyl-glucosaminyl-phosphatidylglyceroylalkylamine.

The radiation-resistant bacterium *Deinococcus radiodurans* is the type species of a genus representing an ancient branch in bacterial phylogeny (5, 6, 27). In support of the taxonomic basis of the *Deinococcus* genus are analyses of 16S rRNA (6, 27), DNA G+C content (17, 23), peptidoglycan (24, 31), cell wall structure (4, 15, 26, 29, 30, 32), and lipid composition (2, 3, 13, 19, 28). Although the unusual lipid composition of members of this genus has been used as a taxonomic means of comparison, structural identification of the unique lipid forms of *D. radiodurans* has so far been achieved for only two polar lipids (3, 13) and one neutral lipid, menaquinone MK8 (33). In addition to their use as taxonomic markers, unconventional lipids continue to arouse interest as to their possible role in the unusual biological properties of the deinococci, such as their high resistance to radiation (1) and desiccation (22). Although lipids do not appear to have a role of shielding against radiation in *D. radiodurans* (20, 25), they may contribute to radiation resistance by functioning as antioxidants (7) or as factors contributing to the maintenance of membrane impermeability to ions (16).

Fundamental to the identification of structure-function relationships in the membrane lipids of *D. radiodurans* is an understanding of their precise chemical structures and of the pathways involved in their biosynthesis. Our previous studies have elucidated the structures of two major phosphoglycolipids from *D. radiodurans* (3, 13). Relevant structures are shown in Fig. 1. The present study relates these two phosphoglycolipids to a novel phospholipid, phosphatidylglyceroylalkylamine, and demonstrates a biosynthetic pathway connecting them.

MATERIALS AND METHODS

Growth of *D. radiodurans* and extraction of lipids. Cultures of *D. radiodurans* Sark were grown at 35°C as rotary-shaken (200 rpm) aerobic suspension cultures in 0.5% tryptone–0.3% yeast extract–3 mM $CaCl_2$. Total lipids were extracted from packed cell pellets by a modified procedure of Folch et al. (12) as previously described (28).

TLC. Lipids were resolved on Sil Gel G Redi-plates (Fisher Scientific) by two-dimensional thin-layer chromatography (TLC) with chloroform–methanol–28% ammonia (65:35:5, vol/vol/vol) used in the first dimension and chloroform-acetone-methanol-acetic acid-water (10:4:2:2:1, vol/vol/vol/vol/vol) used in the second dimension.

Radiolabeling of *D. radiodurans*. For an examination of the kinetics of lipid synthesis, a 100-ml culture of *D. radiodurans* was grown to an optical density at 600 nm of 0.5 and then supplemented with [^{14}C]acetate (0.5 μCi/ml) and incubated at 35°C. At 10 min, 30 min, 90 min, 2.5 h, 4.5 h, and 6.5 h, aliquots were removed from the culture and lipid extracts were prepared. Aliquots of the lipid extracts (equalized on the basis of counts per minute) were resolved by two-dimensional TLC, and radiolabeled lipids were visualized by autoradiography.

Pulse-chase radiolabeling with [^{32}P]phosphate. A freshly grown 50-ml culture of *D. radiodurans* (optical density at 600 nm, 0.5) was centrifuged, and the cell pellet was resuspended in 1 ml of medium supplemented with [^{32}P]phosphate (1 mCi/ml). Following 2- or 5-min pulse-labeling at 35°C, the mixture was microcentrifuged and washed five times with chase medium at 4°C to remove unincorporated label (chase medium is medium supplemented with 10 mM phosphate). One aliquot (the "pulse" phase) was immediately extracted, whereas other aliquots were incubated for various times at 35°C and then extracted (the "chase" phase). Samples from the lipid extracts were resolved by two-dimensional TLC, and the lipids were visualized by autoradiography and by sulfuric acid charring of the TLC plates.

Purification of lipid 4. Samples of total lipid extract from *D. radiodurans* were applied to homemade silica gel H plates (gel thickness, 1 mm) and developed in chloroform–methanol–28% ammonia (80:20:2, vol/vol/vol). By using a companion TLC plate which was stained with iodine as a guide, the lipid 4 band (R_f, 0.41) was scraped out and eluted with chloroform-methanol (1:1, vol/vol).

