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Glucosyl Diglyceride Lipid Structures in *Deinococcus radiodurans*

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The structures of two lipids from the radiation-resistant bacterium *Deinococcus radiodurans* are reported here: 1,2-diacyl-3-α-glucopyranosyl-glycerol and 3-*O*-[6′-*O*-(1″,2″-diacyl-3″-phosphoglycerol)-α-glucopyranosyl]-1,2-diacylglycerol. These lipids are strikingly different from previously characterized polar lipids from this organism, in that they are not unique to the genus *Deinococcus* and indeed have counterparts in both gram-negative and gram-positive bacteria. Moreover, as examples of glucose-containing lipids, they further illustrate the diversity of carbohydrate-containing lipids in *D. radiodurans*, from which lipids containing galactose and *N*-acetylglucosamine have already been structurally characterized.

The lipid composition of *Deinococcus radiodurans* is complex and unusual (19, 23, 28). Early studies identified a number of glyco- and phosphoglycolipids with little characterization of their precise structures (19, 23, 28). Subsequent analyses of lipid structure in *D. radiodurans* concentrated on a class of three alkylamine-containing phospholipids, i.e., phosphatidylglyceroylalkylamine and its glycosylated derivatives (1, 12, 13). We now focus attention on a different class of lipids, denoted by the lack of alkylamine or glyceric acid components and bearing more resemblance to lipid structures found outside the deinococci.

Bacterial glycolipids are a diverse group of structures found in many different bacteria. Among the most common type of bacterial glycolipid are the diglycosyl diglycerides, which are widespread among both gram-positive and gram-negative organisms (18). Mono- and triglycosyl diglycerides are occasionally found, but in much smaller quantities (27). Diglycosyl diglycerides may also occur in conjugated form, such as in the phosphoglycolipid phosphatidyldiglycosyl diglyceride (27). The broad occurrence of glycosyl diglycerides among bacterial taxa suggests that these lipids play an important, perhaps essential, role in cell physiology. The finding of similar structures in the unusual bacterium *D. radiodurans* might serve to strengthen this conviction.

Preliminary examination by nuclear magnetic resonance (NMR) spectroscopy of the remaining uncharacterized lipids of *D. radiodurans* indicated a number of glycolipid and phosphoglycolipid structures which could contain a glycosyl diglyceride core. Foremost among these were lipids designated 3 and 5 (28), which contained a single carbohydrate component and therefore posed the greatest prospects for structure elucidation. In this report, we describe the complete structures of these two lipids and confirm that glycosyl diglycerides are indeed represented in this taxonomically puzzling bacterium.

Cultures of *D. radiodurans* were grown to early stationary phase, and lipids were extracted as previously described (1). Lipids 3 and 5 were isolated from lipid extracts by preparative thin-layer chromatography (TLC) on homemade Silica Gel H plates (gel thickness, 1 mm) and developed in chloroform–methanol–28% ammonia (80:20:2, vol/vol/vol). By using companion TLC plates which were stained with iodine as a guide, the lipid 3 (R_f = 0.520) and lipid 5 (R_f = 0.23) bands were scraped out and eluted with chloroform-methanol (1:1, vol/vol).

Previous studies have indicated that lipid 3 is a glycolipid and lipid 5 is a phosphoglycolipid (28). In order to establish the chemical structures of both lipids, a series of chemical and spectroscopic analyses was undertaken. Hydrofluoric acid, known to cleave phosphodiester bonds (10), cleaved lipid 5 into two products. One of these products comigrated with diglyceride; the other comigrated with lipid 3 and also with monogalactosyl diglyceride (from wheat flour; Sigma Chemical Co., St. Louis, Mo.) on TLC (Redi/Plt Sil Gel G; Fisher Scientific) in the solvent system chloroform–methanol–28% ammonia (80:20:2, vol/vol/vol). Under these chromatographic conditions, monoglycosyl diglycerides (e.g., containing either glucose, galactose, or other hexoses) migrate at the same R_f (0.49). Acid hydrolysis of lipid 3 yielded fatty acid methyl esters and 1-*O*-methyl-glucoside. 1-*O*-Methyl-glucoside (R_f = 0.52) was identified by TLC on Silica Gel G plates in butanol-ethanol-water (5:2:4, vol/vol/vol) and also by gas chromatographic identification of the acetylated product.

