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MEMBRANE LIPID AND *RECA* GENE STRUCTURE IN *DEINOCOCCUS RADIODURANS*

by

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ABSTRACT

Previous studies with bacteria have indicated an intimate association between membranes and DNA replicative processes. Little is known of such processes in the radiation-resistant bacterium, *Deinococcus radiodurans*. This dissertation aims at elucidating two potentially important features of *D. radiodurans*: (i) structural characterization of the membrane lipids, and (ii) identification and characterization of a DNA repair gene, *recA*, in *D. radiodurans*.

The structures of several previously unidentified lipids were determined utilizing a combination of chemical and NMR spectroscopic analyses. One of these, designated lipid 6, was determined to be 2'-0-(1,2-diacyl-*sn*-glycero-3-phospho)-3'-0-(α-N-acetyl-glucosaminyl)-N-glyceroylalkylamine. Another phospholipid, lipid 4, was shown to be phosphatidylglyceroylalkylamine. Lipid 4 was shown to be the precursor of the complex phosphoglycolipids 6 and 7, i.e. α-N-acetyl-glucosaminyl- and α-galactosyl phosphatidylglyceroylalkylamine. While phosphatidylglyceroylalkylamine was rapidly biosynthesized from inorganic phosphate, its subsequent glycosylation occurred much more slowly. Therefore, the final glycosylation step is probably the rate-limiting event in the biosynthesis of the complex phosphoglycolipids, 6 and 7.

Lipids 4, 6 and 7 contain fatty acids and alkylamines. Both of these constituents were found to comprise a mixture of species, of which C-15, C-16 and C-17 saturated and mono-unsaturated alkyl chains predominated. Alkylamines contained a relatively higher proportion of saturated species. Progression of bacterial growth through the mid-exponential 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. 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 ratio of $^{14}C/^{3}H$ was recovered in both

1 and 9, 10 positions, respectively, the same ratio of $^{14}C/^{3}H$ 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 i.e., alkylamine which to date has not been observed in any other organism.

Two additional polar lipids have been characterized. One of these, lipid 3, is a glycolipid: 1,2-diacyl-3-α-glucopyranosyl glycerol. The other, lipid 5, is a phosphatidylglycolipid: 3-0-[6'-0'(1",2"-diacyl-3"-phosphoglycerol)-α-glucopyranosyl]-1,2-diacylglycerol. Both lipid 3 and lipid 5 have structural counterparts in both Gram-negative and Gram-positive bacteria.

A partial *recA* clone of *D. radiodurans* was isolated and its 363 bp nucleotide sequence was determined. The deduced amino acid sequence was 77% identical with the corresponding sequence of the *Bacillus subtilis* RecA protein and 74% identical with that of the *E. coli* RecA protein. By comparison with other RecA proteins, domains could be identified which are believed to be involved in the binding of duplex and single stranded DNA, and in the binding and hydrolysis of ATP.

In addition to providing novel biochemical and structural insights into *D. radiodurans*, the above data now provide a considerable battery of molecular tools with which to investigate DNA- and membrane-related repair processes which are suspected to be involved in the extraordinary radiation resistance of *D. radiodurans*.

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This work is dedicated

to

my parents

and in special memory

to my father

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LIST OF ABBREVIATIONS

ABBREVIATION	EXPLANATION
ADP	adenosine 5'-diphosphate
ATP	adenosine 5'-triphosphate
Å	angstrom
bp	base pair(s)
°C	degrees centigrade
Ci	Curie
CDP	Cytidine 5'-diphosphate
cpm	counts per minute
g	gravity
gly_2	glycine-glycine
h	hour(s)
J	Joule
k	thousand
kb	kilo base pair(s)
kDa	kilodalton
l	liter
M	molar
mg	milligram
MHz	mega Herz
min	minute

ABBREVIATION	EXPLANATION
ml	milliliter
mM	millimolar
µg	microgram
ml	microliter
NTP	nucleotide triphosphate(s)
Pol	polymerase
ppm	parts per million
r	ribosomal
RT	room temperature
ss	single strand(ed)
Taq	*Thermus aquaticus*
TE	Tris-EDTA (pH and molarities as specified in the text)
TYC	0.5% tryptone, 0.3% yeast extract and 3mM $CaCl_2$
UV	ultraviolet
Umu	UV unmutable
v	volume
wt	weight

CHAPTER 1

INTRODUCTION

1.1 CLASSIFICATION

The bacterium *Deinococcus radiodurans* was originally named *Micrococcus radiodurans* (Anderson *et al.*, 1956; Raj *et al.*, 1960). *M. radiodurans*, strain R, was isolated from cans of meat that had been sterilized by γ radiation (Anderson *et al.*, 1956). At that time, this organism was placed in the genus *Micrococcus* on the basis of its morphological and biochemical characteristics: the bacterium is a red pigmented coccus, is positive for catalase, and has a respiratory and nonfermentative metabolism. Later *D. radiodurans* was differentiated from the genus *Micrococcus* due to variations in cell wall structure, lipid composition and radiation resistance. The genus *Deinococcus* possesses a complex multiple-layered cell envelope (Thornley *et al.*, 1965; Work and Griffiths, 1968; Lancy and Murray, 1978; Baumeister *et al.*, 1981). The lipid layer is characterized by the presence of palmitoleate fatty acids (Brooks *et al.*, 1980) and the absence of conventional phosphatidylglycerolipids (Thompson *et al.*, 1980). Another distinction is *Deinococcus*' ability to withstand doses of 500 krad or 500 J m^{-2} of γ or ultraviolet radiation, respectively. In comparison, members of genus *Micrococcus* have a simple cell envelope consisting of a plasma membrane and a peptidoglycan layer. Its lipid layer contains phosphatidylglycerol-like lipids and no palmitoleate fatty acids. Organisms of this genus, as is true of most Gram-positive eubacteria, are sensitive to γ and UV radiation. The main basis for phylogenetic separation of *D. radiodurans* from the genus *Micrococcus* is the difference in their 16S rRNA sequences (Stackebrandt and Woese, 1979; Brooks *et al.*, 1980). The family Deinococcaceae has been grouped with Thermus as one of the 12 major groups of bacteria family (Brock, *et al.*, 1994). The family Deinococaccae comprises two genera, *Deinococcus* and *Deinobacter*. *Deinococcus* contains four spherical, nonmotile and Gram-positive species, usually found as tetrads (Brooks and Murray, 1981; Murray and Brooks, 1986). *Deinobacter* has only one rod-shaped Gram-

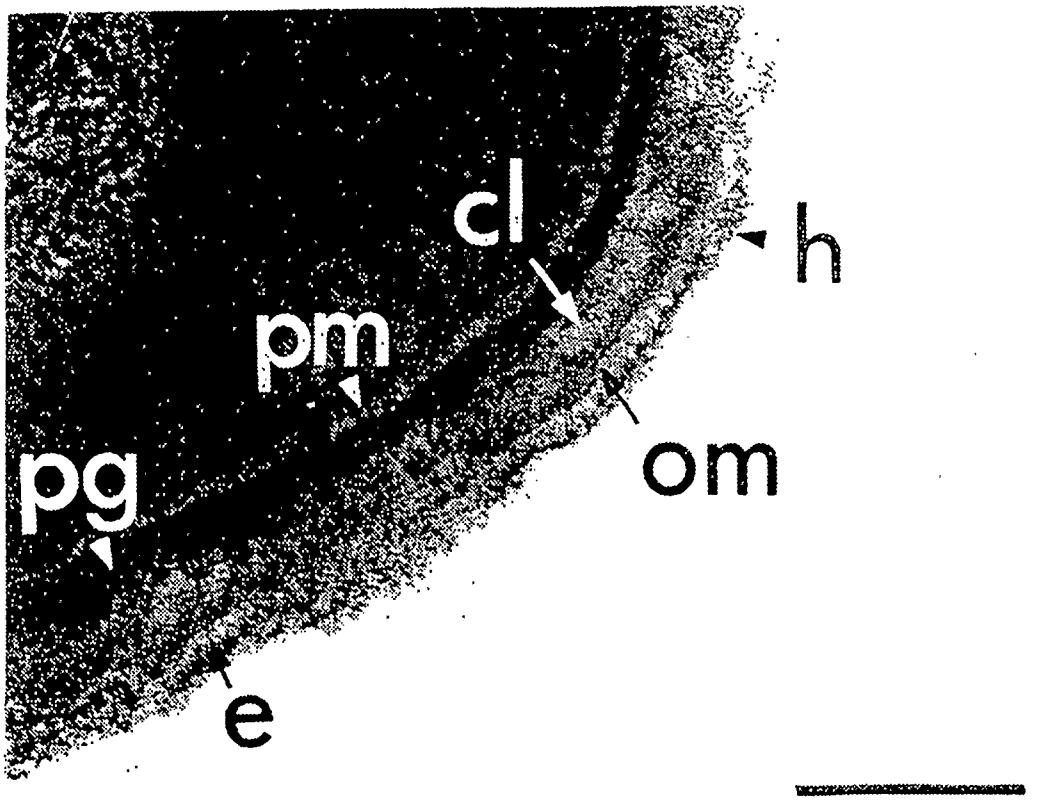
negative species (Oyaizu *et al.*, 1987). The Deinococcaceae have been isolated from diverse environments worldwide such as soil, dried fish, weathered granite, animal faeces, room dust, air contaminant and shielding pool of a Cobalt-60 radiation source (Moseley, 1983; Masters *et al.*, 1991c).

1.2 STRUCTURE AND COMPOSITION OF THE CELL ENVELOPE OF *D. RADIODURANS*

D. radiodurans has a non-conventional cell envelope structure. It contains characteristics of both Gram-positive and Gram-negative bacteria. The cell envelope is composed of seven distinct layers (Thornley *et al.*, 1965; Work and Griffiths, 1968; Lancy and Murray, 1978; Baumeister *et al.*, 1981). They are as follows (see Figure 1, from Thompson and Murray, 1981): the plasma membrane (width 8.1 nm), the peptidoglycan layer (50 nm), the "compartmentalized" layer (30 to 75 nm), the outer membrane (6.5 nm), an electron-transparent zone (10 nm) and a superficial hexagonal protein array also known as the S-layer (10 nm). The outermost layer is a carbohydrate coat (40 nm), not visible in figure 1.

The surface protein forms a hexagonal packed intermediate layer (HPI) (Baumeister *et al.*, 1986). The complete nucleotide sequence of the HPI gene has been determined; it encodes a polypeptide of 1036 amino acids (Peters *et al.*, 1987). The HPI protein is apparently a glycolipoprotein with relatively low carbohydrate content as compared to that of other eubacterial membrane glycoproteins (Peters *et al.*, 1987). These carbohydrates are mainly glucose, with minor components of galactose and mannose as well as trace quantities of N-acetylglucosamine. The fatty acid composition is similar to that of the lipid membrane (Brooks *et al.*, 1980) except that a higher proportion of saturated fatty acids is present. It has been suggested that the fatty acids are located in

Figure 1. **The cell envelope of *D.radiodurans* strain Sark (from Thompson and Murray, 1981).** Abbreviations: h - hexagonal protein array; e - electron transparent zone; om - outermembrane; cl - compartmentalized layer; pg - peptidoglycan layer; pm - plasma membrane. bar = 150 nm.


cl
h
pm
om
pg
e

the N-terminal region of the HPI polypeptide, and that together with the hydrophobic N-terminal they anchor the S-layer to the outer membrane of *D. radiodurans* (Peter *et al.*, 1987). This is consistent with the observation that the HPI layer binds strongly with the outer membrane via hydrophobic bonding (Thompson *et al.*, 1982). The other unusual structural feature of the HPI protein is its N-terminus, which is rich in serine and threonine. Also, the hydrophilic C-terminus is rich in aromatic amino acids. It has been observed that DNA is associated with HPI-layer (Peters *et al.*, 1987). An analogous situation is the hydrophilic and aromatic amino acid rich region of the C-terminus of the DNA-binding protein, DnaB, of *E. coli* which interacts with DNA (Nakayama *et al.*, 1984). Peters *et al.* (1987) speculated that the C-terminus of the HPI protein may interact with DNA.

D. radiodurans has an "outer membrane" which is similar to those of Gram-negative bacteria. It contains lipids and proteins (Thompson *et al.*, 1980; Thompson and Murray, 1981). However, it lacks lipopolysaccharides (Work and Griffiths, 1968; Brooks *et al.*, 1980). Next, there is the "compartmentalized" zone which appears to be divided into compartments between the outer membrane and the peptidoglycan layer. Unlike the Gram-negative bacteria, *D. radiodurans* has a thick peptidoglycan layer. This is likely the reason for its Gram-positive reaction with Gram stain. There are holes 10 to 18 nm apart in this layer. Also, there is no teichoic acid attached to the peptidoglycan layer and L-ornithine replaces the common lysine or diaminopimelic acid in the mucopeptide (Orn-gly_2) (Work, 1964). Gly_2 is the linkage unit between adjacent peptidoglycan strands.