HF treatment of lipid 4. A sample of [^{14}C]acetate-labeled lipid 4 was purified as described above from *D. radiodurans*

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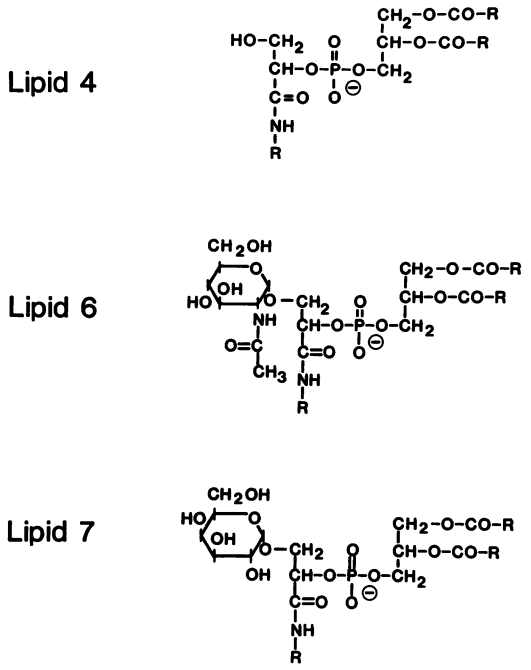

FIG. 1. Structures of phospholipids. Lipids 7 and 6 are α-galactosyl- and α-*N*-acetylglucosaminyl-phosphatidylglyceroylalkylamine, respectively (3, 13). Lipid 4, phosphatidylglyceroylalkylamine, is the subject of the present investigation.

which had been cultured in the presence of [^{14}C]acetate. A portion of this lipid 4 was subjected to HF hydrolysis (11) overnight at 4°C in a polypropylene tube. Cold chloroform (4°C) was added, and the solvents were evaporated under nitrogen, with frequent trituration with chloroform. The residue was taken up in chloroform-methanol (1:1, vol/vol) and examined by TLC. Development was performed sequentially with three solvents: chloroform–methanol–28% ammonia (80:20:2, vol/vol/vol) (one-third development), chloroform-methanol (9:1, vol/vol) (two-thirds development), and hexanes-ether-acetic acid (50:50:1, vol/vol/vol) (complete development). The chromatographic standard, *N*-glyceroylhexadecylamine, was prepared as previously described (3).

Other methods of chemical analysis. Purified lipid 4 was subjected to 5% methanolic HCl hydrolysis at 102°C in sealed glass tubes for 2 h (to liberate fatty acid methyl esters and phosphate) or for 24 h (to liberate alkylamines and glyceroylalkylamines). Phosphorus content was determined by the method of Rouser et al. (21). Fatty acid methyl esters were analyzed by gas chromatography (13). For the analysis of alkylamines, 24-h methanolic HCl hydrolysates were dried, taken up in chloroform, and applied to small (0.5-cm-deep) columns of Bio-Sil A (Bio-Rad) in Pasteur pipettes plugged with glass wool; fatty acid methyl esters were eluted with chloroform, and alkylamines and glyceroylalkylamines were then eluted with chloroform-methanol (3:1, vol/vol). The chloroform-methanol (3:1, vol/vol) eluate was acetylated overnight by reaction with pyridine-acetic anhydride (1:1, vol/vol). The acetylated mixture was dried down, dissolved in chloroform, and analyzed by gas chromatography.