The results given above suggested that lipid 3 is monoglucosyl diglyceride and that lipid 5 consists of monoglucosyl diglyceride linked through a phosphodiester bond to diglyceride. Confirmation of these structures was provided by NMR spectroscopic analysis.

^{1}H NMR spectroscopy of lipids 3 and 5. Analysis using ^{1}H NMR of intact, underivatized lipids 3 and 5 identified the number of sugars and the presence of glycerol moieties in both compounds. Comparison of the ^{1}H NMR spectra of the intact lipids 3 and 5 showed similarity between them. Both spectra showed only a single doublet signal (assigned to the sugar H-1) between δ 4.0 and 5.0 ppm, the window for the anomeric proton resonance. This confirmed that both lipids were monosaccharides. Also, the coupling constants of $J_{1,2}$ = 3.7 Hz for lipid 3 and $J_{1,2}$ = 3.6 Hz for lipid 5 indicated an α configuration for the sugar in both lipids (7, 20). Both lipids also contained glyceride units (including unsaturated fatty acids) as was revealed by comparing the proton signals of CH_3, $C\underline{H}_2CO$, $C\underline{H}_2CH_2CO$, $-C\underline{H}_2-CH{=}CH-C\underline{H}_2-$, and $C\underline{H}{=}C\underline{H}$ with ^{1}H NMR spectra of intact lipids 4, 6, and 7 (1, 10, 12). NMR spectra of lipids 3 and 5 differed in the number of methine (CH) proton resonances corresponding to glycerol–H-2 (two proton equivalents around δ 5.2 ppm for lipid 5, compared with only one proton equivalent around δ 5.2 ppm for lipid 3). These data indicated that there were two glycerol units in lipid 5 and one in lipid 3. The rest of the nonanomeric methine

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TABLE 1. ^{1}H NMR assignments for deacylated, peracetylated lipid 3[a]

Proton assignment	Chemical shift (δ, ppm)[b]	No. of protons	Multiplicity[c]	Coupling constant(s) (Hz)
Glucopyranoside				
H-1	5.11	1	d	$J_{1,2} = 3.7$
H-2	4.86	1	dd	$J_{1,2} = 3.7$; $J_{2,3} = 10.0$
H-3	5.46	1	dd	$J_{3,2} = 10.0$; $J_{3,4} = 9.8$
H-4	5.06	1	dd	$J_{4,3} = 9.8$; $J_{4,5} = 9.9$
H-5	4.01	1	ddd	$J_{5,4} = 9.9$; $J_{5,6A} = 4.6$; $J_{5,6B} = 2.3$
H-6A	4.27	1	dd	$J_{6A,5} = 4.6$; $J_{6A,6B} = 12.3$
H-6B	4.10	1	dd	$J_{6B,5} = 2.3$; $J_{6B,6A} = 12.3$
Glycerol				
1-C$\underline{H}^A$H^{B}OCOR	4.34	1	dd	$J_{A,B} = 11.9$; $J_{A,2} = 4.3$
1-CH$^A\underline{H}^B$OCOR	4.18	1	dd	$J_{B,A} = 11.9$; $J_{B,2} = 5.8$
2-CHOCOR	5.20	1	m	—[d]
3-C$\underline{H}^A$H^{B}O-sugar	3.83	1	dd	$J_{A,2} = 4.3$; $J_{A,B} = 11.3$
3-CH$^A\underline{H}^B$O-sugar	3.65	1	dd	$J_{B,A} = 11.3$; $J_{B,2} = 5.5$

[a] NMR spectra were recorded on a Bruker AM-400 spectrometer. Deacylated, peracetylated lipid 3 was dissolved in $CDCl_3$.
[b] Relative to tetramethylsilane.
[c] Abbreviations: d, doublet (the number of letters corresponds to the number of doublets); m, multiplet.
[d] —, not determined.

sugar protons and the methylene (CH_2) protons of glycerol were overlapped within a relatively small window (δ 3.2 to 3.7 ppm), and it was impossible to make assignments for them.

^{1}H NMR of peracetylated lipids 3 and 5. Because of the limited resolution obtained by ^{1}H NMR spectroscopy of intact, underivatized lipids 3 and 5, we prepared peracetylated derivatives which yielded well-resolved ^{1}H NMR spectra (Tables 1 and 2). Mild alkaline deacylation of lipids was performed according to the procedure of Kates (16). Lipid acetylation was carried out in pyridine-acetic anhydride (1:1, vol/vol) overnight at room temperature. We took advantage of the deshielding effect of the acetoxy group on the sugar protons which results in a downfield shift of ca. 0.5 ppm for methylene protons and ca. 1.1 ppm for methine protons on the pyranose sugar (14).