The lipid composition of the outer and plasma membranes of *D. radiodurans* is complex and unusual. There are 8 neutral and 13 polar lipids present, as revealed by two dimensional thin layer chromatography (Thompson *et al.*, 1980; Reeve *et al.*, 1990). *D.*

radiodurans lacks the common bacterial phospholipids such as phosphatidyl-glycerol, -ethanolamine, -serine and cardiolipin. The polar lipids consist mainly of glyco-, phospho- and glycophospho-lipids as determined by [1-^{14}C]acetate-, [^{35}S]methionine- and [^{32}P]phosphate-labeling studies (Thompson *et al.*, 1980; Huang and Anderson, 1991). There are four glycolipids, one phospholipid, five phosphoglycolipids and one sulfated phospholipid. The polar lipid distributions in the plasma membrane, the outer membrane and the whole cell are listed in Table 1 (Thompson and Murray, 1981). Lipids 3, 10, 11 and 13 are glycolipids. Lipids 5, 6, 7, 9 and 12 are phosphoglycolipids. Lipid 4 is a phospholipid. Lipid 8 is a sulfur-containing phospholipid. The main structural components in the plasma membrane are lipids 6, 7 and 9. Lipids 5 and 9 are the major ones in the outer membrane. Of these only the lipid 7 had been established (see page 38).

The fatty acid composition of the lipids of *D. radiodurans* resembles that of the Gram-negative bacteria, being predominantly even-numbered, with saturated straight chain and unsaturated acids (Lechevalier, 1977; Brooks *et al.*, 1980; Huang and Anderson, 1989). A significant amount of saturated and unsaturated C-15 and C-17 fatty acids is also present. Comparisons of the polar and non-polar lipids have proven useful in the classification of the *Deinococci* (Counsell and Murray, 1986; Embley *et al.*, 1987; Masters *et al.*, 1991c). The polar lipid profiles have helped in the reclassification of the species *Deinococcus erythomyxa* into the *Micrococcus* genus (Counsell and Murray, 1986).

1.3 ROLE OF LIPIDS IN ADAPTATION TO EXTREME ENVIRONMENTS

Although many bacteria inhabit diverse environments, members of the Archaea are particularly noted for their ability to withstand extreme conditions. *D. radiodurans* is not a member of the Archaea. Nevertheless, it is useful to consider structure-function relationships derived from studies of Archaeal lipids, since they provide probably the best

TABLE 1. Proportions [a] of the lipids of *D. radiodurans* strain Sark in the plasma membrane, outer membrane, and whole cell [d].

Lipid No.	Plasma Membrane	Outer Membrane	Whole Cell
1	Tr [b]	Tr	Tr
2	Tr	Tr	Tr
3	1.62	0.86	1.22
4	2.42	2.05	2.37
5	8.83	21.45	11.83
6	11.76	3.58	9.51
7	39.92	3.08	33.28
8	0.52	1.16	0.68
9	18.33	48.45	24.63
10	8.50	8.00	8.4
11	0.34	0.95	0.43
12	3.00	5.48	3.30
13	0.50	1.28	0.70
Total neutral lipids [c]	3.52	3.72	3.57
Carotenoids	2.39	1.61	1.90

[a] Proportions expressed as percent of total lipid.

[b] Trace, less than 0.1% of total lipid

[c] Carotenoids and noncarotenoid neutral lipids

[d] Taken from Thompson and Murray (1981), see also page 74.

examples of how lipids may have evolved to help cells adapt to unusual and extreme environments. The Archaea comprise three groups each inhabiting a different extreme environment: the extreme halophiles, the methanogens and the thermoacidophiles. The polar membrane lipids of members of Archaea are comprised of a variety of phospholipids, glycolipids, phosphoglycolipids and sulpholipids (Kates, 1992). The chemical structures of Archaea lipids are unusual and differ from the lipids found in Bacteria and Eucarya. Archaea lipids are ether lipids. The alkyl chains of the glycerol are highly methyl branched saturated polar isoprenyls of C_{20}, C_{25} or C_{40}. Two unsaturated ether lipids have been identified (Hafenbradl *et al.*, 1993; Nichols and Franzmann, 1992). The stereochemistry of the glycerol moiety is *sn*-2,3-diphytanyl (Kates, 1978). Archaea lipids consist of diphytanylglycerol diether or its dimer dibiphytanyldiglycerol and their derivatives. The polar membrane lipids of Archaea may help these organisms to adapt to their peculiar environments. The presence of saturated alkyl chain and ether linkages to the glycerol units and *sn*-2,3 diphytanyl configuration of the glycerol backbone of Archaea lipids might aid their resistance to anti-oxidation and hydrolysis (e.g., by phospholipase) (Kates, 1978). In thermoacidophiles and some methanogens, the bipolar monolayer membrane formed by dibiphytanylglycerol tetraether with the head groups on the opposite sides of the bilayer would contribute to the stability of the membrane at high growth temperatures (De Rosa *et al.*, 1991). The presence of cyclopentane rings in direct response to the growth temperature of the thermoacidophiles might also increase rigidity of the membrane monolayer (De Rosa *et al.*, 1991). The high content of glycosylated lipids would stabilize the membrane structure of methanogens and thermoacidophiles by hydrogen bonding between the glycosyl headgroups (Kates, 1992). These are just a few examples of the physiological functions that to-date have been attributed to unusual lipids. However, a more complete overview would exceed the scope of this dissertation.

1.4 ROLE OF MEMBRANES IN RADIATION DAMAGE AND PROTECTION

Classically, the pathway leading to cell death by irradiation is commonly considered to consist of two components (Alper, 1963), one which is independent of oxygen concentration (type N damage) and one which is enhanced by oxygen (type O damage). Cellular DNA has generally been accepted as the major N-sensitive target, since inactivation of purified DNA by irradiation is largely unaffected by oxygen (Alper, 1979). Bacq and Alexander (1961) suggested that cell membranes represented another radiation-sensitive site in the cell and Alper (1963) claimed that these were involved in injury of the O type. Particularly, the lipid components of cell membranes are known to undergo increased damage when irradiated in the presence of increasing oxygen concentration, evidently as a result of double-bond peroxidation (Hannan and Boag, 1952; Mead, 1952). Irradiation of cell membranes leads to changes which can be monitored either by electron microscopy (Goldfeder and Miller, 1963; Hugon *et al.*, 1965) or by permeability tests (Barer and Joseph, 1960). Membrane permeability may be due to the formation of pore-like structures at sites at which lipid peroxidation is initiated (Stark, 1991). Permeability changes, eg. ion leakage, may often be observed at radiation doses even lower than those required for lipid peroxidation (Myers and Bide, 1966). Thus, in addition to peroxidative processes, radiation-induced membrane damage may involve other less characterized mechanisms, some of which may be oxygen-independent (Bianchi *et al.*, 1964; Cancelliere *et al.*, 1975).

Bacterial DNA is commonly associated with the plasma membrane (Sueoka and Hammers, 1974). Peroxidative reactions on membrane lipids may generate additional molecular or radical species which in turn act destructively with membrane-bound genetic material (Vaca and Harms-Ringsdahl, 1989). The highly reactive free radicals

(Hutchinson, 1985; Okada, 1970) and non-radical products, malondialdehyde and related aldehydes (Esterbauer *et al.*, 1990; Reiss *et al.*, 1972) of lipid peroxidation are biologically active and can damage cellular DNA (Imlay and Linn, 1988; Joenje *et al.*, 1990). Studies by Cramp *et al.* (1972) suggest that membrane-DNA complexes are primary targets for oxygen-mediated irradiation damage in the relatively resistant *E. coli* B/r. The intimate association of membrane and DNA in the bacterial cell may, in fact, accelerate radiation-mediated DNA damage while minimizing the peroxidative effects on membrane lipids (Pietronigro *et al.*, 1976, 1977). Clearly, the nature of the membrane-DNA complex as well as that of the membrane constituents is of importance in mediating the effects of radiation on cellular DNA. Similar considerations may apply to mitochondria, in which mitochondrial DNA may be particularly susceptible to oxidative damage as a result of its proximity to membrane-localized components of electron transport and attendant increased risks of peroxidative processes (Hruszkewycz, 1992).

Radiation resistance in *D. radiodurans* is usually attributed to efficient DNA repair systems. In contrast, little has been done on the subject of membrane repair. Injury to lipid and protein components of irradiated membranes must be corrected by turnover of damaged molecules and their replacement by newly synthesized ones. By assaying the fragility of spheroplasts prepared from *E. coli* subjected to various irradiation treatments, Yatvin *et al.* (1972) presented evidence that the relatively radioresistant *E. coli* B/r strain is able to undergo membrane repair while the radiosensitive B/s-1 strain could not. It was found to precede ssDNA repair and the speculation was made (Yatvin, 1976) that the relative inefficiency of *E. coli* B/s-1 to repair ssDNA breaks might be a consequence of its inability to repair damaged membrane or DNA-membrane complexes.

Correlations have been made between radiation resistance of a bacterium and resistance of its cell membranes to withstand radiation-induced permeability changes. Using potassium efflux as an indicator of membrane permeability, Harrison *et al.* (1958)

showed that the induction of permeability changes in *E. coli* B/r required higher doses of X-irradiation than was the case for a radiation-sensitive strain. Merrick and Bruce (1965) used similar techniques to demonstrate that membrane permeability in *D. radiodurans* was much less susceptible to radiation effects than the radiation-sensitive bacterium, *Sarcina lutea*. Interestingly, the radiation dose required to effect a doubling in the rate of potassium leakage from *D. radiodurans* was similar to the D_{50} (radiation dose required to kill 50% of the population).

1.5 A ROLE FOR MEMBRANE LIPIDS IN RADIATION RESISTANCE

By culturing auxotrophic *E. coli* in the presence of various fatty acids (thereby modifying membrane lipid composition), Yatvin (1976) and Redpath and Patterson (1978) noted an influence of fatty acid composition on the survival rate of irradiated cultures which they interpretted as showning that the more fluid the membrane the greater the resistance to radiation. The same conclusion was reached by Suzuki and Akamatsu (1978).

Of all natural lipids for which there has been postulated a role in radiation protection, carotenoids are the key members. Any radioprotective role for carotenoids is likely related to their ability to scavenge reactive oxygen species which are generated by exposure of aqueous systems to ionizing radiation. Carotenoids are able to quench singlet oxygen (Foote and Denny, 1968) and oxidative free radicals (Burton and Ingold, 1984) which have been implicated in cell destruction (Fridovitch, 1981) and tumor promotion (Estensen et al., 1974, Hoffman and Autor, 1982; Kensler *et al.*, 1983, Slaga *et al.*, 1981). Carotenoids have been shown in bacterial (Anwar *et al.*, 1977, Sistrom *et al.*, 1956) and yeast (Moore *et al.*, 1989) systems to be protective against photo-oxidative damage and in certain kinds of skin photosensitivity in animals (Mathews, 1964; Mathews-Roth and

Pathak, 1975) and humans (Kligman *et al.*, 1984; Mathews-Roth *et al.*, 1977). Cloned carotenoid genes expressed in *E. coli* have been reported to confer protection against near-UV (320-400 nm) radiation (Tuveson *et al.*, 1988).

Carotenoid-like pigments have been detected in *D. radiodurans* (Krabbenhoft *et al.*, 1967; Lee *et al.*, 1963; Lewis *et al.*, 1973) and efforts have been made to determine their possible contribution to radiation-resistance. These efforts, using non-pigmented mutants (Mathews and Krinsky, 1965; Moseley, 1963), or cells in which pigment synthesis had been inhibited by drugs (Lewis *et al.*, 1973) or altered growth medium (Krabbenhoft *et al.*, 1967), have produced mixed results. One problem common to such studies is the inability to eliminate the overriding cell-regenerative ability of the efficient DNA repair systems. There is evidence that carotenoids in *D. radiodurans* may function as antioxidants and thereby possibly contribute to radiation resistance (Carbonneau *et al.*, 1989).

In addition to carotenoids, other compounds such as ascorbic acid and α-tocopherol act as important antioxidants. It is important to recognize that even with such numerous defenses, cellular macromolecules, including DNA, are prone to oxidative damage, even among higher organisms and in the absence of radiation (Ames *et al.*, 1993).

Although membrane lipids can contribute to radiation resistance of radiation-sensitive targets (Anderson *et al.*, 1987), it is important to note that membrane lipids of *D. radiodurans* are not likely to shield the cell's DNA from damage by radiation. This conclusion stems from findings which showed that DNA from *D. radiodurans* is only three-times more resistant to UV-induced thymine dimer formation than the DNA from *E. coli* (Setlow and Duggan, 1964). Should lipid constituents play a role in radiation-resistance, such a role probably operates at the level of the membranes themselves in limiting the harmful effects of radiation on membrane function. One indication that *D. radiodurans* may possess cell membranes capable of withstanding the damaging effects of

radiation is the previously mentioned study (Merrick and Bruce, 1965) indicating high resistance to X-ray-induced membrane permeability to potassium ions.

Studies have shown that of all the lipids in *D. radiodurans*, the most significant UV-absorbing lipid is vitamin MK8 (Reeve *et al.*, 1990). Lipids such as this could possibly contribute to radiation stability of the membrane in which they are located.

1.6 DNA REPAIR MECHANISMS IN *E. COLI*

In addition to the above-described involvement of cell membranes in radiation resistance, there is the well-studied area of DNA repair processes which play a leading role in the ability of various organisms to withstand otherwise lethal doses of radiation. The DNA of all living cells is constantly being damaged by radiation (ionizing or ultraviolet) and chemical agents, and resulting in DNA lesions causing altered DNA structure. DNA lesions must be repaired, otherwise they will eventually cause cell death. All living systems maintain several mechanisms for DNA repair (Sancar and Tang, 1993). The DNA repair systems in *E. coli* have been intensively studied. In this section the process of DNA damage by UV radiation and its repair in *E. coli* will be reviewed.