Gas chromatography. A Hewlett-Packard 5890 gas chromatograph, equipped with a 25-m 5% phenylmethyl silicone capillary column, was used. Fatty acid methyl esters were analyzed by using a temperature program of 180 to 290°C (temperature increased at a rate of 5°C/min). Acetylated alkylamines and glyceroylalkylamines were analyzed by using a two-step temperature program (240°C for 20 min, followed by an increase to 290°C at a rate of 5°C/min). Confirmation of gas chromatographic assignments was ob-

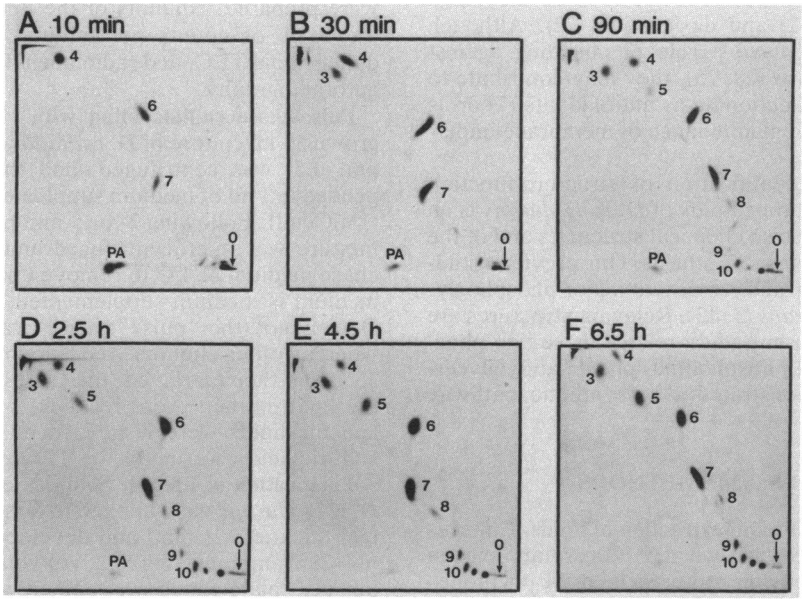

FIG. 2. Kinetics of lipid synthesis in *D. radiodurans*. A 100-ml culture of *D. radiodurans* with an optical density at 600 nm of 0.5 was supplemented with [^{14}C]acetate, and aliquots were removed after the indicated labeling times. Lipids were extracted and subjected to autoradiographic two-dimensional TLC. Numerical lipid designations are as previously assigned (28). Abbreviations: PA, phosphatidic acid; O, origin.

tained by coupled gas chromatography-mass spectrometry as described previously (2).

NMR spectroscopy of lipid 4. Proton nuclear magnetic resonance (NMR) was performed on a Bruker AM-400 spectrometer. Chemical shifts are expressed as parts per million (δ) relative to tetramethylsilane. Coupling constants (*J*) are given in hertz.

RESULTS

Kinetics of lipid synthesis in *D. radiodurans*. Despite the complexity of the lipids found in *D. radiodurans* (19, 28), we were able to demonstrate distinct differences in their rates of synthesis as indicated by the incorporation of [^{14}C]acetate. Only four lipids (lipids 4, 6, and 7 and phosphatidic acid) were significantly labeled when *D. radiodurans* was cultured for a short (10-min) interval in the presence of [^{14}C]acetate (Fig. 2). With increasing labeling times, radiolabel appeared in the remaining lipids; after about 6.5 h, the distribution of radiolabel resembled that obtained from equilibrium labeling conditions (28). Under conditions of pulse-labeling (Fig. 2A), the rapid incorporation of radiolabel into lipids 6 and 7 is not surprising since they constitute large proportions of the total lipid. However, the appearance of abundant radiolabel in lipid 4 was unexpected since lipid 4 is a relatively minor constituent of *D. radiodurans* (28). The results thus suggest that, along with phosphatidic acid, lipids 4, 6, and 7 are rapidly synthesized and/or turned over in *D. radiodurans*.

