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Fatty Acids Are Precursors of Alkylamines in *Deinococcus radiodurans*

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Deinococcus radiodurans **contains novel phospholipids of which the structures of three have been previously described. These three lipids contain both fatty acids and alkylamines. Both the fatty acid and alkylamine constituents were found to be composed of a mixture of species, of which C_{15}, C_{16}, and C_{17} saturated and monounsaturated alkyl chains predominated. Alkylamines contained a relatively higher proportion of saturated species. Progression of bacterial growth through the mid-log to stationary phases was accompanied by an increase in the proportions of C_{15} and C_{17} alkyl chains in both fatty acid and alkylamine constituents. Radiolabeled palmitic acid was found to be rapidly incorporated into both fatty acid and alkylamine components of phosphatidylglyceroylalkylamine, which is the precursor of the more-complex phosphoglycolipids found in major amounts in *D. radiodurans*. After culturing *D. radiodurans* in the presence of a mixture of palmitic acids labeled with ^{14}C and ^{3}H in the 1 and 9,10 positions, respectively, the same $^{14}C/^{3}H$ ratio was recovered in both fatty acid and alkylamine constituents, strongly suggesting that alkylamines are derived from intact fatty acids rather than by a de novo pathway. The results identify a novel product of fatty acid metabolism which has not to date been observed in any other organism.**

The radiation-resistant bacterium *Deinococcus radiodurans* contains a complex cell wall structure in which two lipid membranes consisting of unusual lipid structures are a feature. The structures of some of these unusual lipids have been previously elucidated (3, 4, 19, 20). Three of these lipids contain both fatty acids and alkylamines, the latter of which have not to date been observed outside members of the family *Deinococcaceae*. Alkylamine-containing phospholipids may be of special interest in *D. radiodurans* since they appear to represent a subclass of membrane lipids which is characterized by a high rate of turnover (20). The biosynthesis of the alkylamine moieties, heretofore unique in nature, remains unexplored.

Comparative analyses of polar and nonpolar lipids has proven very useful in the classification of deinococci (9, 12, 30). Although a complete chemical analysis has not been carried out on all strains, comparative thin-layer chromatographic (TLC) analyses of a large number of isolates of *D. radiodurans* indicate the presence of two major phosphoglycolipids (9, 12) with characteristics similar to those of the glycosylated phosphatidylglyceroylalkylamines previously characterized in *D. radiodurans* Sark (4, 19). It is therefore likely that alkylamines will prove to be an almost universal constituent of the unique lipids of *D. radiodurans* despite its isolation from a diversity of environments worldwide (2, 8, 22, 23, 27).

MATERIALS AND METHODS

Cell culture and lipid isolation. Cultures of *D. radiodurans* Sark were grown at 35°C as rotary-shaken aerobic suspension cultures (200 rpm) in 0.5% tryptone–0.3% yeast extract–3 mM $CaCl_2$. Lipid extractions of packed cell pellets of *D. radiodurans* were performed by using a modified procedure of Folch et al. (13) as previously described (34).

Analytical methods. Pyrrolidyl esters of fatty acids were obtained from fatty acid methyl esters by the procedure of Andersson and Holman (7) by using pyrrolidine (99% purity; Aldrich) and acetic acid (10:1 [vol/vol]) (100°C for 30 min in sealed tubes). For the preparation of fatty acids and alkylamines, purified lipids were subjected to 5% methanolic HCl hydrolysis at 102°C in sealed glass tubes for 2 h (to liberate fatty acid methyl esters) or 24 h (to liberate alkylamines and glyceroylalkylamines). 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 glass-wool-plugged Pasteur pipettes; first, fatty acid methyl esters were eluted with chloroform and then alkylamines and glyceroylalkylamines were eluted with chloroform-methanol (3:1 [vol/vol]). The chloroform-methanol (3:1 [vol/vol]) eluates were acetylated overnight by reaction with pyridine-acetic anhydride (1:1 [vol/vol]). The acetylated mixtures were dried, dissolved in chloroform, and analyzed by gas chromatography.