TABLE 2. ^{1}H NMR assignments for peracetylated lipid 5[a]

Proton assignment	Chemical shift (δ, ppm)	No. of protons	Multiplicity[b]	Coupling constant(s) (Hz)
Glucopyranoside				
H-1	5.03	1	d	$J_{1,2} = 3.6$
H-2	4.78	1	dd	$J_{1,2} = 3.6$; $J_{2,3} = 10.0$
H-3	5.39	1	dd	$J_{3,2} = 10.0$; $J_{3,4} = 9.7$
H-4	5.08	1	dd	$J_{4,3} = 9.7$; $J_{4,5} = 9.5$
H-5	~3.9	1	m	—[c]
H-6A6B	~3.9	2	m	—
O-acetate	2.024	3	s	—
	2.019	3	s	—
	1.967	3	s	—
Glycerol 1				
1-C$\underline{H}^A\underline{H}^B$OCOR	~4.32	1	m	—
	~4.11	1	m	—
2-C$\underline{H}$OCOR	5.18	1	m	—
3-C$\underline{H}_2$OP	~3.9	2	m	—
Glycerol 2				
1-C$\underline{H}^A$H^{B}OCOR	~4.32	1	m	—
1-CH$^A\underline{H}^B$OCOR	~4.11	1	m	—
2-C$\underline{H}$OCOR	5.18	1	m	—
3-C$\underline{H}^A$H^B-*O*-sugar	3.80	1	dd	$J_{A,B} = 11.6$; $J_{A,2} = 4.7$
3-CH$^A\underline{H}^B$-*O*-sugar	3.58	1	dd	$J_{B,A} = 11.6$; $J_{B,2} = 5.5$
Fatty acid				
-C$\underline{H}$═CH-	5.3	—	m	—
-C$\underline{H}_2$-CH═CH-C$\underline{H}_2$-	2.0	~10[d]	m	—
-C$\underline{H}_2$CO	2.29	4	t	$J_{2,3} = 7.4$
	2.27	4	t	$J_{2,3} = 7.4$
-C$\underline{H}_2$CH$_2$CO	1.57	8	m	—
-C$\underline{H}_3$	0.85	12	m	—

[a] NMR spectra were recorded as described in Table 1, footnotes *a* and *b*. Peracetylated lipid 5 was dissolved in $CDCl_3$-CD_3OD (3:1, vol/vol).
[b] Abbreviations: s, singlet; d, doublet (the number of letters corresponds to the number of doublets); t, triplet; m, multiplet.
[c] —, not determined.
[d] Roughly consistent with >60% monounsaturation in fatty acyl chains (see Table 4).

TABLE 3. ^{13}C NMR assignments for lipid 5, deacylated lipid 5, lipid 3, and galactosyldiglyceride[a]

Carbon	Chemical shift (δ, ppm) of:			
	Lipid 5 ($J_{31P,13C}$ [Hz])	Deacylated lipid 5 ($J_{31P,13C}$ [Hz])	Lipid 3	β-D-Galactosyldiglyceride
Glucopyranoside				
C-1	99.28	100.69	99.07	103.87
C-2	71.54	73.75	72.00	71.24
C-3	72.51	74.95	73.88	73.18
C-4	69.62	72.36	70.11	70.23
C-5	71.16	72.76[c] (7.6)	71.79	74.77
C-6	63.70[b] (4.4)	67.60[e] (5.9)	61.70	61.84
Glycerol 1				
1-$\underline{C}H_2OCOR$	62.36	64.14[d]	—	—
2-$\underline{C}HOCOR$	69.98 (8.6)	72.60[c] (7.3)	—	—
3-$\underline{C}H_2OP$	63.35[b] (4.4)	65.77[e] (5.1)	—	—
Glycerol 2				
1-$\underline{C}H_2OCOR$	62.36	63.92[d]	62.35	62.64
2-$\underline{C}HOCOR$	68.27	71.29	69.84	68.89
3-$\underline{C}H_2O$-sugar	66.12	71.02	66.18	67.90
Fatty acid				
-$\underline{C}$═O	173.74	—	173.80	173.83
	173.22		173.42	171.69
-$\underline{C}H$═$\underline{C}H$-	129.69	—	129.92	130.14
	129.35		129.60	129.90
				127.98
				127.78
-$\underline{C}H_2CO$	33.89	—	34.15	34.16
	33.76		34.00	33.99
-$\underline{C}H_2$-CH═CH-$\underline{C}H_2$	26.86	—	27.06	27.08
-$\underline{C}H_2CH_2CO$	24.54	—	24.75	24.73
-$\underline{C}H_3$	13.72	—	13.95	13.91