Identification of lipid 4 as phosphatidylglyceroylalkylamine. Lipids 6 and 7 have been previously identified as glycosylated phosphatidylglyceroylalkylamines (3, 13). In order to establish the structure of lipid 4, we undertook a series of chemical and spectroscopic analyses. Lipid 4 was found to contain fatty acid, alkylamine, and phosphorus in a molar ratio of 2:1:1. When HF was used to specifically cleave phosphoester bonds (11), lipid 4 yielded two products which were identified by TLC as diglyceride and *N*-glyceroylalkylamine (Fig. 3). (Diglyceride was present as a mixture of 1,2 and 1,3 isomers, which is consistent with the known facile interconversion of diglycerides in general [18]). *N*-Glyceroylalkylamine was identified following acetylation and analysis by coupled gas chromatography-mass spectrometry as previously described (3). These results thus suggested that lipid 4 consisted of diglyceride and glyceroylalkylamine linked by a phosphodiester bond. Analysis of the fatty acids, obtained as methyl esters from methanolic HCl hydrolysis of lipid 4, yielded a fatty acid composition similar to that previously reported for lipids 6 and 7 (13). The alkylamines and glyceroylalkylamines were liberated from lipid 4 by methanolic HCl hydrolysis. Even after 24 h of methanolic HCl hydrolysis, approximately 50% of the total alkylamines were still present in the glyceroyl form. The mixture of alkylamines and glyceroylalkylamines was separated from fatty acid methyl esters by Bio-Sil A column chromatography, acetylated with pyridine-acetic anhydride, and examined by gas chromatography. Both the acetylated alkylamines and the acetylated glyceroylalkylamines were resolved as mixtures consisting of different alkylamine species. The alkylamines had C_{15} to C_{18} hydrocarbon chains, with $C_{16:0}$, $C_{16:1}$, $C_{17:0}$, and $C_{17:1}$ predominating.

NMR spectroscopy of lipid 4. Confirmation that lipid 4 is phosphatidylglyceroylalkylamine was obtained by proton NMR spectroscopy (Fig. 4). The proton assignments of lipid 4 were made as follows. Glycerate: H^2 (δ 4.55, m, $J31_{P,H}$ = 7.2 Hz, $J_{2,A}$ = 3.0 Hz, $J_{2,B}$ = 6.8 Hz), H^{3A} (δ 3.93, 1H, dd, $J_{A,B}$ = 12.5 Hz, $J_{A,2}$ = 3.0 Hz), H^{3B} (δ 3.74, 1H, dd, $J_{A,B}$ = 12.5 Hz, $J_{B,2}$ = 6.8 Hz). Glycerol: H^{1A} (δ 4.36, 1H, dd, $J_{A,B}$ = 12.0 Hz, $J_{A,2}$ = 3.4 Hz), H^{1B} (δ 4.14, 1H, dd, $J_{A,B}$ = 12.0 Hz, $J_{B,2}$ = 6.7 Hz), H^2 (δ 5.22, 1H, m, $J_{2,A}$ = 3.4 Hz, $J_{2,B}$ = 6.7 Hz), H^{3A} and H^{3B} (δ ~ 3.98). Fatty acid: the quartet at δ 2.30 (-$CH_2C\underline{H}_2CO$) represents two overlapping triplets, one from each of the fatty acids (δ 2.31, 2H, t, $J_{2,3}$ = 8.0 Hz; δ 2.29, 2H, t, $J_{2,3}$ = 8.0 Hz); -$C\underline{H}_2CH_2CO$ (δ 1.59, 4H, m); $(CH_2)_n$ (δ 1.2-1.4, ~60H, m); $\underline{C}H_3$ (δ 0.85-0.95, 9H [three protons belong to terminal methyl group of alkylamine], $J_{\omega,\omega-1}$ = 6.7); the overall unsaturation (50%) of fatty acid and alkylamine is reflected in the assignments at -CH=CH-(δ 5.34, 3H, m) and -CH_2-CH=CH-CH_2-(δ 2.01, 6H, m). Alkylamine: -NHC$\underline{H}_2$-(δ 3.22, 2H, m); -$NHCH_2C\underline{H}_2$-(δ 1.51, 2H, m).