1.6.1 Photoreactivation Repair in *E. coli*

Ultraviolet light causes a variety of DNA damage (Freidberg, 1985). The maximum DNA absorption of UV light occurs at a wavelength of 260 nm. This wavelength of light provides the formation energy for pyrimidine dimers, which are bulky lesions in the DNA. In the process of photoreactivation this damage is reversed by converting the pyrimidine dimers back to monomers. In *E. coli* the photoreactivation repair is a two step process involving a DNA photolyase (a flavoprotein) and two noncovalently bound cofactors. One cofactor is a fully reduced flavin adenine dinucleotide

($FADH^-$) and the other is either a methenyltetrahydrofolate (MTHF) or an 8-hydroxyldeazaflavin (8-HDF) which acts as a light harvesting molecule (Kim and Sancar, 1993). The MTHF or 8-HDF cofactor is capable of absorbing light in the 300 to 500 nm (visible to near-UV) wavelength range. In the first step of the repair process, the DNA photolyase binds to the pyrimidine dimer. The second step is light dependent catalysis, involving both cofactors. The $FADH^-$ cofactor absorbs weakly at wavelengths of 300 to 500 nm. Acting as a light harvesting molecule the MTHF or 8-HDF cofactor absorbs light and transfers the energy to $FADH^-$, after which the excited $FADH^-$ transfers an electron to the pyrimidine dimer to form an unstable dimer-anion radical. The dimer-anion radical undergoes spontaneous reversal to the original monomer configuration, thus repairing the DNA lesion. The electron is then transferred back to the $FADH^{\cdot}$ to regenerate the active $FADH^-$. (Kim and Sancar, 1993). The crystal structure of substrate free DNA photolyase of *E. coli* has been determined (Park *et al.*, 1995). The structure provides further insight into the enzyme catalysis of a radical repair mechnism. This photolyase contains two major domains, an α/β domain and a helical domain. There is a cleft between the two domains which can fit the light harvasing cofactor MTHF. On the surface of the helical domains, there is a hole which connects to the FADH binding site. This hole has the right diameter and the van der Waal's contact distance between a pyrimidine dimer and the cofator FADH. Park *et al.* (1995) suggested that the pyrimidine dimer flips out of the helix into the hole and the electron transfers back and forth between the FADH and pyrimidine dimer during the repair process .

1.6.2 Nucleotide Excision Repair in *E. coli*

In the process of nucleotide excision repair the DNA lesion is removed, generating a single-strand gap which is filled using the complementary strand as a template. There are two types of excision repair. The first type, base excision repair, removes the modified

DNA bases as free bases (Sancar and Sancar, 1988). The key reaction in this process is the hydrolysis by DNA glycosylase of the N-glycosydic bond which joins the base to the sugar. This reaction is followed by the removal of apurinic or apyrimidinic (AP) sites by AP endonuclease. The gap is filled by PolI and ligase. The enzyme involved in this base repair operates in an ATP-independent fashion.

The second type, nucleotide excision repair, removes the modified DNA bases as an intact oligonucleotide and can excise up to a 12-13 mer, making it a major DNA repair system. It also repairs other types of DNA lesions. Nucleotide excision repair involves a multi-step ATP-driven process (Sancar and Tang, 1993). The excision nuclease incises both 5' and 3' end lesions. In *E. coli*, six proteins are involved in the nucleotide excision repair: UvrA (ATPase), UvrB (ATPase), UvrC, UvrD, PolI and ligase (Husain *et al.*, 1985). UvrA binds to UvrB to form a dimer which is a damage recognition complex (Orren and Sancar, 1989). This complex "walks" along the DNA and binds to any damaged area it encounters (Koo *et al.*, 1991). Then the complex dissociates and catalyzes the formation of a stable UvrB-DNA complex which can remain at the damaged site for 2 to 3 hours. The next step in the repair process is the binding of UvrC to the UvrB-DNA complex, inducing a conformational change that enables UvrB to cut the 3' end of the damaged DNA strand. This incision in turn induces a conformational change which allows UvrC to make an incision at the 5' end. The post-incision complex, UvrB-UvrC-DNA, remains at the repair site and with the help of UvrD (Helicase II), PolI and possibly other factors catalyze the removal of the damaged bases (Sancar and Tang, 1993). Only Helicase II is capable to remove the oligomer and UvrC from the post-incision complex (Orren *et al.*, 1992). After the displacement of UvrC by helicase II, the 5' nick is accessible to PolI. PolI fills in the gap and displaces UvrB. Immediately, the nick at the 3' incision site is filled in by ligase, thus preventing PolI from nick translation. Thus, the DNA lesion is repaired (Sancar and Tang, 1993).

1.6.3 Recombinational Repair and SOS Repair (SOS Mutagenesis)

Neither of these types of repair mechanisms remove the DNA lesion. In recombinational repair, the DNA lesion is "diluted out" (Streips and Yasbin, 1991). SOS mutagenesis is an error-prone process. This process allows continuation of DNA synthesis without gap formation (Friedberg, 1985). RecA plays an important role in recombinational repair and SOS mutagenesis in *E. coli*. Repair mechanisms will be discussed in the RecA section below.

1.7 DNA REPAIR MECHANISMS IN *D. RADIODURANS*

1.7.1 Photoreactivation and Error-prone (SOS) repair

Photoreactivation experiments did not show any increase in the survival rate of *D. radiodurans* (Moseley and Laser, 1965) or of its UV-sensitive mutants *rec*30 and UV17 (Moseley and Copland, 1975; Moseley, 1983). It was concluded that *D. radiodurans* does not possess a photoreactivation DNA repair mechanism (Moseley 1983).

Studies showed that no UV induced mutant could be found in *D. radiodurans* (Sweet and Moseley, 1974; Tempest, 1978). It was concluded that *D. radiouran* lacks the error-prone repair mechanism (Tempest and Moseley, 1982). Moseley (1983) speculated that *D. radiodurans*, which has multiple genomic copies (Hansen, 1980), is capable of using the genomic information from the other genomic copies to repair the single strand gaps opposite the pyrimidine dimer.

1.7.2 Nucleotide Excision Repair

D. radiodurans has two independent but functionally overlapping repair pathways for the removal of pyrimidine dimers (Evans and Moseley, 1983; Moseley and Evans, 1983). One excision pathway is initiated by endonuclease α, which is the gene product of *mtcA*. Based on the high sequence homology of *mtcA* gene and *uvr*A gene of *E.coli*, *mtcA* from *D. radiodurans* was renamed *uvrA* (H. Agostini and K. Minton, unpublished observations, from Minton, 1994). The other pathway is initiated by endonuclease β, which is composed of the gene products of *uvsC*, *uvsD* and *uvsE*. Only double mutants of both UV endonucleases are completely defective in their excision repair activity, as either endonuclease can substitute for the other. It is believed that neither the α or β endonucleases involved in excision repair in *D. radiodurans* are glycosylases (Evans and Moseley, 1985, 1988; Tempest and Moseley, 1980).

1.7.3 Base Excision Repair

In contrast to nucleotide excision repair, very little is known about base excision repair. Some enzymes which are involved in *D. radiodurans* base excision repair have been investigated. They are UV or ionizing radiation induced enzymes such as thymine glycosylase (Targovik and Hariharan, 1980), apyrimidinic/apurinic (AP) endonuclease, uracil DNA glycosylase, DNA deoxyribophosphodiesterase, and thymine glycol glycosylase (Masters *et al.*, 1991a; Mun *et al.*, 1994).

1.7.4 Recombinational Repair

D. radiodurans is highly resistant to UV and γ radiation. This is partly due to an efficient recombinational repair mechanism. At the time my research project was initiated, very little was known of how this repair mechanism operates. At a dose of 1.2 krad of γ radiation or 1.8 Jm^{-2} of UV radiation the recovery time due to recombination repair in *D.*

radiodurans ts1 at 30°C is about one minute (Moseley and Copland, 1976). A recombination deficient mutant, *rec*30, is 15 and 100 times more sensitive to UV and γ radiation, respectively than the wild type strain (Moseley and Copland, 1976).

Recombinational repair in *D. radiodurans* is somewhat less well characterized than excision repair (Grimsley *et al.*, 1991; Masters *et al.*, 1991b; Moseley, 1983; Moseley and Copland, 1975). It has been remarked (Gutman *et al.*, 1993) that strains of *D. radiodurans* which are deficient in recombination-dependent transformation are sensitive to DNA-damaging agents, suggesting a role for recombination in DNA repair. Recombinational repair was not defective in a mutant of *D. radiodurans* deficient in a DNA polymerase gene which was recently found to play a major role in resistance to γ radiation (Gutman *et al.*, 1993).

1.7.5 Role of DNA Polymerase

As mentioned above, recent studies by Gutman *et al.* (1993) suggest a major role for a DNA polymerase in radiation resistance of *D. radiodurans*. Examination of a mutant (designated UV17) first studied in 1967 (Moseley, 1967) indicated a DNA repair mechanism that was independent of known excision and recombination processes. Early studies showed that while excision of pyrimidine dimers proceeded normally in the mutant, subsequent repair of excision gaps was delayed (Moseley, 1967). Bonura and Bruce (1974) concluded from their studies that UV17 had a Pol defect in analogy with the *E. coli polA* (PolI) gene. Gutman *et al.* (1993) noted further that the UV-sensitivity of UV17 was identical to that of a *D. radiodurans* strain which had a double mutation in the α and β endonuclease arms of the excision pathway. They suggested the possibility that the DNA polymerase identified in UV17 may be required for both of these excision-mediated events. By expressing *E. coli* PolA in *pol D. radiodurans* mutants, Gutman *et al.* (1994b) demonstrated that *E. coli* DNA PolI can fully restore *pol D. radiodurans*

mutants resistance to radiation and chemical damage. They concluded that *D. radiodurans* DNA Pol is not essential for the extraordinarily efficient DNA damage repair of *D. radiodurans*.

1.8 OVERVIEW OF THE RECA PROTEIN

In the search for a recombination deficient mutant, Clark and Margulies (1965) discovered the *recA* gene of *E. coli*. Extensive biochemical and genetic studies, both *in vitro* and *in vivo* showed that the RecA of *E. coli* is a multifunctional protein. It has a central role in genetic recombination, DNA repair, SOS induction and mutagenesis

1.8.1 Functions of the RecA Protein of *E. coli*

1.8.1.1 Role in Homologous Recombination

The RecA protein plays a direct role in homologous recombination and recombinational DNA repair. Homologous recombination is an enzyme mediated process in which two homologous DNA molecules come together and exchange segments of DNA. The RecA protein promotes a variety of ATP-dependent events including homologous pairing and strand exchange between DNA molecules. The complex mechanisms of these processes appear to proceed by multistep pathways (Kowalczykowski, 1994; Clark and Sandle, 1994). The best studied *in vitro* model involves three strands: a circular single-strand and a linear duplex strand, designated a "joint molecule".

RecA is an allosteric protein. The crucial event in DNA strand exchange is ATP binding to RecA. ATP binding induces a conformational change in RecA. This results in an increase in the affinity of RecA for DNA. This event provides the free energy for polymerization of RecA and ssDNA to form a helical nucleoprotein filament. The RecA

filament is the active species during the homologous search and DNA strand exchange. Studies have shown that the RecA filament recognizes partial homology in duplex DNA via non-Watson-Crick hydrogen bondings in base triplets (Rao et *al.*,1995). The RecA filament aligns and pairs itself paralled to the like strand of duplex DNA. Subsequently, the unsuitable matches are filtered out by the mismatch repair system of *E.coli* (Rayssiguier *et al.*, 1989: Worth *et al.*, 1994). After localizing a homologous sequence, the RecA filament invades the homologous region of duplex DNA, exchanges homologous strands, diplaces one of the existing strands and forms a joint molecule (heteroduplex). Then RecA mediates branch migration to create a Holliday junction. The final step is the resolution of the Holliday junction by endonuclease cleavage and ligation. RecA as an ATPase hydrolyzes ATP to ADP. This process results in the induction of a conformational change which promotes dissociation of RecA protein from the heteroduplex. Dissociation is unidirectional in the 5' to 3' direction relative to the displaced strand of the heteroduplex region in the joint molecule. The specific mechanism of energy transduction in ATP hydrolysis is still disputable (Kowalczykowski, 1994; Stasiak and Egelman,1994). In the presence of the poorly hydrolyzed ATP analogue, ATP-γ-S or an allosteric effector, a noncovalent complex of ADP and aluminum fluoride, ADP·AlF4 $^-$, a substantial strand exchange still proceeds (Menetski *et al.*, 1990; Kowalczykowski and Krupp, 1995). These results demonstrate that RecA-promoted DNA pairing and strand exchange requires an allosteric transition induced by ATP binding, but does not require the free energy derived from ATP hydrolysis (Rehrauer and Kowalczykowski, 1993; Kowalczykowski, 1995). Based on biochemical and electron microscopy studies, Rosseli and Stasiak (1990) proposed that the increase in homologous pairing and the increasing stability of RecA-DNA complexes during subsequent stages of the reaction promote the strand exchange to proceed. The hydrolysis of ATP is only needed to dissociate the RecA protein from these stable complexes. So why does RecA

constantly hydrolyze ATP during the reaction, instead of having an ATPase activity induced only after completion of the strand switch process? The answer is not yet certain. It remains a controversial issue.