Gas chromatography. A Hewlett-Packard model 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 (at 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 5°C/min). Confirmation of gas chromatographic assignments was obtained by coupled gas chromatography-mass spectrometry (GC-MS) as described previously (3). Percentage compositions were determined by using a Hewlett-Packard model 3392A integrator. Fatty acid pyrrolidides were analyzed by GC-MS with a Kratos 80 mass spectrometer equipped with a 30-m DB-1 capillary column, temperature programmed from 50 to 180°C (at 10°C/min) and then from 180 to 250°C (at 5°C/min). GC-MS analyses of fatty acid pyrrolidides permitted the identification of positional isomers of the monounsaturated fatty acids (7).

Radiolabeling of *D. radiodurans*. Radiolabeled palmitic acids were all purchased from New England Nuclear and

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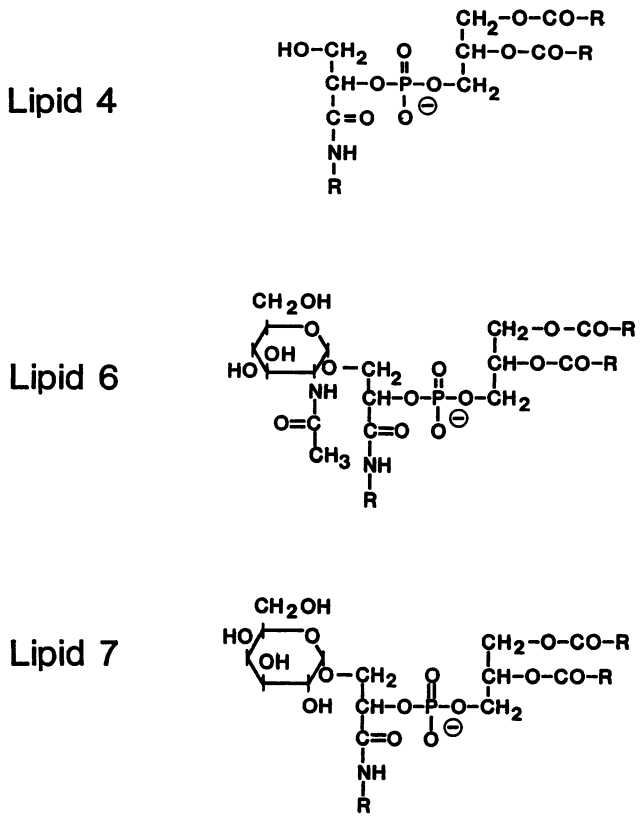

FIG. 1. Structures of phospholipids referred to in this article.

were added as ethanol solutions to growth media consisting of 0.5% tryptone, 0.3% yeast extract, and 3 mM $CaCl_2$. A 10-ml culture of *D. radiodurans* was incubated with [1-^{14}C]palmitic acid (1 μCi/ml) at 35°C. Aliquots of 1 ml were removed after 5, 10, 15, 20, 30, and 40 min and chilled on ice, and cells were harvested by centrifugation for 10 min at 13,000 × *g* in a microcentrifuge at 4°C. For dual labeling, a 50-ml culture of *D. radiodurans* (optical density at 600 nm, 0.6) was incubated with a mixture of [1-^{14}C]palmitic acid (0.2 μCi/ml) and [9,10-^{3}H]palmitic acid (0.1 μCi/ml) for 15 min at 35°C. The culture was harvested by centrifugation (4,000 × *g* at 4°C for 10 min). Packed cell pellets were lipid extracted and examined by TLC as described above.

TLC. Analytical TLC was performed by using Sil Gel G Redi-plates (Fisher Scientific). Total lipid extracts from *D. radiodurans* were resolved in chloroform–methanol–28% ammonia (65:35:5 [vol/vol/vol]). Lipid hydrolysates containing mixtures of fatty acid methyl esters, alkylamines, and glyceroylalkylamines were resolved by successive development in three solvents: one-third development in chloroform–methanol–28% ammonia (80:20:2 [vol/vol/vol]); two-thirds development in chloroform-methanol (15:1 [vol/vol]); and full development in chloroform-ether (85:15 [vol/vol]).