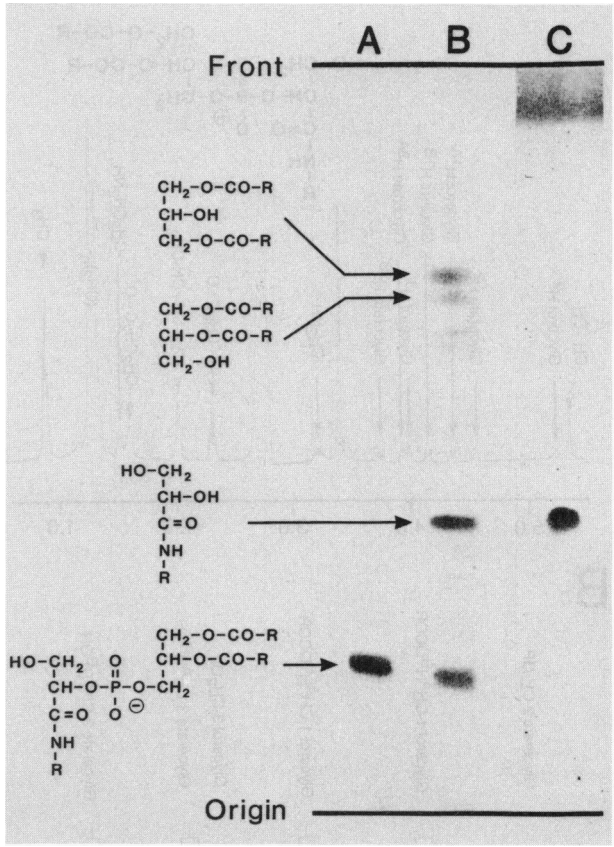

FIG. 3. Products of HF hydrolysis of lipid 4. Pure lipid 4, isolated from [^{14}C]acetate-labeled *D. radiodurans*, was subjected to HF hydrolysis and to analysis by TLC. Lane A, Pure lipid 4; lane B, lipid 4 following HF treatment; lane C, synthetic glyceroyl hexadecylamine. Lanes A and B were visualized by autoradiography; lane C was visualized by sulfuric acid charring.

The key proton assignments listed above were verified by spin decoupling as follows. Glycerate: irradiation of δ 4.55 ppm (H^2) collapsed two sets of doublet-doublets at δ 3.93 and 3.74 ppm (3-CH^AH^BOH) to two sets of doublets ($J_{A,B}$ = 12.5 Hz). The coupling constant, $J_{31_{P,H}}$ = 7.2 Hz, was determined by irradiating at either δ 3.93 or δ 3.74 ppm. Both of these irradiations collapsed the multiplet at δ 4.55 ppm (H^2) to a doublet-doublet. Glycerol: irradiation at δ 5.22 ppm (2-CHOCOR) collapsed two sets of doublet-doublets at δ 4.36 and δ 4.14 ppm (1-CH^AH^BOCOR) to two sets of doublets ($J_{A,B}$ = 12.0 Hz) and also collapsed the "quartet"