In vivo, the recombination reaction is to exchange strands between partially heterologous DNA moleculs. This reaction is not isogenic. Therefore, more energy is needed to proceed the strand exchange (Bianchi and Radding, 1983). In the presence of the poorly hydrolysable analog, ATP-γ-S, Kim *et al.*, demonstrated that ATP hydrolysis was needed for reciprocal strand exchange between two double-stranded DNA molecules. *In vivo* it is necessary to displace the proteins bound to DNA and to unwind DNA during the strand exchange. Therefore it is likely that the constitutive ATPase activity of RecA protein is required to supply the needed energy (Honigberg and Radding, 1988). Adams *et al.* (1994) argued that ATP hydrolysis alone does not provide enought energy for dissociation of the RecA protein from the reaction complex. They demonstrated that in addition to ATP, RuvA and RuvB are required for the dissociation of the RecA protein from supercoiled DNA. Furthermore, studies showed that the *E. coli* RuvA, RuvB, RuvC and RuvG proteins are required for processing of the joint molecule. *In vitro*, RuvA protein binds to the joint molecule guiding RuvB (an ATPase) to the site of junction and promotes ATP-dependent branch migration in 5' to 3' direction (Parsons and West,1993; West, 1994). RuvG protein (an ATPase) also binds to the joint molecules. In the presence of ATP, RuvG protein promotes the branch migration in 3' to 5' direction (Lloyd and Buckman, 1991). RuvC acts independently to resolve recombination intermediates by specific endonucleolytic cleavage of the joint molecule (Dunderdale *et al.*, 1991).

1.8.1.2 Role in Recombinational DNA Damage Repair

UV and γ irradiation, oxidative damage and chemical agents generate single and double-stranded DNA breaks. In *E.coli*, this damage can be repaired by two RecA-dependent recombinational repair pathways (Clark and Sandler, 1994: Smith and Wang, 1989). During the recombinational repair a gap in one strand can be repaired using the undamaged strand as a template. One of these pathways is the RecBCD pathway and the other is the RecF pathway. These pathways are differentiated by the resistance (RecBCD) or sensitivity (RecF) to the suppression of recBC (sbcB) gene function. In the RecBCD pathway , the RecBCD protein complex (ExonucleaseV), which is a helicase and a nuclease, recognizes dsDNA with flush or nearly flush dsDNA ends and initiates a nick at 3' end of Chi sequence (the recombination hot spot) and produces a ssDNA "tail". This "tail" provides the site for binding of single strand binding protein. This binding further facilitates the binding of the RecA protein. *In vitro*, the Chi sequence reduces nuclease activity of RecBCD and maintains the ssDNA. Single stranded DNA binding protein traps the ssDNA produced by RecBCD to promote binding by the RecA protein. (Kowalczykowalski, 1994). In the RecF pathway, it was proposed that RecJ nuclease, in conjunction with a helicase (RecQ?), generates a terminated ssDNA for binding of RecA protein (Lovett and Kolodner, 1989). Based on biochemical studies, Clark and Sandler (1994) proposed that RecF, as a molecular "matchmaker" guides RecO and RecR proteins to the suitable substrate such as gapped duplex DNA. The binding of RecFOR complex assists the displacement of single stranded DNA binding protein in gapped duplex DNA by RecA protein. Thus, RecFOR complex converts replication templates to recombination intermediates, "ssDNA.RecA". The primary function of the RecA protein is to repair the damaged DNA and its role in recombination is secondary (Roca and Cox, 1990).

1.8.1.3 Role in Gene Regulation

RecA is one of the major regulators of damage-induced genes which contribute to the multigene SOS response in *E. coli* (Walker, 1984). LexA normally (i.e. uninduced) represses a network of SOS genes and permits only a low-level synthesis of SOS gene products, including RecA and LexA. In damaged cells (i.e. induced), the RecA protein becomes activated in response to a metabolic signal such as ssDNA. The active form of RecA is a triple complex of the nucleoprotein filament, consisting of RecA, ssDNA and a nucleotide triphosphate. Then, the nucleoprotein filament promotes the autoproteolytic cleavage of the LexA protein. Derepression by LexA allows the transcription of approximately 20 genes (including *recA*, *umuC*, *umuD*) which participate in DNA repair; collectively these genes make up the SOS response (Walker, 1984 and 1985). The activated RecA also acts as a co-protease to promote self-cleavage of the bacteriophage lambda repressor and the repressors of other phages (Little, 1984).

1.8.1.4 Role in Mutagenesis

RecA protein has two regulatory functions in SOS mutagenesis. Mutagenesis may be a mode of damage tolerance. SOS mutagenesis is one of the cellular processes controlled by the derepression of the SOS genes, *recA* and *umuDC*. First, proteolytic cleavage of the SOS repressor, LexA, by RecA, allows expression of the *umuDC* genes (Walker, 1984). *In vitro* and *in vivo* studies have shown that transcription of the *umuDC* genes is necessary but not sufficient for SOS mutagenesis. Second, the activated RecA promotes the proteolytic cleavage of UmuD to produce a fragment (UmuD') that is the active species in mutagenesis (Nohmi *et al.*, 1988; Shinagawa *et al.*, 1988; Burckhardt *et al.*, 1988). Woodgate *et al.* (1989), based on direct evidence from physical interaction, showed that the UmuD'C complex is the fundamental unit for mutagenesis. In addition, Sweasy *et al.* (1990) showed that UmuD'C does not determine the mutation rate

independently of RecA and that RecA has a third essential role in SOS mutator activity. This role is based on measuring spontaneous mutability in a family of LexA-defective strains, each having a different *recA* allele. Each strain was transformed with plasmids that overproduced either UmuD' alone or both UmuD' and UmuC, where the controls were strains without plasmids. Bailone *et al.* (1991) postulated that the third role for RecA protein in SOS mutagenesis is to place the UmuD'C complex at DNA lesions and allow translesion DNA replication. Using purified protein, Woodgate *et al.* (1989) and Rajagopalan *et al.* (1992) demonstrated that the replicative bypass of a DNA lesion required UmuD'C, RecA and polymerase III (PolIII), a damage-localized nucleoprotein complex (a "mutasome"). It has been suggested that this multiprotein complex might induce a conformational change at the template, thus allowing base insertion by PolIII, inhibiting PolIII from editing (normally PolIII removes mispaired bases), facilitating rapid replication by PolIII and relaxing PolIII's requirements for precise base pairing (Woodgate *et al.*, 1989). Furthermore studies showed that the binding capacity of the free epsilon subunit of PolIII, not its 3' to 5' editing activity, has the ability to counteract SOS mutagenesis (Kanabus *et al.*, 1995). Thus they suggested that the epsilon subunit of PolIII may influence the interaction between the UmuD'C-RecA complexs and PolIII and the outcome of the SOS mutagenesis.

1.8.1.5 Unknown roles of RecA protein

There are other SOS-inducible activities which require functions of activated RecA. DNA replication is profoundly altered in SOS-induced cells. Induced stable DNA replication (ISDR) does not require new protein synthesis for the reinitiation of a new round of replication at secondary origins (*oriM*'s) (i.e., many rounds of replication can occur in the presence of the antibiotic chloramphenicol). A possible role of activated

RecA with its helicase-like activity could be to open these regions, making them accessible to the primosome (Quinones *et al.*, 1987).

Another type of SOS-inducible activity requiring activated RecA is induced replisome reactivation (IRR), which enables cells to recover a normal rate of DNA synthesis after blockage of replication by UV photoproducts or other bulky lesions. Khidhir *et al.* (1985) showed that IRR requires the amplification of RecA and synthesis of at least one other protein. Other studies also demonstrated that RecA is the only LexA-controlled protein that needs to be amplified for restarting replication (Witkin *et al.*, 1987).

1.8.2 Structure and Function of RecA Protein

1.8.2.1 Primary Structure

The *E. coli recA* gene encodes a protein of 352 amino acids with a predicted molecular weight of 37,842 Da. (McEntee *et al.*, 1976). All known RecA proteins have similar molecular weights.

Comparisons of the RecA amino acid sequences between organisms have shown that there are highly conserved regions within RecA, with 105 invariant residues in 16 bacterial species (Roca and Cox, 1990). The invariant residues are mainly located in the middle functional domain (residues 31-260 of the *E. coli* RecA protein). These invariant residues are responsible for recombination, UV resistance and formation of an active oligomer of RecA proteins (Ogawa *et al.*, 1992).

The C-terminus of the RecA proteins is the least conserved between species.

1.8.2.2 Three-dimensional Crystal Structure of the RecA Protein of *E. coli*

Figure 2 shows the three-dimensional structure of the RecA protein (from Story *et al.*, 1992). The 2.3 Å crystal structure of the RecA/ADP polymer comprises a major center, containing nearly two-thirds of the mass, and two subdomains at the N- and C-termini which protrude from the protein and stabilize the helical polymer subunits and the interpolymer bundles of the protein. Each monomer of the RecA/ADP polymer contains 10 α-helices, 11 β-sheets, disordered loops L1 (residues 157-164) and L2 (residues 195-209), and two disordered chain termini at residues 1-3 and 328-353. The RecA polymer has a pitch of around 85-100 Å and 6 monomers per turn . These values are in agreement with the low resolution electron microscopic image of the ATP-bound RecA/DNA filament that has a pitch of approximately 95 Å and 6.1-6.2 monomers per turn (Egelman and Stasiak, 1986; Yu and Egelman, 1990).

1.8.2.3 The RecA Protein Structure/Function Relationship

Before the three dimensional structure of RecA protein of *E. coli* became available, the structural and functional correlations in RecA homologues were derived mainly from primary sequence analysis of mutants and truncated proteins, as well as chemical modification analyses. The postulated functional domains within the primary structure of the RecA protein are shown in Fig. 3 (from Roca and Cox, 1990). Most data are derived from closely related enteric species. RecA gene sequences from more distantly related genera are beginning to emerge, such as Gram positive bacterial, eukaryotic and Archaea RecA analogues. These primary sequences will provide insight into both the structure/function relationship and evolutionary conservation of the RecA protein. With the availability of data from various studies, including the three dimensional

Figure 2. **Structure of the RecA monomer (taken from Story *et al.*, 1992).** β-strands are shown as striped arrows and are numbered 0-10. α-helices are labeled A-J according to their relative position to the N-terminus. Bound ADP is shown in bold near helix C. The two disordered loops (L1 and L2, drawn as dashed lines) and helix G are proposed to be involved in DNA binding.

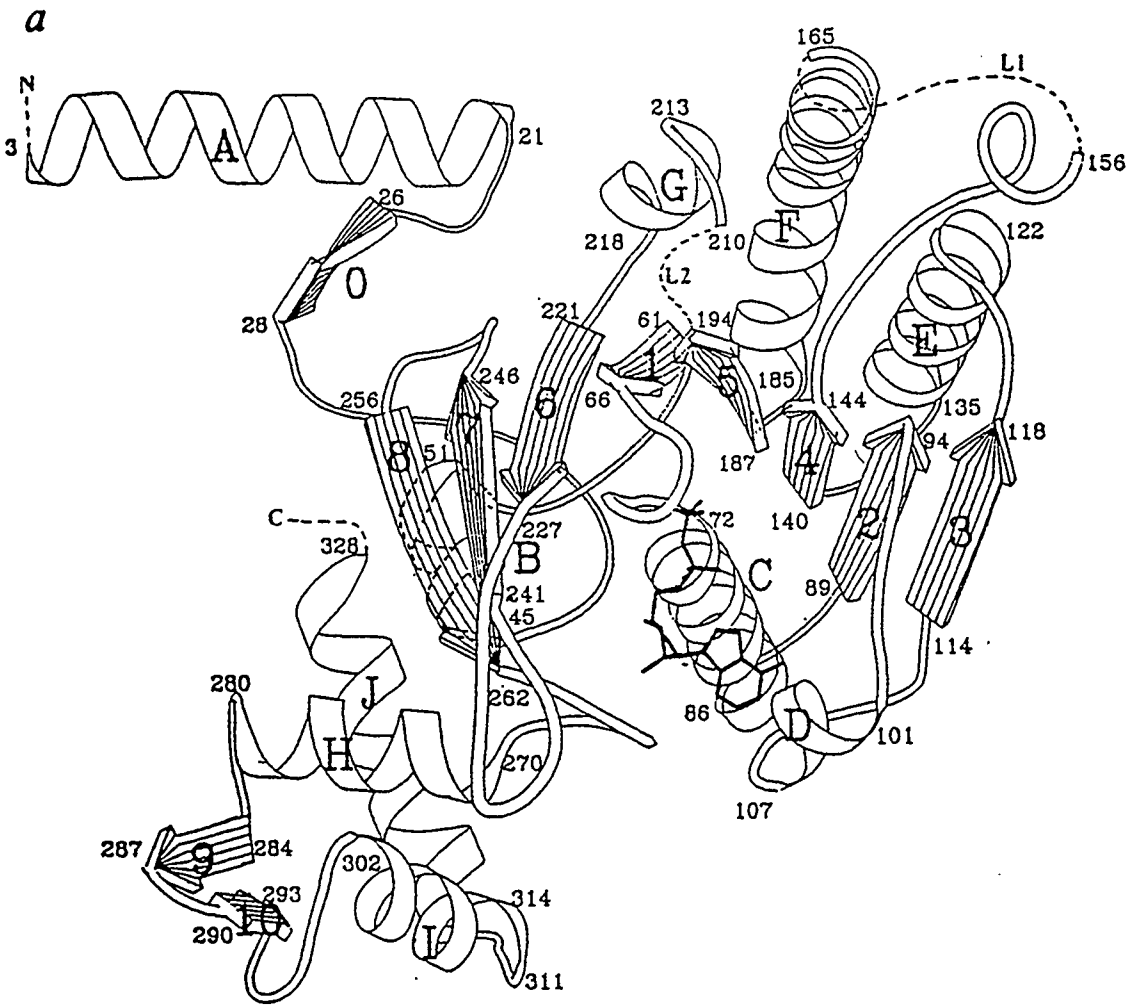
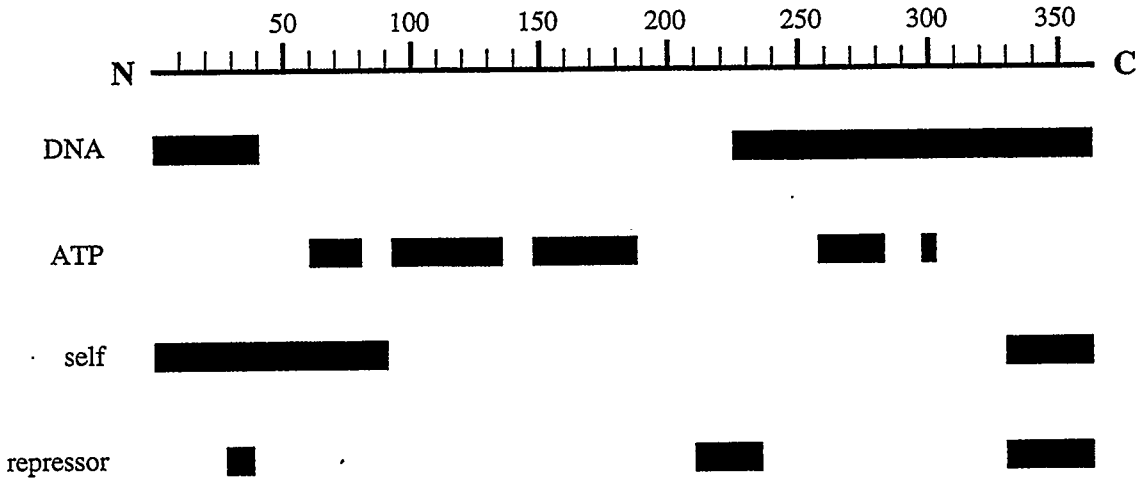