RESULTS

Fatty acid and alkylamine composition of major phosphoglycolipids of *D. radiodurans*. Three lipids of *D. radiodurans* contain alkylamine constituents, namely, lipids 4, 6, and 7, the structures of which are shown in Fig. 1. Lipid 6 is 2′-*O*-(1,2-diacyl-*sn*-glycero-3-phospho)-3′-*O*-(α-*N*-acetylglucosaminyl)-*N*-glyceroylalkylamine (19), lipid 7 is 2′-*O*-(1,2-diacyl-*sn*-glycero-3-phospho)-3′-*O*-(α-galactosyl)-*N*-glyceroylalkylamine (4), and lipid 4 is phosphatidylglyceroylalkylamine (20). While lipid 4 is a relatively low-abundance constituent, lipids 6 and 7 are major components of *D. radiodurans* (34). Samples of lipids 6 and 7 were isolated by preparative TLC from cultures grown to the mid-log, early stationary, and late stationary phases. The results shown in Fig. 2 indicate several interesting features, which are described below.

The major alkyl chains of both fatty acid and alkylamine constituents were C_{15}, C_{16}, and C_{17} in which both saturated and monounsaturated species were observed as well as small amounts of *iso* fatty acids, particularly *i*-17:0 and *i*-17:1. The alkylamines were relatively enriched for saturated alkyl chains (mainly C_{16}), whereas monounsaturated (especially 16:1) species predominated among the fatty acids.

The most striking feature of the fatty acid or alkylamine composition was the marked dependence on the bacterial growth phase. Mid-log-phase cells showed predominantly C_{16} fatty acids and alkylamines. By early stationary phase, the proportions of C_{16} species were reduced in favor of C_{15} and C_{17}. This trend increased further, so that by late stationary phase, C_{15} and C_{17} alkyl chains made up more than 50% of the total composition in both fatty acid and alkylamine constituents.

The features noted above applied to both of the major alkylamine-containing phosphoglycolipids examined. Fatty acid compositions of lipids 6 and 7 were remarkably similar to one another and showed parallel variations in response to growth phase. This was also true for the alkylamine composition of the two lipids, although some relative enrichment for certain species, particularly 17:1, was evident in lipid 7 in cells harvested at the early and late stationary phases.

Incorporation of radiolabel from [1-^{14}C]palmitate into alkylamines. The observed similarities in fatty acid and alkylamine compositions suggested that both might be derived from a common biosynthetic pathway. As an initial test of this possibility, a culture of *D. radiodurans* was incubated in the presence of [1-^{14}C]palmitate for short periods of time (ranging from 5 to 40 min) at 35°C. As shown in Fig. 3, radiolabel was incorporated rapidly into cellular lipids. Within 5 min of exposure to [1-^{14}C]palmitate, the radiolabel was found in lipid 4, which has been previously identified as phosphatidylglyceroylalkylamine (20). Lipid 4 is the precursor to the complex phosphoglycolipids 6 and 7 (20). Consistent with its role as an intermediate in the biosynthesis of lipids 6 and 7 is the observed rise and fall of incorporated radiolabel in lipid 4 along with the progressive increase in the levels of radiolabeled lipids 6 and 7 (Fig. 3). (The structures of lipids 3 and 5 have not yet been characterized.)