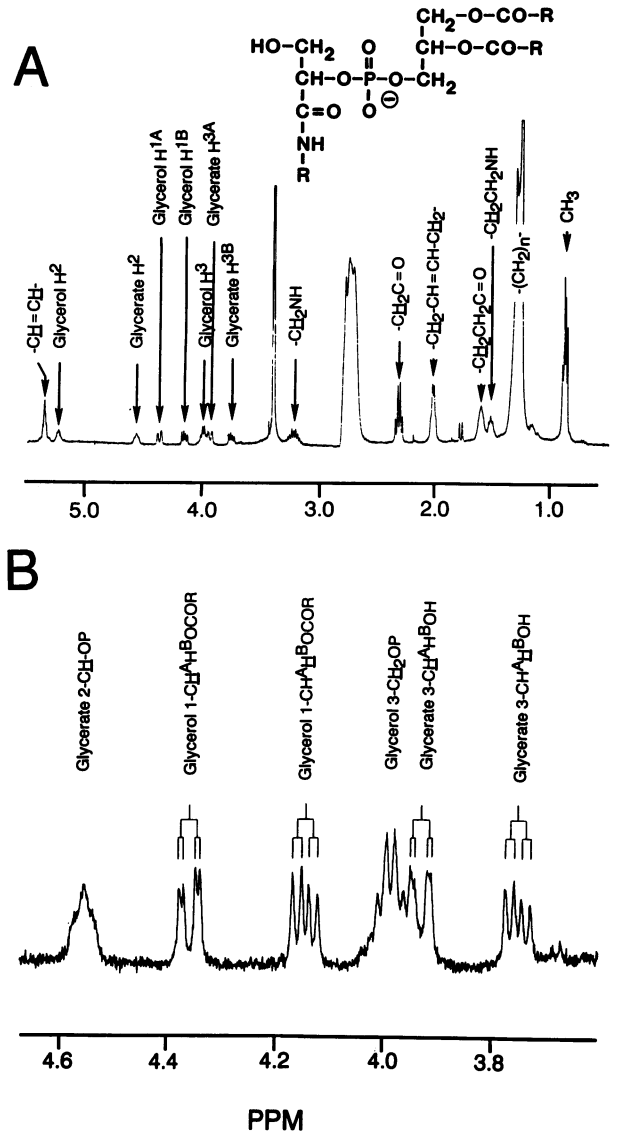

FIG. 4. ^1H NMR spectrum of lipid 4 dissolved in $CDCl_3$-CD_3OD (10:1, vol/vol). (A) Complete spectrum; (B) partial, expanded spectrum showing splitting patterns of glycerol and glycerate protons. Signals at 3.39 and 2.74 ppm correspond to CD_2HOD and HDO, respectively. The doublet at 1.76 ppm is derived from an impurity present in the $CDCl_3$ solvent.

at δ 3.98 ppm (3-CH^AH^BOP) to a triplet. H^{3A} and H^{3B} are diastereotopic and form an ABMX (M = P, X = H) system, where $\Delta\delta_{A,B}$ is small and consequently the outer AB lines are difficult to measure. The separation of the large inner lines is about the same as the $J_{M,A}$, $J_{M,B}$, $J_{X,A}$, and $J_{X,B}$ coupling constants ($J \sim 6$ to 7 Hz). Hence, the splitting pattern in the spectrum appears as a quartet. Fatty acid: irradiation of δ 2.01 ppm (-CH_2-CH=CH-CH_2-) collapsed the multiplets at δ 5.34 (-CH_2-CH=CH-CH_2-) to a singlet. Irradiation at 1.59 ppm (-CH_2CH_2CO-) collapsed the quartet at δ 2.30 ppm (-CH_2CO-) to a doublet at δ 2.31 and 2.29 ppm, each corresponding to one fatty acid chain. Alkylamine: irradiation at δ 3.22 ppm (-$NHCH_2$-) simplified the multiplet at δ 1.51 ppm (-$NHCH_2CH_2$-).

Lipid 4 is a precursor to lipids 6 and 7. The structural

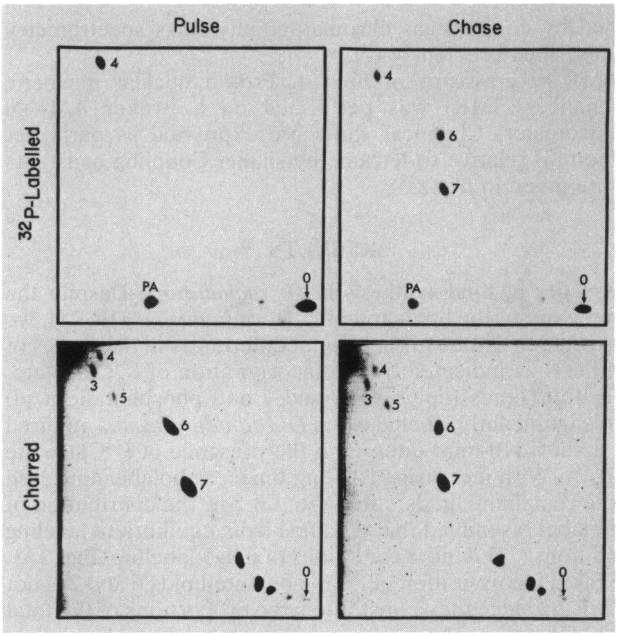

FIG. 5. Lipid 4 is a precursor to lipids 6 and 7. A 1-ml culture of *D. radiodurans* was pulse-labeled for 5 min with [^{32}P]phosphate (1 mCi/ml). Following washing of the cells, an aliquot (pulse phase) was immediately extracted, whereas the remainder was incubated at 35°C for 60 min and then extracted (chase phase). Samples from the lipid extracts were resolved by TLC. The TLC plates were examined both by autoradiography and by charring. Numerical lipid designations are as previously assigned (28). Abbreviations: PA, phosphatidic acid; O, origin.