a
N
3
A
21
26
0
28
165
L1
213
156
G
F
218
210
122
L2
221
61
194
E
246
256
6
66
1
5
185
144
135
8
51
7
187
4
94
118
C
328
227
B
72
140
2
3
241
45
C
89
262
114
280
J
86
D
101
H
270
107
287
9
284
302
293
314
290
10
I
311

Figure 3. **Postulated functional domains within the primary structure of RecA.** Domain assignments are based on data derived from interspecies comparisons, chemical modification, and mutant analysis. Note that there is a conflict in the assignments of DNA binding domains by these analyses and three-dimensional-analyses which places the DNA binding domains between residues 157-164 and 195-209 (see Section 1.8.2.3). Also photochemical cross-linking analysises show that the DNA-binding sites are in the region of residues 89-106 and 178-183 (Morimatsu and Horii, 1995). This figure was modified from Roca and Cox, 1990.


50
100
150
200
250
300
350
N
C
DNA
ATP
self
repressor

RecA structure, the functional domains of the RecA protein can be summarized as follows:

The RecA protein has two DNA binding sites. One binds to a single stranded DNA to form RecA/DNA filaments. The other binds to a homologous duplex DNA. Story *et al.*, (1992) proposed that the residues in or near the disordered loop L1 (156-165) are the binding site for the homologous duplex DNA and residues in or near disordered loop L2 (195-209) and Helix G are the single stranded DNA binding site. The disordered loops L1 and L2 are located near the polymer axis. Thus these sites are ready accessible to single or double stranded DNA. In the RecA polymer, the disordered loop L2 sites direct above the ATP binding site (Fig. 2). Analyses of the RecA mutants in these regions showed that the single or double stranded DNA binding activities of RecA protein are weakened or inhibited (Menetski and Kowalczykowski, 1990; Cazaux *et al.*, 1991; Kawashima *et al.*,1984). Helix G is on the most conserved region in bacterial RecA proteins (Roca and Cox, 1990). In this helix there are two glycines (residues 211 and 212) which are capable of interact with DNA phosphates (Wierenga *et al.*, 1985). These two glycines are also conserved in the bacteriophage and yeast RecA homologues (Fujisawa *et al.*, 1985; Bishop *et al.*, 1992). Based on these observations Story *et al.*, (1992) suggested that Helix G is likely involved in single-stranded DNA binding.

Based on mutational studies as well as the known three-dimensional structure, Story *et al.* (1992) proposed that the repressor binding site was located in the `notch' region between adjacent lobes of the RecA polymer. Mutational studies revealed that residues which effect the cleavage of the LexA and UmuD proteins, as well as λ and $\phi 80$ phage repressors, are located on the surface at the edge of the RecA 'notch' (Duteix *et al.*, 1989; Ogawa and Ogawa, 1986). Furthermore, this 'notch' is able to fit a small protein (Story *et al.*, 1992).

Story *et al.*, (1992) concluded that the crystal structure of RecA/ADP polymer is quite similar to the RecA/ATP/DNA filament (Egelman and Stasiak, 1986; Yu and Egelman, 1990). However they do not agree that the DNA binding sites are at the residues near the amino terminus (Zlotnick and Brenner, 1989) or residues 243-257 (de Jong *et al.*, 1989). They argued that these regions are far away from the polymer axis.

1.8.3 RecA Homologues

Since the identification of the *recA* gene of *E. coli* (Clark and Margulies, 1965), the gene and its product RecA have been well characterized. The crucial role of the RecA protein in homologous recombination and DNA-damage repair has stimulated numerous studies directed at identifying and characterizing homologous *recA* genes in prokaryotes and eukaryotes.

In prokaryotes, more than 40 *recA* genes have been cloned (references in Miller and Kokjohn, 1990; Roca and Cox, 1990), nearly all using the interspecies complementation method (Eitner *et al.*, 1981 and 1982). The genomic library of each organism was transformed into an *E. coli recA* mutant and the *recA* clone was selected by its ability to complement one or more *recA* phenotypes, such as resistance to the DNA alkylating agent, methyl methane sulfonate (MMS). Most of the *recA* genes which have been cloned utilizing this method are from closely related enteric species. However, data from distantly related genera are beginning to emerge. *recA* homologues from Gram positive bacterial species have been cloned and completely sequenced. These include *Bacillus subtilis* (Marrero and Yasbin, 1988), *Mycobacterium tuberculosis* (Davis *et al.*, 1991), *Lactococcus lactis* (Duwat *et al.*, 1992), and *Acholeplasma laidlawii* which is phylogenetically related to Gram-positive bacteria (Dybvig and Woodard, 1992).

1.8.3.1 A Bacteriophage RecA Homologue

The bacteriophage T4 UvsX protein is distantly related to the bacteria RecA protein with 23% invariant residues which are comparable to those of the *E. coli* RecA protein (Fujisawa *et al.*, 1985). Although the UvsX protein is a DNA-dependent ATPase, it catalyzes the strand exchange reaction in a more dynamic manner when compared to the RecA protein. The protein-DNA filament is constantly collapsing, and reforming in a cycle coupled to ATP hydrolysis. Furthermore, Formosa and Alberts (1986) and Kodadek *et al.* (1988) demonstrated that in order to achieve a greater strand exchange activity, the UvsX protein needed to interact with UvsY and the gene 32 protein (helix-destabilizing protein) to form a protein complex. The helical conformation of the nucleoprotein filament formed by UvsX on DNA is very similar to the RecA/DNA filament of the *E. coli* RecA protein. The DNA is uncoiled to about 19 bp per turn and stretched to about 5 Å per base-pair (Yu and Egelman, 1993a). The yeast Rad51 protein also induces this DNA structure within the helical filament. Ogawa *et al.* (1993) speculated that the formation of the RecA filament may be a universal structural element in homologous recombination.

1.8.3.2 Eukaryotic RecA and Archaean RecA Homologues

Eukaryotic RecA homologues have also been characterized. They are from the plastids of higher plants (*Arabidopsis thaliana*; Cerutti *et al.*, 1992), yeast Dmc1 (encoded by a meiosis-specific gene from *S. cerevisiae*; Bishop *et al.*, 1992), Rad51 (encoded by a mitotic gene in *S. cerevisiae*; Shinohara *et al.*, 1992, a mitotic gene in *S. pombe*; Muris *et al.*, 1993), the chicken Rad51 (Bezzubova *et al.*, 1993) and Rad51 from a human testis cDNA library (Yoshimura *et al.*, 1993). Like the bacterial RecA proteins, the primary amino acid sequences of eukaryotic RecA homologues contain a middle core domain, flanked by N-terminal and C-terminal domains. Comparison of the structures of the RecA proteins suggests that the prokaryotic and eukaryotic RecA proteins are more distantly

related to each other. This is to be expected based on the phylogenetic separation of these groups. The predicted functional domain for the RecA protein ATP binding `motif A' (GDEFRTGKT) and `motif B' (LLIVD) (Walker *et al.*, 1982), as well as the DNA binding site are largely conserved (Bishop *et al.*, 1992; Yoshimura *et al.*, 1993; Aboussekhra *et al.*, 1992; Bezzubova *et al.*, 1993). One distinct feature of eukaryotic RecA homologues compared to the *E. coli* RecA protein is that they have longer N-amino and shorter C-terminal domains. The C-terminal domain of these eukaryotic RecA homologues is rich in acidic residues. Ogawa *et al.* (1993) demonstrated that yeast Rad51 polymerized on double stranded DNA to form a nucleoprotein filament whose low resolution three-dimensional structure is nearly identical to that of *E. coli* RecA protein. Bezzubova *et al.* (1993) speculated that the chicken Rad51 also formed nucleofilaments based on the extensive structural similarity with yeast Rad51 and RecA. Sequence analysis revealed that eukaryotic RecA homologues share stronger structural sequence similarities among themselves then with RecA protein of *E.coli*. Chicken Rad51 shares 68% and 49% identical amino acids with *S. cerevisiae* Rad51 and Dmc1 gene products, respectively (Bishop *et al.*, 1992). Human Rad51 is about 83% homologous (67% identical) to yeast Rad51 protein (Yoshimura *et al.*, 1993) and 56% homologous (30% identical) to *E. coli* RecA (Horii *et al.*, 1980).

The first Archaean RecA homologue *RadA* has been characterized from *Sulfolobus solfataricus* (Clark and Sandler, 1994). The RadA amino acid sequence is about 40% identical to the yeast *S. cerevisiae* Rad51 and Dmc1 protein and 23% identical to *E.coli* RecA protein. This finding further supports the universality of the RecA protein.

1.9 PROJECT AIM

In addition to being the two major "targets" for radiation, membranes and DNA play important roles in the relative tolerance to and in the repair processes of radiation-induced cell damage. Despite the recognition of the extreme radiation resistance among the *Deinococci*, surprisingly little is known of the processes by which membrane- and DNA-dependent repair events may contribute to the high degree of radiation resistance in this organism. In contrast to organisms such as *E. coli*, *D. radiodurans* remains largely uncharacterized with regard to its unique membrane constituents and to its DNA repair functions. The specific goals of this project were (i) to characterize the structure of polar lipids in the membranes of *D. radiodurans*, begining with lipid 6; (ii) to study their biosynthetic pathways; and (iii) to clone the *recA* gene of *D. radiodurans* to gain a better understanding of the role of this protein in DNA repair.

CHAPTER 2

STRUCTURE OF A NOVEL GLUCOSAMINE-CONTAINING PHOSPHOGLYCOLIPID FROM *D. RADIODURANS*

2.1 INTRODUCTION

In spite of the fact that the lipids of *D. radiodurans* are recognized as being unusual, the complete chemical structures of only two of these have been definitively described to date (see table 1). One of these is the menaquinone, MK8 which is also found in other bacterial species (Yamada *et al.*, 1977). The other is the phosphoglycolipid, 2'-0-(1,2-diacyl-*sn*-glycero-3-phospho)-3'-(α-galactosyl)-N-glyceroyl alkylamine (Lipid 7), which as of this date has only been reported in *D. radiodurans* (Anderson and Hansen, 1985). Some tentative assignments have been given to various lipids of *D. radiodurans* (Melin *et al.*, 1986), although conclusive chemical structural analysis has been lacking.

In this chapter, the complete structure of a second novel phosphoglycolipid isolated from *D. radiodurans* is reported.