[a] NMR spectra were recorded on a Bruker AM-400 spectrometer. Chemical shifts are relative to tetramethylsilane. Solvents used were $CDCl_3$-CD_3OD (3:1, vol/vol) for lipid 3, lipid 5, and β-D-galactosyldiglyceride and CD_3OD for deacylated lipid 5. —, not determined.
[b,c,d,e] Like letters indicate that assignments may be reversed.

Pyranosyl methine proton signals of H-2, H-3, and H-4 (in the δ 3.2- to 3.7-ppm region) in lipid 3 were shifted downfield to δ 4.86, 5.46 and 5.06 ppm, respectively, in deacylated, peracetylated lipid 3 (Table 1). This confirmed the presence of acetylable hydroxyl groups at these positions. In addition, the methylene proton signals of H-6 in the intact unmodified lipid 3 in the δ 3.2- to 3.7-ppm region were shifted downfield to δ 4.10 and 4.27 ppm upon acetylation. Therefore, lipid 3 has four hydroxyl groups on the pyranose ring and is a monosubstituted sugar. A total of six *O*-acetyl ($OCOC\underline{H}_3$) resonances were observed around 2.0 ppm (δ 2.024, 2.045, 2.075, 2.085, 2.105, and 2.109 ppm [data not shown]). This is consistent with four acetylable hydroxy groups on the glucopyranosyl C-2, C-3, C-4, and C-6 and another two acetylable hydroxy groups on C-1 and C-2 of glycerol. Individual assignments were not made because of the observed small differences in chemical shift (11). The data are consistent with lipid 3 being an α-glucopyranosyl diglyceride.

Comparison of the ^1H NMR spectra of underivatized lipid 5 and the peracetylated lipid 5 showed that upon acetylation, the methine proton resonances attributed to the H-2, H-3, and H-4 of the sugar had shifted from the δ 3.2- to 4.2-ppm region to δ 4.78, 5.39, and 5.08 ppm, respectively (Table 2). In addition, there were only three signals corresponding to *O*-acetyl ($OCOC\underline{H}_3$) groups at δ 1.967, 2.019, and 2.024 ppm, none of which were seen in the ^1H NMR spectrum of the intact, unmodified lipid 5. This confirmed the presence of only three free hydroxyl groups in lipid 5, and these could be assigned to positions 2, 3, and 4 of the sugar moiety (definitive assignments were confirmed as described in the next paragraph). This, in turn, confirmed that the glucose in lipid 5 was 1,6 disubstituted.

Two-dimensional chemical shift-correlated spectroscopy. Verification of the sugar proton assignments made above was provided by two-dimensional chemical shift-correlated spectra, which identify coupling of protons within a molecule (15). By this method, the chemical shifts of all the protons of deacylated, peracetylated lipid 3 and peracetylated lipid 5 were assigned as shown in Tables 1 and 2. The chemical shifts of the diastereotopic H-6 protons of deacylated, peracetylated lipid 3 were further confirmed by spin-decoupling experiments. The signals of the four methylene protons (CH^AH^B) of the two glycerol units of peracetylated lipid 5 were assigned by comparison of their chemical shifts with those of dioleoylphosphatidylcholine (Sigma Chemical Co.) and lipids 3, 4, 6, and 7 of *D. radiodurans* (1, 12, 13).

^{13}C NMR spectroscopy of lipids 3 and 5. To further confirm the chemical structures of lipids 3 and 5, ^{13}C NMR analyses of lipid 3, lipid 5, and deacylated lipid 5 were performed. The ^{13}C NMR data (Table 3) permitted the assignment of all the carbon centers. Distortionless enhancement by polarization transfer NMR spectra of lipid 3 and lipid 5 were also obtained to verify the CH_3, CH_2, and CH carbons. The downfield shift of the anomeric carbon from δ 92.9 to 99.07 ppm in lipid 3 and to δ 99.28 ppm in lipid 5 confirmed the presence of substitution on C-1 of the pyranoside (21). The ^{13}C NMR data of lipid 3 were in agreement with the reference compounds, α-methyl-D-glucopyranoside, dissolved in D_2O (21), and β-galactopyranosyl diglyceride, dissolved in $CDCl_3$-CD_3OD (3:1, vol/vol). Thus, lipid 3 is a 1,2-diacyl-3-α-glucopyranosylglycerol (Fig. 1).