similarities between lipid 4 and the previously identified lipids 6 and 7 suggested an obvious precursor-product relationship. This was verified by means of a pulse-chase procedure using [^{32}P]phosphate. Pulse-labeling of *D. radiodurans* for 5 min resulted in the incorporation of label only into lipid 4 and phosphatidic acid (Fig. 5). (^{32}P label at the origin did not char and is probably residual [^{32}P]phosphate carried through the lipid extraction procedure.) A subsequent 60-min chase caused the displacement of radiolabel into lipids 6 and 7 concomitant with a loss of label from lipid 4 and phosphatidic acid. Given the ubiquitous primary role of phosphatidic acid in phospholipid biosynthesis, the results indicate a clear biosynthetic pathway through lipid 4 to the complex phosphoglycolipids 6 and 7.

Glycosylation of lipid 4 is the rate-limiting step in the biosynthesis of the complex phosphoglycolipids 6 and 7. A closer examination of the interrelationship between lipid 4 and the glycosylated lipids 6 and 7 was performed through additional pulse-chase techniques. Incorporation of [^{32}P] phosphate into lipid 4 could be achieved within 2 min of pulse-labeling (Fig. 6). The subsequent glycosylation of lipid 4 occurred more slowly, as determined by the appearance of ^{32}P label in lipids 6 and 7 during the chase period. With a 2-min ^{32}P pulse, radiolabeled lipid 4 began to be converted to lipid 7 within 10 min and to lipid 6 within 20 min. With a 5-min ^{32}P pulse, both lipids 6 and 7 were labeled within 10 min. It was noted, however, that the displacement of radiolabel from lipid 4 to lipids 6 and 7 continued to occur throughout the chase period (60 min), indicating that lipid 4 glycosylation is a relatively slow process. It may also be mentioned that glycosylation of lipid 4 appears to favor