2.2 MATERIALS AND METHODS

2.2.1 Cell Culture, Lipid Isolation and Analytical Methods

Cultures of *D. radiodurans* (strain Sark) were grown at 35°C as rotary shaken, aerobic suspension cultures (200 rpm) in 0.5% tryptone, 0.3% yeast extract, 3mM $CaCl_2$ (TYC). The cells were harvested after overnight growth (OD_{600}=0.5) or in defined growth phases, as specified in the individual experiments. Total lipids were extracted from packed cell pellets of *D. radiodurans* using a modified Folch (Folch *et al.*, 1957) and Thompson *et al.*, (1980) procedure procedure as previously described (Thompson *et al.*, 1980). The cell pellets were suspended in chloroform ($CHCl_3$)/methanol (CH_3OH) (2:1, v/v) for 12 h. After collecting the supernatant, the cell pellets were reextracted with $CHCl_3/CH_3OH$ (1:2, v/v) for 12 h. The combined supernatant was concentrated and redissolved in $CHCl_3/CH_3OH$ (1:1, v/v). This procedure extracted both polar and neutral

lipids (Thompson *et al.*, 1980). Lipids 6 and 7 (as designated in Thompson *et al.*, 1980) which comprise 9.5% and 33.3% of the total lipid, respectively, (Thompson and Murray, 1981) were purified by preparative thin layer chromatography (TLC) on silica gel H after developing in chloroform/methanol/28% ammonia (65:35:5, v/v/v). TLC of carbohydrates was performed on Sil Gel G Redi-plates (Fisher Scientific) developed in n-butanol/ethanol/28% ammonia (67:20:20, v/v/v). Mild alkaline hydrolysis of individual lipids was performed according to Kates (1986). Lipid, dissolved in chloroform, was hydrolyzed with 0.2 N methanolic sodium hydroxide, mixed and allowed to stand at RT for 1 h. Then, the solution was mixed with prewashed Rexyn 101(H) (Fisher Scientific) until pH was less than 7 and filtered through glass wool. Hydrofluoric acid (HF) hydrolysis of phosphodiester bonds was carried out according to Fischer *et al.* (1973). Individual lipid was hydrolyzed with HF overnight at 4°C in a polypropylene tube. Cold chloroform (4°C) was added and the solvents evaporated under nitrogen, with frequent trituration with chloroform. The residue was dissolved in chloroform/methanol (1:1, V/V). Phospholipase A_2 digestion was performed according to the method of Fischer (1977). Lipid, dissolved in ether/chloroform (98:2, V/V) was mixed with phospholipase A_2 (porcine pancreas; Sigma Chem. Co.) at a concentration of 0.5 mg/ml in borate buffer. The mixture was stirred magnetically for three hours at RT, then dried under nitrogen and the residue taken up in chloroform/methanol (1:1, V/V).

For the preparation of fatty acid methyl esters (FAME), lipids were treated with 5% methanolic HCl for 2 h at 80°C and extracted with hexanes following the addition of one-tenth volume of water. Pyrrolidyl esters of fatty acids were obtained from fatty acid methyl esters by the procedure of Andersson and Holman (1974) using pyrrolidine (99%, Aldrich Chem. Co.) and acetic acid (10:1, v/v) (100°C for 30 min in sealed tubes).

2.2.2 Gas Chromatography/Mass Spectrometry (GC/MS) Analysis of Fatty Acids

Fatty acid methyl esters were analyzed using a Hewlett-Packard 5890 Gas Chromatograph equipped with a 25m 5% phenyl methylsilicone capillary column, temperature programmed from 180-240°C (at 5°C/min). Fatty acid compositions were determined using a Hewlett-Packard 3392A Integrator.

Fatty acid pyrrolidides were analyzed by GC/MS using a Kratos 80 Mass Spectrometer equipped with a 30 m DB-1 capillary column, temperature-programmed from 50-180°C (at 10°C/min) followed by 180-250°C (at 5°C/min). GC/MS analyses of fatty acid pyrrolidides permitted the identification of positional isomers of the monounsaturated fatty acids (Andersson and Holman, 1974).

2.2.3 Nuclear Magnetic Resonance (NMR) Spectroscopy

^{1}H-NMR spectra were obtained using a Bruker AM-400 Spectrometer. Chemical shifts (δ) are expressed as ppm relative to tetramethylsilane as an internal standard. Coupling constants (J) are given in Hz. ^{13}C-NMR spectra were recorded on a Bruker AC-200 Spectrometer. Chemical shifts are expressed as ppm relative to tetramethylsilane.. The number of protons attached to carbon atom was determined by DEPT (Distortionless Enhancement by Polarization Transfer) measurement (Sanders and Hunter, 1988) with Bruker instrument company microprogram.. The reference compounds, dioleoylphosphatidyl choline, N-acetylglucosamine and 1-0-methyl-α-D-N-acetylglucosamine, were obtained from Sigma Chem. Co. The peracetylated derivative of 1-0-methyl-α-D-N-acetylglucosamine was obtained by treating the compound with pyridine/acetic anhydride (1:1, v/v) and stirring overnight at room temperature.

2.3 RESULTS

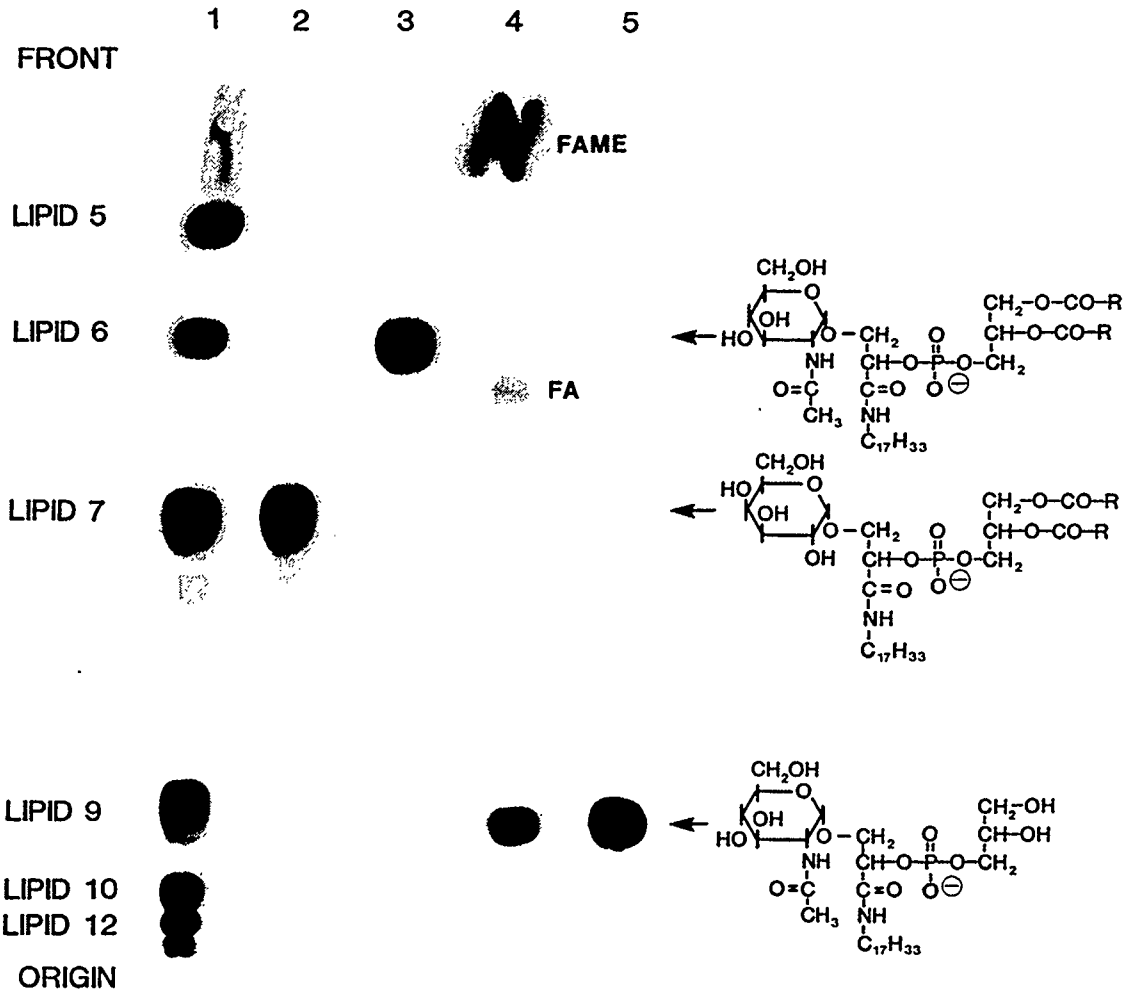
Lipid 6 comprises a substantial proportion (9.5%) of the total lipids of *D. radiodurans* (Thompson and Murray, 1981). Preliminary results indicated structural similarities between lipid 6 and the previously characterized lipid 7 (Anderson and Hansen, 1985). Both lipids are phosphoglycolipids (Thompson *et al.*, 1980), share similar infrared and NMR spectroscopic characteristics and contain an alkylamine constituent (unpublished observations and data presented below).

2.3.1 Infrared Spectroscopy (IR) of lipid 6

Lipid 6 showed infrared spectral characteristics similar to those previously reported for lipid 7 [2'-0-(1,2-diacyl-*sn*-glycero-3-phospho)-3'-0-(α-galactosyl)-N-glyceroyl alkylamine] (Anderson and Hansen, 1985), with major absorption bands at: 2920, 2850 and 1480 cm^{-1} (CH_2 and CH_3), 3400 cm^{-1} (OH), 1740 and 1170 (shoulder) cm^{-1} (carbonyl ester), 3420 (shoulder), 1665 and 1530 cm^{-1} (secondary amide) and 1260 and 1090 cm^{-1} (phosphate). Treatment of lipid 6 with mild methanolic alkali (Kates, 1972) yielded fatty acid methyl esters and a deacylated product with R_f=0.13 on TLC in chloroform/methanol/28% ammonia (65:35:5, v/v/v; Fig. 4, lanes 4 and 5). Infrared spectroscopy of the purified deacylated lipid 6 verified the absence of fatty acid esters (no 1740 cm^{-1} absorbance) and indicated the presence of hydroxyl, secondary amide and phosphate groups as indicated above.

Also shown in Fig. 4 are the chemical structures for lipid 7 (Anderson and Hansen, 1985) and for lipid 6 and its deacylated product. The latter structures are derived from consideration of the data presented below.

Figure 4. **Thin layer chromatogram developed in chloroform/methanol/28% ammonia (65:35:5, v/v/v) of total *D. radiodurans* lipids (lane 1), isolated lipids 7 (lane 2), and 6 (lane 3), mild alkaline deacylation products of lipid 6 (lane 4), and purified deacylated lipid 6 (lane 5).** Lipids were visualized by spraying with 25% sulfuric acid and charring. FA and FAME are abbreviations for fatty acids and fatty acid methyl esters, respectively. The lipid structures were derived from Anderson and Hansen (1985) and from consideration of the data presented in this chapter. The bottom lipid structure represents deacylated lipid 6. The chain length for the alkylamine moiety ($C_{17}H_{33}$) represents the most abundant species (see Anderson, 1983).


1
2
3
4
5
FRONT
FAME
LIPID 5
LIPID 6
FA
LIPID 7
LIPID 9
LIPID 10
LIPID 12
ORIGIN

2.3.2 Characterization of Phosphatidate Portion of Lipid 6

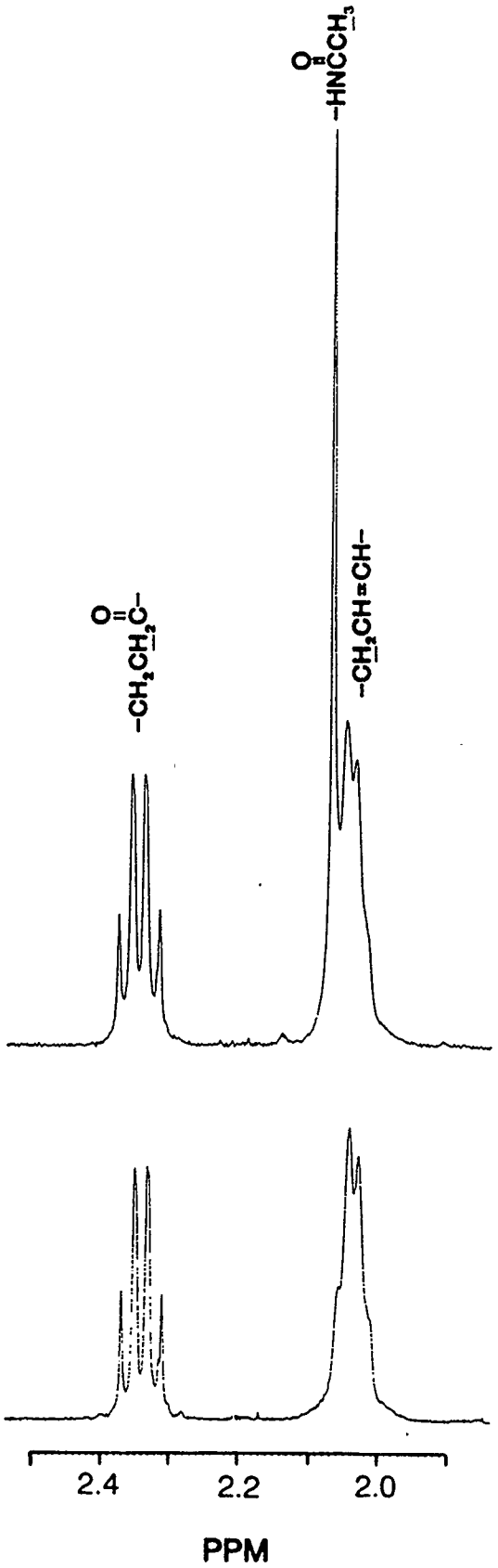
Lipid 6 was shown by two methods to contain a phosphatidate backbone. First, hydrolysis of lipid 6 with HF, known to cleave phosphodiester bonds, (Fischer *et al.*, 1973) gave rise to two products, as shown by TLC. One of these products, designated HF-6, was characterized below. The other product was diglyceride as shown by comigration with the diglyceride standard on TLC in hexane/ether/acetic acid (70:30:1, v/v/v). Thus lipid 6 contains a diglyceride moiety linked to phosphate. The second method used was phospholipase A_2 digestion of lipid 6 which yielded fatty acid as well as a mono-deacylated product (designated lyso-6). Since liberation of fatty acid from a lipid by phospholipase A_2 requires an *sn*-glycerol 3-phosphate backbone (De Haas *et al.*, 1968) it may be concluded that lipid 6 contains a diacylated *sn*-glycerol 3-phosphate constituent.