To determine whether the incorporated radiolabel was associated with the fatty acid or alkylamine constituents of lipid 4, samples of lipid 4 were preparatively isolated by TLC and subjected to acid methanolysis. The resultant products of methanolysis were resolved on TLC and autoradiographed. As shown in Fig. 4, most of the incorporated radiolabel was found in fatty acid methyl esters. However, a considerable amount was also seen in the alkylamine and glyceroylalkylamine products of methanolysis. Interestingly, the proportions of alkylamine-associated radiolabel to fatty acid-associated radiolabel remained quite constant over the time period examined (5 to 40 min). By densitometric scanning of the autoradiogram shown in Fig. 4, the radiolabeled fatty acid/alkylamine ratio (FAME/[alk + GA-alk]) was 3.5 ± 0.3 (mean ± standard deviation) for the six time points examined. This observation along with the finding of

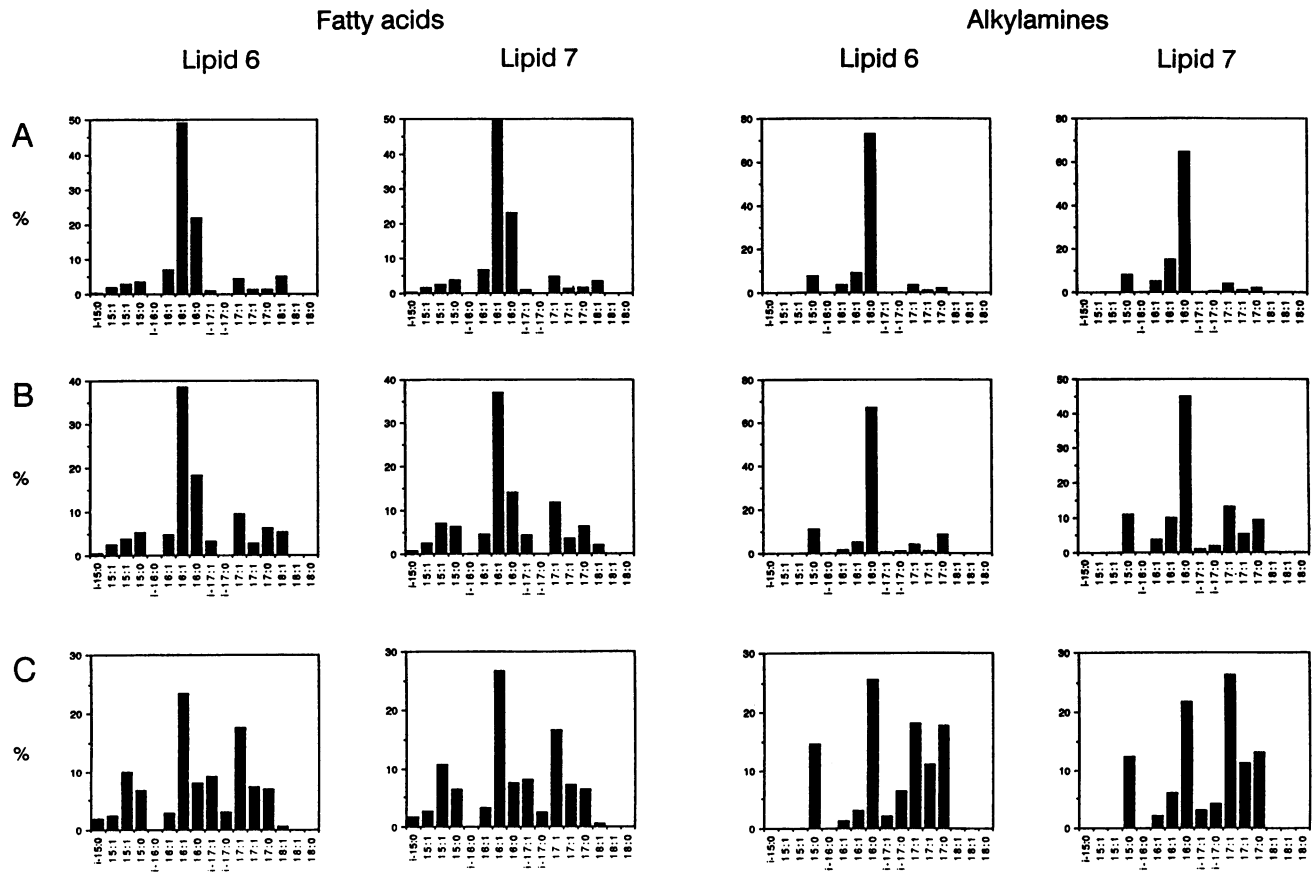

FIG. 2. Fatty acid and alkylamine compositions of the major phosphoglycolipids, lipids 6 and 7, isolated from cultures of *D. radiodurans* harvested at mid-log (22 h) (A), early stationary (36 h) (B), and late stationary (70 h) (C) stages. Lipids were isolated by preparative TLC and subjected to acid methanolysis, and the resultant fatty acid methyl esters and alkylamines (plus glyceroylalkylamines) were recovered by preparative TLC. Fatty acid methyl esters and acetylated alkylamines (plus glyceroylalkylamines) were resolved by gas chromatography as detailed in Materials and Methods. Monounsaturated alkyl chains constitute at least two isomeric populations with respect to the location of the double bond (Δ), namely (reading from left to right on the *x* axes), 15:1Δ7, 15:1Δ9, 16:1Δ7, 16:1Δ9, 17:1Δ9, 17:1Δ11, 18:1Δ9, and 18:1Δ11. Abbreviation: *i*, *iso*.

radiolabel in alkylamine as early as 5 min suggested that palmitic acid was the major precursor pool for alkylamine biosynthesis. If the ^{14}C label from the palmitic acid were being significantly recycled through acetate or other intermediates, the radiolabeled fatty acid/alkylamine ratio would be expected to vary as a result of changing specific activities of these additional intermediate pools.