The ^{13}C NMR assignments for lipid 3, lipid 5, and deacylated lipid 5 (Table 3) were made by comparison with ^{13}C NMR spectra of 1,6-diphosphoglucopyranoside (cyclohexyl ammo-

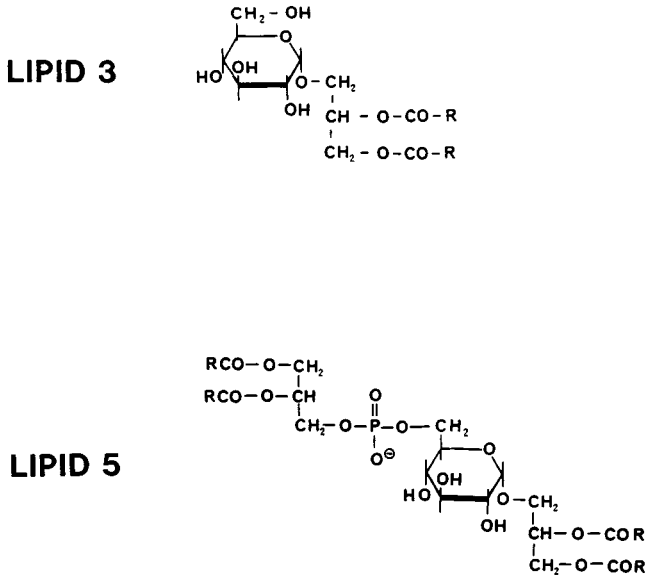

FIG. 1. Chemical structures of lipid 3 and lipid 5.

nium salt [Aldrich Chemical Co., St. Louis, Mo.] dissolved in CD_3OD), dioleoylphosphatidylcholine (dissolved in $CDCl_3$-CD_3OD, 3:1, vol/vol), and lipid 6 from *D. radiodurans* (12) (dissolved in $CDCl_3$-CD_3OD, 3:1, vol/vol). The differences in the chemical shifts between lipid 5 and deacylated lipid 5 are likely due to solvent effects ($CDCl_3$-CD_3OD, 3:1, vol/vol, versus CD_3OD) and different chemical environments (absence of fatty acids in the latter). Distortionless enhancement by polarization transfer analyses revealed five methylene (CH_2) carbon centers in lipid 5. Four methylene signals were assigned to C-1 and C-3 of the two glycerol units, and one methylene signal was assigned to the C-6 of the pyranoside (Table 3). The observation of the ^{31}P-^{13}C coupling confirmed the presence of the phosphodiester linkage and the location of this linkage on C-6 of the glucopyranoside (no ^{31}P-^{13}C coupling was observed on C-1 of the glucopyranoside). Thus, lipid 5 is 3-*O*-[6′-*O*-(1″,2″-diacyl-3″-phosphoglycerol)-α-glucopyranosyl]-1,2-diacylglycerol (Fig. 1).

Fatty acid composition of lipids 3 and 5. Fatty acid analysis of lipids 3 and 5, isolated from early-stationary-phase cultures of *D. radiodurans*, was performed by procedures described in reference 2. Both lipids contained predominantly C_{15}, C_{16}, and C_{17} fatty acids, although lipid 3 contained proportionately higher levels of C_{15} and C_{17} and lower levels of C_{16} (Table 4). The differences may reflect differences in the fatty acid composition of the glucosyldiglyceride (present in both lipids 3 and 5) vis-à-vis the phosphatidate (present only in lipid 5) components. The low concentration of lipid 3 (28) in the total cell and the fact that it is a chemical constituent of lipid 5 suggest its possible role as a biosynthetic or catabolic (or even breakdown) intermediate. In other microorganisms, monoglycosyl diglycerides are biosynthetic precursors of diglycosyl diglycerides, and they do not generally accumulate in significant amounts (25). Diglycosyl diglycerides are not present in significant amounts in *D. radiodurans*, as indicated by TLC (28). Thus, if lipid 3 has a role as a biosynthetic intermediate, it is probably with respect to the formation of lipid 5 rather than diglycosyl diglyceride.