2.3.3 Identification of the Carbohydrate Moiety of Lipid 6

Acid hydrolysis (18 h at 105°C in 2N HCl) of lipid 6 yielded a water-soluble compound which co-migrated with standard glucosamine (R_f=0.26) after TLC in n-butanol/ethanol/28% ammonia (67:20:20, v/v/v). Since acetyl groups of amino sugars are easily removed during acid hydrolysis, NMR spectroscopy of the intact (unhydrolyzed) lipid 6 molecule was used to determine whether or not the glucosamine had a free or an acetylated amino group. ^{1}H-NMR spectroscopy of lipid 6 showed a clear NHCOC$\underline{H}_3$ signal at δ 2.06 ppm (compared to δ 2.08 ppm for standard N-acetyl glucosamine NHCOC$\underline{H}_3$). No similar signal was apparent in the NMR spectrum of lipid 7 (Fig. 5) which contains a non-acetylated galactose (Anderson and Hansen, 1985).

Parenthetically, it should be noted that the apparent quartet at δ 2.34 ppm ($CH_2C\underline{H}_2CO$) actually represents two overlapping triplets, one from each of the

Figure 5. **Partial 400 MHz ^{1}H-NMR spectrum of the N-acetyl(CH_3) group in intact lipid 6 (upper tracing) compared with the same region in lipid 7 (lower tracing).** Samples were dissolved in $CDCl_3/CD_3OD$ (3:1, v/v).


O=HNCCH3
O=CH2CH2C
CH2CH=CH
2.4
2.2
2.0
PPM

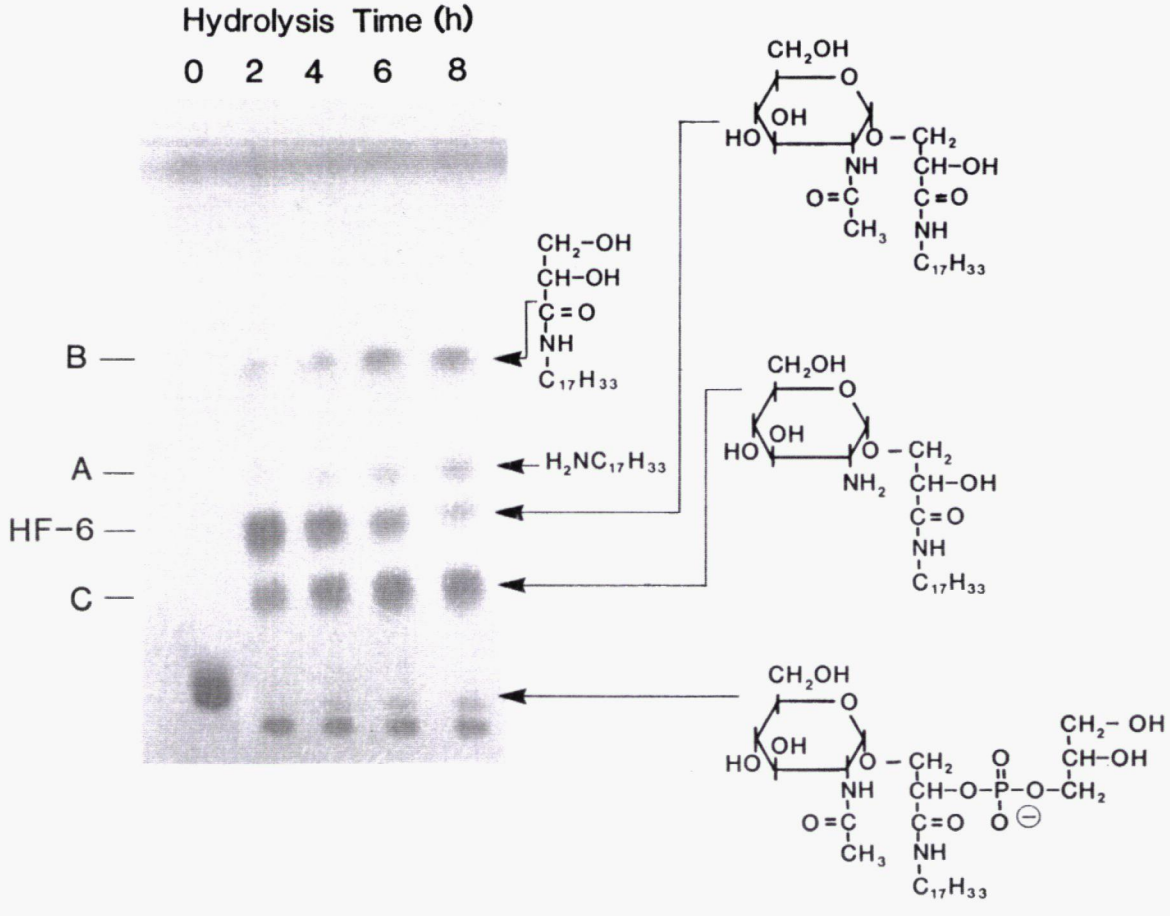
two fatty acids. This was verified by decoupling experiments, involving irradiation at δ 1.62 ppm ($C\underline{H}_2CH_2CO$) which resulted in the collapse of the multiplet into two singlets (δ 2.33 and 2.35 ppm) corresponding to the α-CH_2 groups of each fatty acid.

Further confirmation that lipid 6 contains N-acetylglucosamine as the sole carbohydrate constituent is presented below with regard to the ^1H and ^{13}C-NMR spectra.

2.3.4 Time-Controlled Acid Hydrolysis of Deacylated Lipid 6

In order to identify the structural constituents of lipid 6 a time-controlled hydrolysis was performed. Lipid 6 was first treated with mild methanolic NaOH (Kates, 1972) to yield the deacylated derivative, which was purified by preparative TLC, as shown in Fig. 6, lane 0. Treatment of the deacylated lipid 6 with methanolic HCl for various times at 80°C gave rise to a series of four products as shown in Fig. 6. The most abundant product formed after only 2 h of hydrolysis was the same as that obtained after HF hydrolysis and is designated HF-6 (HF-6 was acetylated and characterized by ^1H-NMR spectroscopy as described below). The relative amount of HF-6 decreased with increasing time of hydrolysis. Concomitant with this was an increase in two products designated B and C in Fig. 6. Product B was shown to be glyceroyl alkylamine, which was also obtained from hydrolysis of lipid 7 (Anderson and Hansen, 1985). Product B had an R_f of 0.62 on self-prepared silica gel H and an R_f of 0.49 on commercial silica gel 60 (Merck) TLC plates (Anderson and Hansen, 1985). Its identity was confirmed by cochromatography with authentic glyceroyl hexadecylamine (synthesized as described in Anderson and Hansen, 1985) and by NMR analysis of its acetylated derivative (NMR assignments as in Anderson and Hanson, 1985). Product C was observed to arise at the expense of HF-6 (Fig. 6, lanes 2-8) and most likely reflects removal of the

Figure 6. **Thin layer chromatogram of purified deacylated lipid 6 (0 h) and products of methanolic HCl hydrolysis obtained after 2, 4, 6, and 8 h at 80°C.** The chromatogram was developed halfway in chloroform/methanol/28% ammonia (65:35:5, v/v/v) and then fully in chloroform/methanol (9:1, v/v). Lipids were visualized by spraying with 25% sulfuric acid and charring.


Hydrolysis Time (h)
0 2 4 6 8
B
A
HF-6
C
$H_2NC_{17}H_{33}$

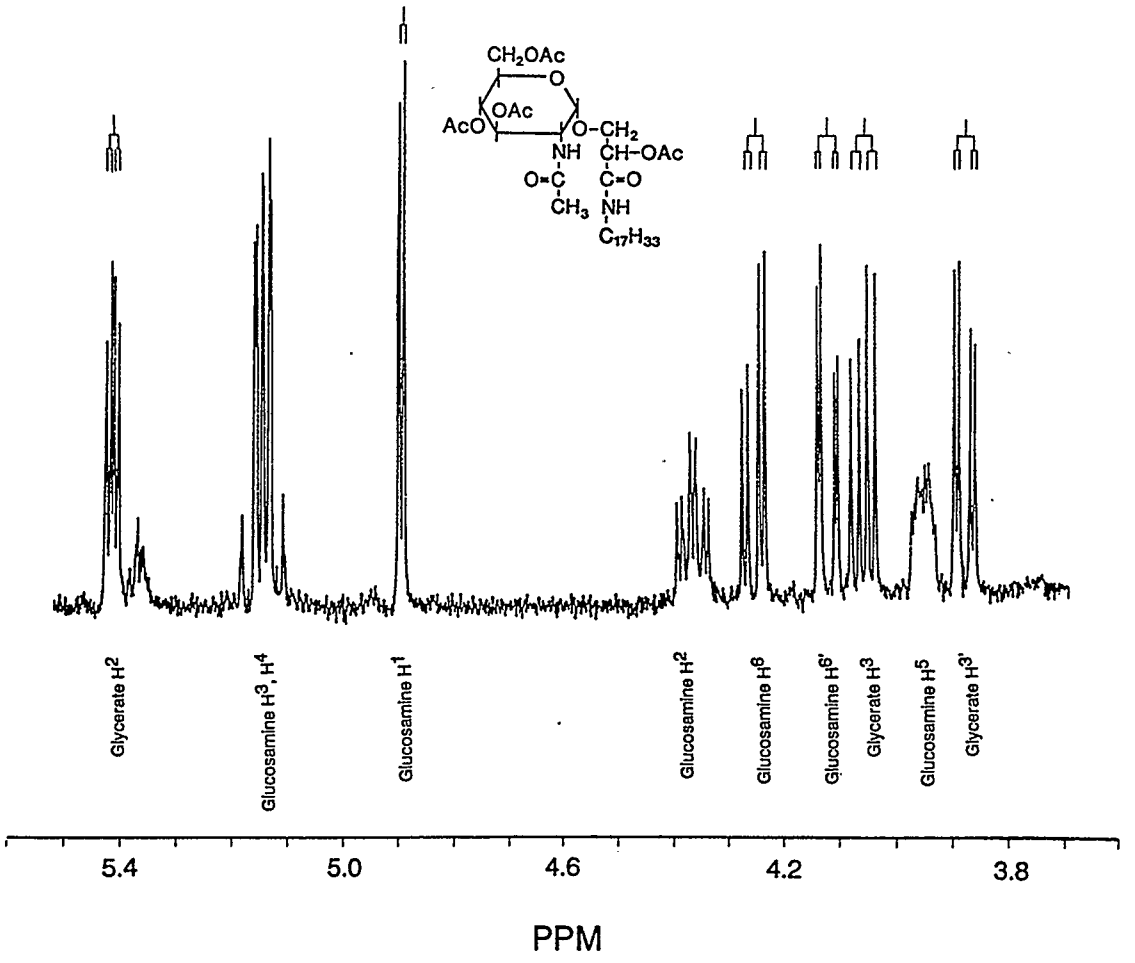
relatively acid-labile N-acetyl group on glucosamine. A fourth, low abundant product (designated A) gradually increased in amount over the 8 h time course. Product A was acetylated and analyzed by GC/MS which showed it to be a mixture of alkylamines of which heptadecenylamine was a major component. Alkylamines have already been reported as structural constituents of lipid 7 (Anderson, 1983; Anderson and Hansen, 1985).

2.3.5 ^{1}H-NMR Spectroscopy of Acetylated HF-6

Acetylation of the HF hydrolysis product of lipid 6 yielded a single compound (R_f=0.70) by TLC in chloroform/methanol (15:l, v/v). This product, designated acetylated HF-6, showed a clear, highly resolved NMR spectrum (Fig. 7). This was particularly useful in the verification of the carbohydrate component of this lipid and in determining its anomeric identity.