Radiolabeling of alkylamine with dual-labeled palmitic acid. To confirm and extend the above findings, a culture of *D. radiodurans* was incubated for 15 min with a mixture of [1-^{14}C]palmitic acid and [9,10-^{3}H]palmitic acid. Lipids were extracted from harvested cells and resolved on a TLC plate which was autoradiographed, and the radiolabeled lipid 4 was preparatively isolated by scraping and by eluting the gel with chloroform-methanol (1:1). The purity of lipid 4 was verified by TLC (data not shown). The eluted lipid 4 was subjected to acid methanolysis and TLC analysis of the resultant products which were detected by autoradiography. The $^{14}C/^{3}H$ ratios in fatty acid methyl esters, alkylamines, and glyceroylalkylamines were determined by scintillation counting of the excised and eluted products from the TLC plate. The results (Fig. 5) showed similar $^{14}C/^{3}H$ ratios in the three products examined, suggesting that the radiolabeled palmitic acid was likely incorporated intact (exclusive of the carboxy OOH) into alkylamine. Recycling of radiolabel into alkylamine via products of fatty acid catabolism is therefore unlikely.

DISCUSSION

In this study, we have presented evidence that the alkylamine moieties present in lipids from *D. radiodurans* are derived biosynthetically from fatty acids. The evidence for this is threefold. First, although quantitative differences are evident, the alkyl chain compositions of both fatty acids and alkylamines are qualitatively remarkably similar and show parallel changes in response to bacterial growth phase. Second, radiolabel from [1-^{14}C]palmitate is incorporated very rapidly (within 5 min) into alkylamines. Finally, the $^{3}H/^{14}C$ ratio was found to be the same in both fatty acids and alkylamines isolated from phosphatidylglyceroylalkylamine (lipid 4) from *D. radiodurans* cultured in the presence of an exogenously supplied mixture of [1-^{14}C]- and [9,10-^{3}H]palmitic acids. This latter result is more consistent with a biosynthetic pathway leading from intact fatty acids to alkylamines rather than a pathway involving fatty acid degradation and recycling of radiolabel into alkylamine precursors. Fatty acid degradation and then de novo alkyl-