D. radiodurans contains two lipid membranes (29). While lipid 3 is found in both the plasma membrane and the outer membrane in almost equal amounts, lipid 5 is preferentially sequestered in the outer membrane, where it composes about 21% of the lipid composition of the outer membrane (29). This may indicate conversion of lipid 3 to lipid 5 concomitant with or following transport from the plasma membrane to the outer membrane. The biosynthesis of lipid 3 itself likely proceeds by glycosylation of diacyl glycerol (22). Free diacyl glycerol can be readily detected in lipid extracts from *D. radiodurans* (2) and is thus a logical candidate for the biosynthesis of lipid 3.

TABLE 4. Fatty acid compositions of lipids 3 and 5

Fatty acid	% (Mean ± SD) of:	
	Lipid 3	Lipid 5
iso-15:0	2.0 ± 0.9	1.0 ± 0.1
15:1Δ7	2.1 ± 1.1	3.9 ± 1.4
15:1Δ9	5.4 ± 1.9	7.8 ± 3.9
15:0	12.7 ± 4.6	6.6 ± 2.4
iso-16:0	0.6 ± 0.4	0.3 ± 0.2
16:1Δ7	1.7 ± 1.5	3.9 ± 1.0
16:1Δ9	25.9 ± 9.7	37.0 ± 7.7
16:0	13.8 ± 6.7	13.3 ± 7.4
iso-17:1	0.0 ± 0.0	0.0 ± 0.0
iso-17:0	1.8 ± 0.6	0.6 ± 0.1
17:1Δ9	14.9 ± 4.8	9.3 ± 4.0
17:1Δ11	6.9 ± 4.7	3.2 ± 2.2
17:0	8.6 ± 5.2	2.5 ± 1.5
18:1Δ9	0.5 ± 0.2	0.6 ± 0.2
18:1Δ11	1.3 ± 0.6	0.7 ± 0.6
18:0	0.8 ± 0.2	1.7 ± 1.2
Unknown	1.1 ± 1.1	7.6 ± 4.6

Lipids with the same structures as those of lipids 3 and 5 have been identified in other bacteria. Glycosyl diglycerides (including lipid 3) are a fairly common constituent of bacteria, especially gram-positive bacteria (9, 18, 25). Phosphatidylglucosyl diglyceride (lipid 5) appears to be less widespread but has been detected in the gram-negative *Pseudomonas diminuta* (30) and *Pseudomonas vesicularis* (31) as well as the gram-positive *Streptococcus haemolyticus* (8). In *P. diminuta*, phosphatidylglucosyl diglyceride is synthesized from glucosyl diglyceride via an enzyme-catalyzed transphosphatidylation reaction in which phosphatidylglycerol donates a phosphatidyl group (24). Phosphatidylglycerol has so far not been detected in *D. radiodurans*, and it is unlikely that it occurs in significant quantities in this organism (28). Further studies will be necessary to elucidate the biosynthetic pathway of lipid 5 in *D. radiodurans.*

Finally, the results obtained in this study are pertinent to the utility of lipids for bacterial taxonomy studies. The use of lipid analyses in bacterial taxonomy has long been recognized (for example, see references 18 and 26). The genus *Deinococcus* (3, 4) was established on the basis of several criteria, including G+C content of DNA, rRNA homologies, peptidoglycan chemical structure, and lipid composition. The lipid composition of *D. radiodurans* (19, 23, 28) and of other deinococci (5, 6) is complex and characterized by numerous unconventional lipids, the structures of some of which are so far unique in nature (1, 12, 13). The identification of lipids 3 and 5 (as glucosyl diglyceride and its phosphatidylated derivative) demonstrates that *D. radiodurans* also contains examples of more conventional lipid structures. Our studies thus define two major classes of lipid structures in *D. radiodurans*, i.e., a novel class of alkylamine-containing phospholipids (lipids 4, 6, and 7) and a more conventional class of glycosyl diglyceride-based glyco- and phosphoglycolipids (lipids 3 and 5). The first class tends to firmly entrench *D. radiodurans* as a relatively unique

taxonomic group, while the second suggests links with divergent bacteria, including both gram-positive and gram-negative organisms. We suggest that glycosyl diglyceride structures represent a highly conserved element among bacteria and likely serve essential structural or functional roles. Note also that even the phylogenetically distinct archaea possess glycolipid structures which are ether-linked analogs of glycosyl diglycerides (reviewed by Kates [17]).

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