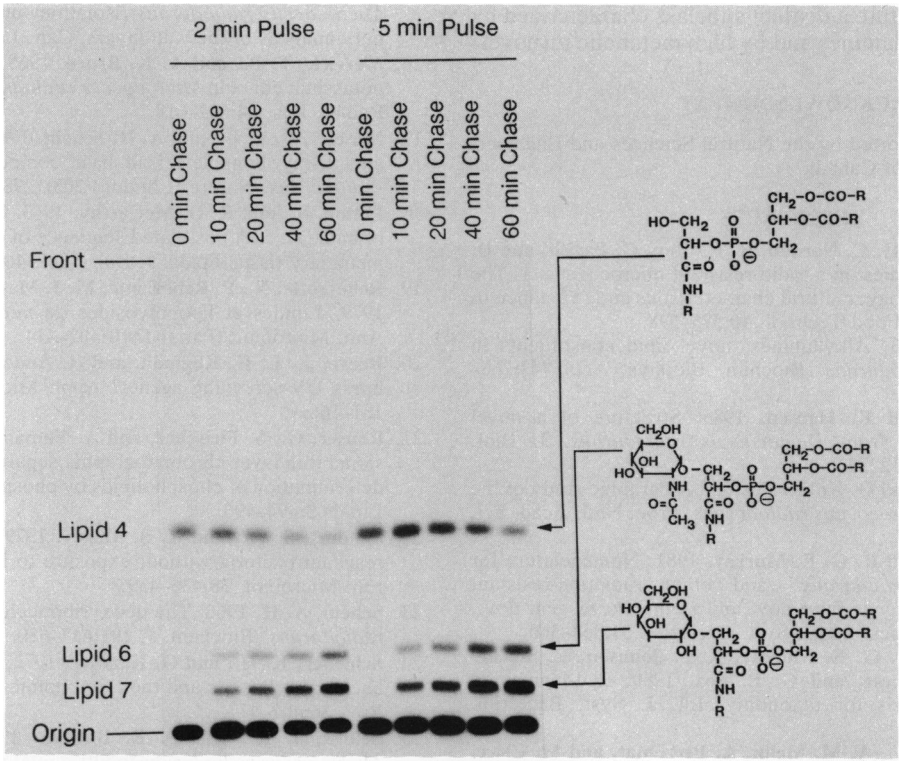

FIG. 6. Kinetics of glycosylation of lipid 4. One-milliliter cultures of *D. radiodurans* were pulse-labeled for either 2 or 5 min with [^{32}P]phosphate (1 mCi/ml), washed, and either extracted immediately (pulse phase) or incubated at 35°C for the times indicated before extraction (chase phase). Lipid extracts were resolved by TLC in chloroform–methanol–28% ammonia (80:20:2, vol/vol/vol). Radiolabeled lipids were detected by autoradiography.

galactose (thus generating lipid 7), which likely accounts for the greater abundance of lipid 7 relative to lipid 6 found in *D. radiodurans* (28).

DISCUSSION

In this report, we have described another novel lipid from *D. radiodurans* and have provided some insights into the biosynthesis of complex phosphoglycolipids in this organism. Lipid 4 (phosphatidylglyceroylalkylamine) is synthesized rapidly (within 2 min by using radiolabeled phosphate as a precursor). In contrast, the subsequent glycosylation of lipid 4 to produce lipids 6 and 7 occurs much more slowly and appears to be the rate-limiting step in the biosynthesis of these complex phosphoglycolipids.

Under conditions of short pulse-labeling (2 to 5 min), [^{32}P]phosphate is incorporated into only two phospholipids, lipid 4 and phosphatidic acid. Radiolabeling of these two lipids was also prominent when a 10-min pulse of [^{14}C] acetate was used (Fig. 2). The rapid incorporation of either of these radiolabeled lipid precursors into phosphatidic acid by such pulse-labeling techniques suggests that phosphatidic acid is an important intermediate in phospholipid biosynthesis and is likely the source of the 1,2-diacylglycero backbone found in lipids 4, 6, and 7. We have previously shown that the glycerol moiety of lipids 6 and 7 are of the 1,2-diacyl-*sn*-glycero-3-phospho configuration (3, 13). Thus, despite the largely unconventional nature of *D. radiodurans* lipids, the commonly configured *sn*-1,2-phosphatidic acid backbone found almost ubiquitously in bacterial lipids is conserved in *D. radiodurans*. In contrast, only members of the *Archaebacteria* have been found to contain glycerolipids of an *sn*-2,3 stereochemistry (for a review, see references 9 and 14).

It is of interest that all polar lipids (lipids 4, 6, and 7) from *D. radiodurans* identified to date contain the unusual lipid constituents glyceric acid and alkylamine. By short-pulse-labeling methods, we were unable to demonstrate appreciable incorporation of [^{32}P]phosphate into any lipids except lipid 4 and phosphatidic acid. In particular, we did not detect a radiolabeled lipid which might be phosphatidylglycerate, a potential precursor of lipid 4. It therefore seems unlikely that phosphatidylglycerate is a precursor of lipid 4, but rather it seems likely that the biosynthesis of lipid 4 proceeds via condensation of phosphatidic acid (or its dephosphorylated product, diacylglycerol) and glyceroylalkylamine (possibly involving liponucleotide derivatives thereof). Studies are currently under way to verify such a biosynthetic pathway.

Efforts have been made to use the unusual lipid composition of *D. radiodurans* as a taxonomic means of identification (8, 10). On the basis of lipid patterns defined by TLC, *D. radiodurans* could be readily differentiated from other bacteria, including other *Deinococcus* species (8, 10). On the other hand, the lipid patterns of nine isolates of *D. radiodurans* were very similar (8), particularly with regard to the two major alkylamine-containing phosphoglycolipids, lipids 6 and 7, which have been previously identified (3, 13). In contrast to lipids 6 and 7 and their precursor (lipid 4), our preliminary analyses indicate that the remaining uncharacterized major lipids of *D. radiodurans* do not contain alkylamines. Thus, of all the lipids of *D. radiodurans*, lipids 4, 6,

and 7 seem to constitute a distinct subclass characterized by the presence of alkylamines and by high metabolic turnover.

ACKNOWLEDGMENT

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