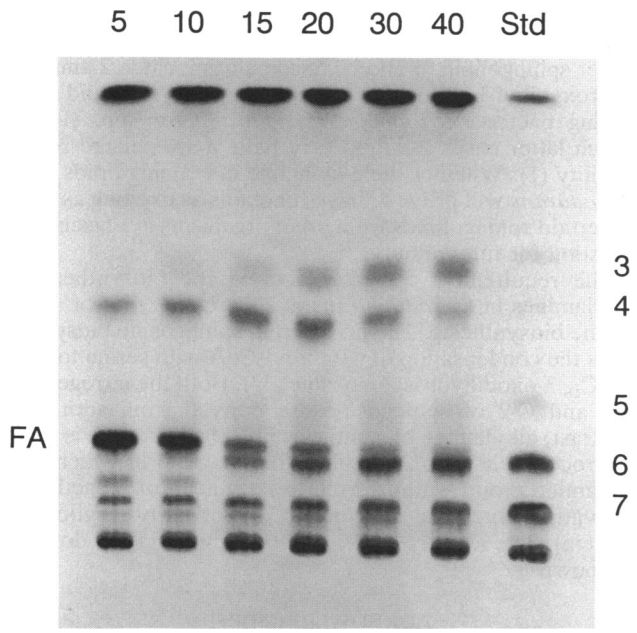

FIG. 3. Incorporation of [1-^{14}C]palmitic acid into lipids of *D. radiodurans*. A 10-ml culture of *D. radiodurans* was incubated with [1-^{14}C]palmitic acid at 35°C. Aliquots of 1 ml were removed after 5, 10, 15, 20, 30, and 40 min and microcentrifuged; cell pellets were lipid extracted, and aliquots of the extracts were run on a TLC in chloroform–methanol–28% ammonia (80:20:2 [vol/vol/vol]) and autoradiographed. Lipid designations are those described by Thompson et al. (34). FA, fatty acid; Std (standard), a lipid extract from [^{14}C]acetate-labeled *D. radiodurans*.

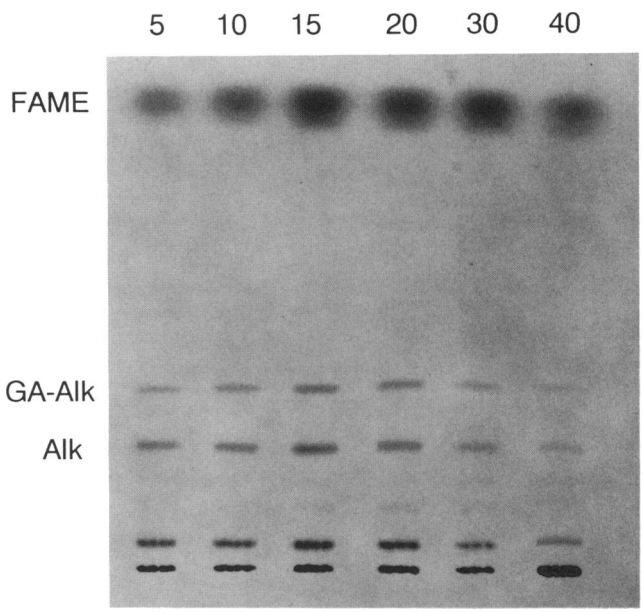

FIG. 4. Incorporation of radiolabel from [1-^{14}C]palmitic acid into both fatty acid and alkylamine moieties of lipid 4, phosphatidylglyceroylalkylamine. Radiolabeled lipid 4, isolated from preparative TLC of the samples shown in Fig. 3, was subjected to acid methanolysis for 24 h at 102°C. The resultant methanolysates were dried and applied to TLC (one-third development in chloroform–methanol–28% ammonia [80:20:2, by volume]; two-thirds development in chloroform-methanol [15:1, by volume]; full development in chloroform-ether [85:15, by volume]). An autoradiograph is shown. Major products of methanolysis: FAME, fatty acid methyl esters; Alk, alkylamines; GA-Alk, glyceroylalkylamines. The radiolabeled band near the origin was not characterized but is likely a side product of methanolysis.

amine synthesis from acetate would likely yield an alkylamine product enriched in ^{14}C for the following reasons. Since the oxidative degradation of fatty acids proceeds sequentially from the carboxyl end, the most abundant product would be acetate derived from C-1 and C-2 (and containing the ^{14}C label from [1-^{14}C]palmitate). Moreover, since each step of oxidative degradation involves extensive dehydrogenation (33), there would be considerable depletion of ^{3}H from [9,10-^{3}H]palmitate and consequently any derived acetates therefrom. The possibility of a partial degradation of fatty acid followed by reelongation to alkylamines has admittedly not been excluded but is unlikely given our finding of very similar ^{3}H/^{14}C ratios in both fatty acid and alkylamine moieties derived from the dual-radiolabeling protocol.

The fatty acid compositions of deinococci have been recognized as unusual in that they include various isomers, i.e., both straight- and branched-chain, saturated and monounsaturated species (12). The results presented here extend such isomeric diversity to the alkylamines of the type member of this genus, *D. radiodurans*, and indicate that alkylamine composition is determined largely, but not absolutely, by the composition of the precursor fatty acids.

The observed growth-dependent alterations in alkyl chain composition for both fatty acids and alkylamines may best be summarized as a progressive shift from even-numbered straight-chain alkyl chains (16:0 and 16:1) to odd-numbered straight- and branched-chain alkyl chains (15:0, 15:1, 17:0, 17:1, *i*-17:0 and *i*-17:1). This shift, which is most apparent during the stationary phase, may be due to a number of factors. Growth phase-dependent alterations in fatty acid composition have been described for other bacteria, the best characterized of which is an increase in cyclopropane fatty acids during the transition from the exponential to the stationary phase demonstrated in *Escherichia coli* (10, 26, 28, 29) and in several other bacteria (for examples, see references 11, 24, 25, and 28). A variety of factors, including oxygen tension and changes in nutrient concentrations and in secreted metabolites, have been implicated in the growth-dependent increase in cyclopropane fatty acids, which are biosynthetically derived by transmethylation of monoenoic fatty acids from *S*-adenosylmethionine (reviewed in reference 14). As a consequence, levels of monoenoic fatty acids are concomitantly reduced. As indicated in the present study, the growth-dependent changes observed in the fatty acid composition of *D. radiodurans* differ from the pattern described in the preceding examples. Cyclopropane fatty acids are absent from *D. radiodurans*, which instead shows a growth-dependent shift from even-numbered to odd-numbered alkyl chains. The observed changes in alkyl chain composition in both fatty acids and alkylamines could have their basis in altered extra- or intracellular levels of the respective fatty acid precursor substrates (e.g., acetate, propionate, or isobutyrate) during bacterial growth. (In this connection, note that exogenous propionate can stimulate synthesis of odd-numbered fatty acids in *E. coli* [21].) Alternatively, there might be a growth-dependent shift in the amount, activity, or preference of the bacterial fatty acid synthetases for primer substrates other than acetyl coen-

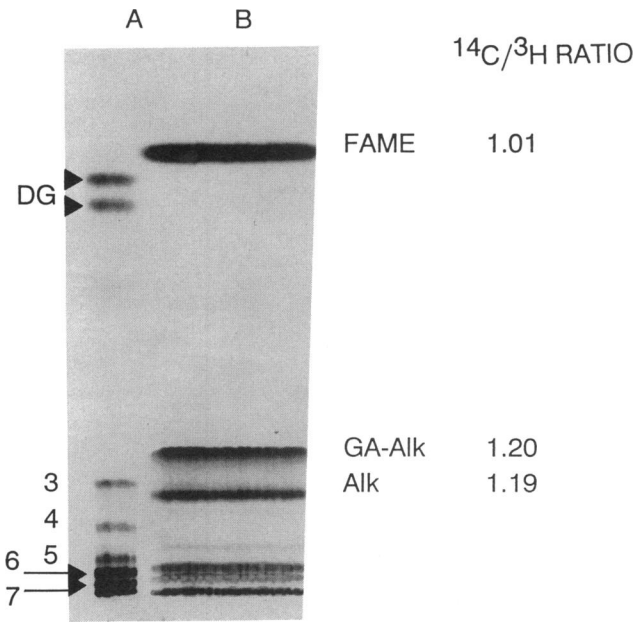

FIG. 5. Incorporation of radiolabel from [1-^{14}C]palmitic acid and [9,10-^{3}H]palmitic acid into both fatty acid and alkylamine moieties of lipid 4, phosphatidylglyceroylalkylamine. A culture of *D. radiodurans* was incubated for 15 min at 35°C in the presence of both of the radiolabeled palmitic acids and subsequently harvested for lipid extraction. Lipid 4 was isolated by preparative TLC and subjected to methanolic HCl hydrolysis, and the resultant methanolysate was resolved on TLC (lane B). A lipid extract of [^{14}C]acetate-labeled *D. radiodurans* (lane A) is included as a standard marker. DG is diglyceride (consisting of 1,2 and 1,3 isomers). Also shown are the ^{14}C/^{3}H ratios recovered in fatty acid methyl ester (FAME), alkylamine (Alk), and glyceroylalkylamine (GA-Alk) components.

zyme A (acetyl-CoA) i.e., propionyl-CoA or isobutyryl-CoA, which would promote the synthesis of odd-numbered straight- or branched-chain fatty acids. Collectively, the above findings augment the existing evidence that growth-dependent alterations in fatty acid composition are widespread among divergent bacteria even though quite distinct mechanisms may be employed.

Although, as shown here, alkylamine biosynthesis in *D. radiodurans* proceeds from fatty acid precursors, it is evident that cellular processes exist for the maintenance of distinct compositions within the fatty acid and alkylamine constituents of the major lipids of this organism. For example, although there is a growth-dependent trend towards greater proportions of C_{15} and C_{17} species in both fatty acids and alkylamines, the alkylamines retain a relatively greater proportion of saturated alkyl chains than unsaturated alkyl chains. Subject to this bias, we suggest that at any given phase of bacterial growth, alkylamines are largely derived, in a fairly nonselective manner, from fatty acid substrates depending on the prevailing fatty acid composition in the cell at that time.

The occurrence of both fatty acids and alkylamines in *D. radiodurans* is an interesting example of structural and possibly functional dichotomy in alkyl chain biosynthesis. The closest analogs to alkylamines so far detected in nature are probably the sphingosines, which are found predominantly in eukaryotes, including animals, plants, and fungi. Recent research has begun to establish some functions for certain sphingolipids, particularly in cell regulatory processes (15, 17, 18). Studies with *Saccharomyces cerevisiae* have indicated a vital requirement for sphingolipids (31). Other sphingolipid or alkylamine analogs include 2-amino-3-hydroxyalkyl-1-sulfonates found in diatoms (5, 6) and in the gliding bacteria *Cytophaga* and *Flexibacter* spp. (16), in which latter cases the lipid may have a specialized role in motility (1). Whether the alkylamine-containing lipids of *D. radiodurans* will prove to have functions as exciting as those of certain sphingolipids from other organisms is a fascinating question for future study.

The requirement for fatty acid in the biosynthesis of alkylamines in *D. radiodurans* is also reminiscent of sphingosine biosynthesis. The first step in sphingosine biosynthesis is the condensation of palmitoyl-CoA with serine to yield the C_{18} 3-oxodihydrosphingosine (32). Both the nitrogen and C-1 and C-2 of sphingosine are derived from serine. In contrast, alkylamine biosynthesis in *D. radiodurans* is likely to proceed via a transaminationlike reaction in which only the amino group is transferred to fatty acid (or an activated derivative, such as fatty acyl-CoA or fatty acyl-carrier protein). The identity of the putative amino group donor is unknown.

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