By NMR analysis (and spin decoupling) of acetylated HF-6 and of standard reference compounds (e.g., l-0-methyl-α-D-glucosamine tetraacetate) it was possible to unequivocally make the following assignments (refer to Fig. 7): glycerate: H^2 (δ 5.40, 1H, dd, $J_{2,3}$=5.7 Hz, $J_{2,3'}$=3.4 Hz); H^3 (δ 4.05, 1H, dd, $J_{3,2}$=5.7 Hz, $J_{3,3'}$=11.3 Hz); $H^{3'}$ (δ 3.83, 1H, dd, $J_{2,3'}$=3.4 Hz, $J_{3',3}$=11.3 Hz); glucosamine: H^1 (δ 4.88, 1H, d, $J_{1,2}$=3.5 Hz); H^2 (δ 4.36, 1H, dddd, $J_{2,1}$=3.5 Hz, $J_{2,NH}$=9.5 Hz, $J_{2,3}$=10.6 Hz, $J_{2,4}$~1.0 Hz); H^3 (δ~5.15, 1H, dd, $J_{3,2}$=10.6 Hz, $J_{3,4}$~9.8 Hz); H^4 (δ~5.13, 1H, ddd, $J_{4,3}$~9.8 Hz, $J_{4,5}$~8.7 Hz, $J_{4,2}$~1.0 Hz); H^5 (δ 3.93, 1H, ddd, $J_{5,4}$~8.7, $J_{5,6}$=4.3 Hz, $J_{5,6'}$=2.3 Hz); H^6 (δ 4.24, 1H, dd, $J_{6,5}$=4.3 Hz, $J_{6,6'}$=12.4 Hz); $H^{6'}$ (δ 4.11, 1H, dd, $J_{6',5}$=2.3 Hz, $J_{6',6}$=12.4 Hz). Although not shown in the region of the spectrum illustrated in Fig. 7, significant additional assigments were: glucosamine NH (δ 6.03, 1H,d $J_{NH,2}$=9.5 Hz), and alkylamine NH (δ 6.17, 1H, t, $J_{NH,CH2}$=6.0 Hz). For clarification of some of the key NMR assignments, the following points are relevant:

Figure 7. **Partial 400 MHz ^{1}H-NMR spectrum of acetylated HF-6 (in $CDCl_3$).**


CH2OAc
O
OAc
AcO
O-CH2
NH
CH-OAc
O=C
C=O
CH3
NH
C17H33
Glycerate H2
Glucosamine H3, H4
Glucosamine H1
Glucosamine H2
Glucosamine H6
Glucosamine H6'
Glycerate H3
Glucosamine H5
Glycerate H3'
5.4
5.0
4.6
4.2
3.8
PPM

1) The chemical shifts and coupling constants of H^3 and H^4 (which overlap due to the shielding effect of the aglycan) were determined using spin-decoupling and spin-simulation computer analysis (based on an ABMX spin system).

2) With regard to the anomeric proton (H^1), it is evident that the coupling constant ($J_{1,2}$=3.5 Hz) is diagnostic of an α-linkage (Minniken, 1972; Falk *et al.*, 1979).

3) In addition to interaction with H^1, NH and H^3, the H^2 proton also shows long-range coupling with H^4, as indicated by spin-decoupling experiments ($J_{2,4}$~1.0 Hz).

4) The chemical shifts and coupling constants observed for the carbohydrate moiety of lipid 6 are compatible only with N-acetylglucosamine and with no other amino-sugar.

As a further verification of the structure of lipid 6, a complete ^{1}H-NMR spectrum was obtained from the intact, underivatized lipid (Fig. 8). By means of decoupling experiments, the proton assignments were made as shown in Table 2. The chemical shifts for the glucosamine protons were clustered much more closely together than the corresponding signals observed in the acetylated HF-6 derivative (Fig. 7), thereby rendering decoupling difficult and permitting only partial assignment of some of the N-acetylglucosamine protons, particularly H^3, H^5 and H^6.

Figure 8. **400 MHz ^{1}H-NMR spectrum of intact lipid 6 (in $CDCl_3/^{12}CD_3OD$, 3:1, v/v).**

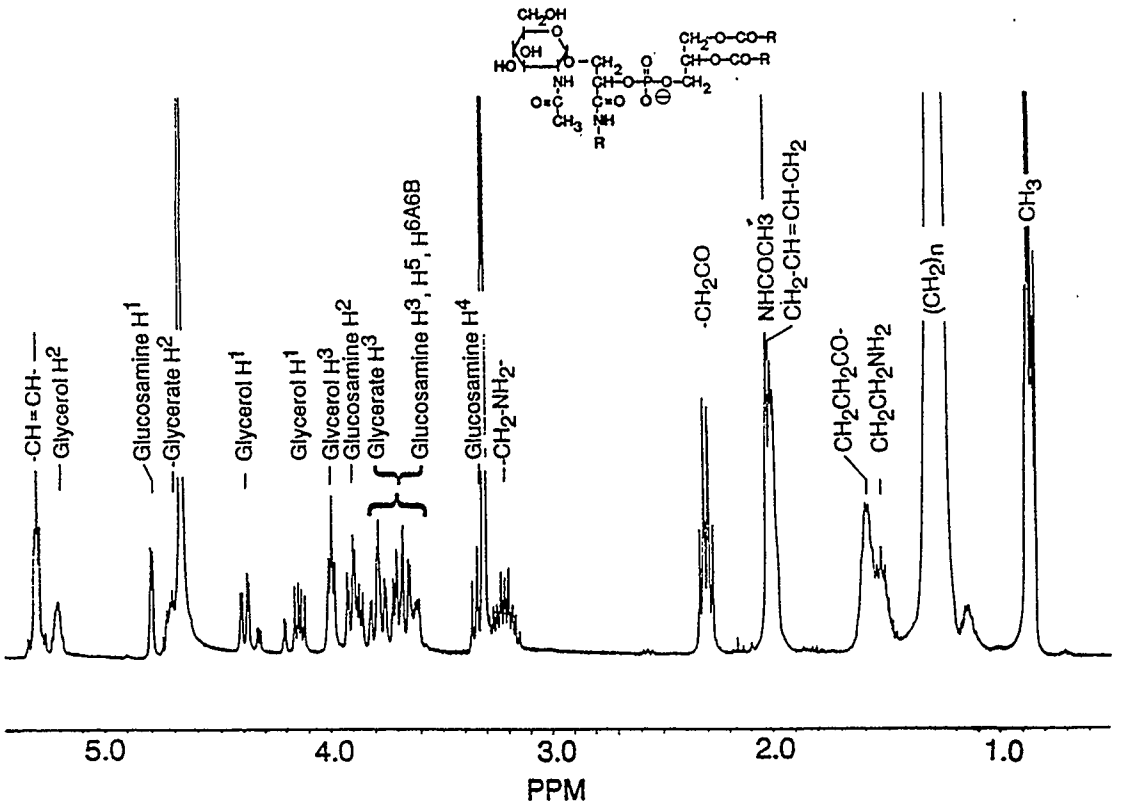

-CH=CH-
Glycerol H2
Glucosamine H1
Glycerate H2
Glycerol H1
Glycerol H1
Glycerol H3
Glucosamine H2
Glycerate H3
Glucosamine H3, H5, H6A6B
Glucosamine H4
-CH2-NH2-
-CH2CO
NHCOCH3
CH2-CH=CH-CH2
CH2CH2CO-
CH2CH2NH2
(CH2)n
CH3
5.0
4.0
3.0
2.0
1.0
PPM

TABLE 2. ^{1}H-NMR assignment for lipid 6.

Proton Assignment	Chemical Shift (ppm)	No. of Protons	Multi-plicity	Coupling Constant (Hz)
Glycerol				
1-CH^AH^BOCOR	4.43	1	dd	$J_{A,B} = 12.0$ $J_{A,2} = 3.2$
	4.18	1	dd	$J_{A,B} = 12.0$ $J_{B,2} = 6.8$
2-CHOCOR	5.25	1	m	$J_{A,2} = 3.2$ $J_{B,2} = 6.8$ $J_{2,3} = 5.7$
3-CH_2OP-	4.03	2	t	$J_{3,2} = 5.7$ $J_{31P,H} = 5.7$
Fatty acid				
-CH=CH-	5.34	3[a]	m	
-$C\underline{H}_2$-CH=CH-$C\underline{H}_2$-	2.04	6[a]	m	
-CH_2CO-	2.33 2.35	2 2	t t	$J_{2,3} = 7.4$ $J_{2,3} = 7.4$
-$C\underline{H}_2CH_2CO$-	1.62	4	m	
-$(CH_2)_n$-	1.1 - 1.4	~60	m	
-CH_3	0.87 - 0.92	9	t (x 3)	$J_{\omega,\omega-1} = 6.7$
Glycerate				
3-CH^AH^B	3.92	1	dd	$J_{A,B} = 11.0$ $J_{A,2} = 6.3$
	3.81	1	dd	$J_{A,B} = 11.0$ $J_{B,2} = 2.0$
2-CHOP-	4.74	1	m	

TABLE 2. (cont'd.)

Proton Assignment	Chemical Shift (ppm)	No. of Protons	Multi-plicity	Coupling Constant (Hz)
Alkylamine				
-NHC$\underline{H}_2$-	3.24	2	m	
-NHCH$_2$C$\underline{H}_2$-	1.55	2	m	
Glucosamine				
H^1	4.84	1	d	$J_{1,2} = 3.4$
H^2	3.94	1	ddd	$J_{1,2} = 3.4$ $J_{2,3} = 10.7$
H^4	3.38	1	dd[b]	
H^5 H^3 $H^{6A,6B}$	3.64-3.85	4		
-NHCOC$\underline{H}_3$	2.06	3	s	

NH or OH proton signals were not observed due to deuterium exchange with solvent $CDCl_3/^{12}CD_3OD$ (3:1, v/v). Abbreviations: s, singlet; d, doublet; t, triplet; m, multiplet.

[a] Consistent with ~50% monounsaturation, and ~50% saturation in fatty acid and alkylamine chains.

[b] Partially obscured by solvent signal.

2.3.6 ^{13}C-NMR Spectroscopy of Lipid 6

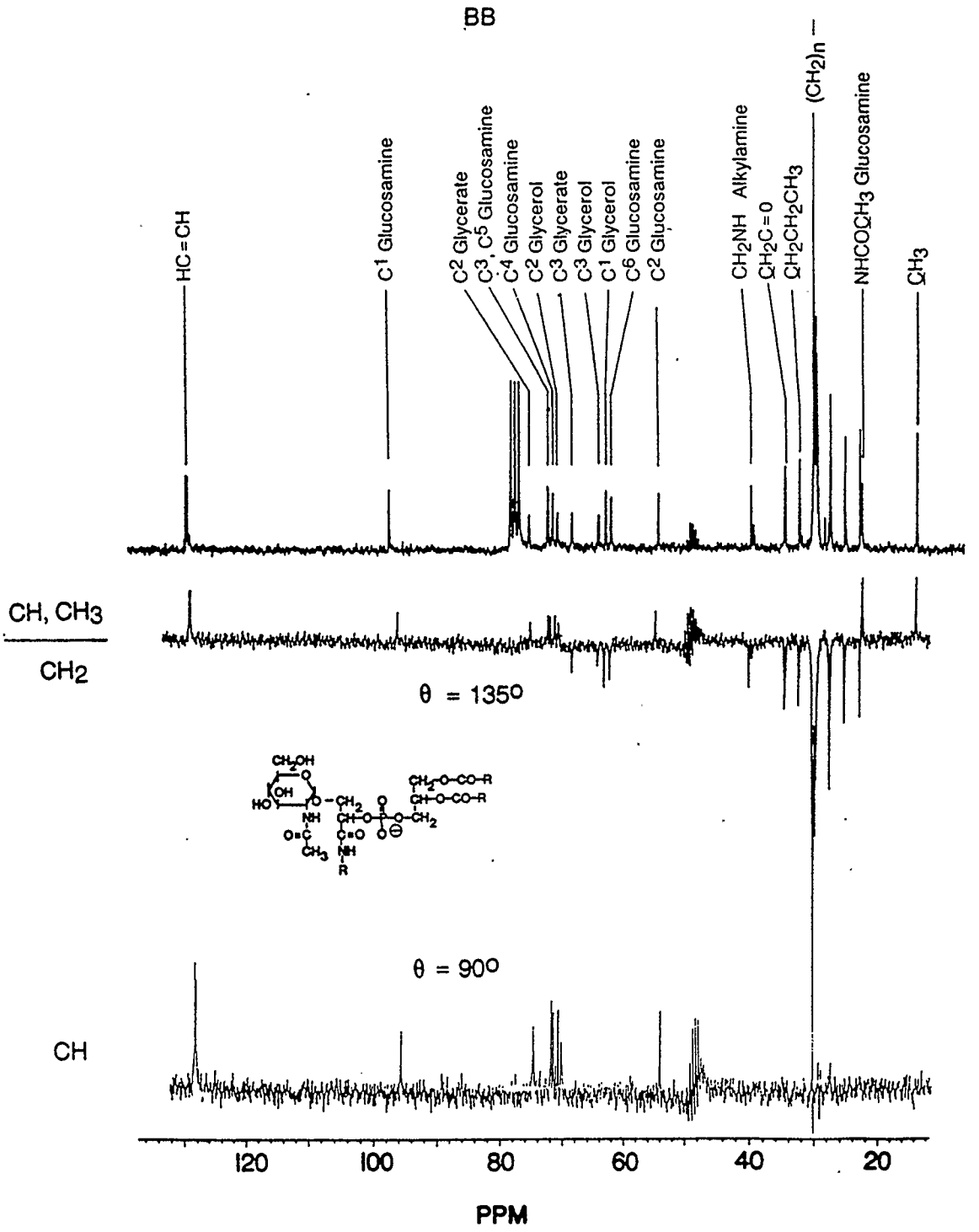
As a final check on the derived structure for lipid 6, a ^{13}C-NMR spectrum of the intact lipid was obtained (Fig. 9, upper panel). This permitted the assignment of all carbon centers. Comparisons were made with ^{13}C-NMR spectra of dioleoylphosphatidyl choline and 1-0-methyl-α-D-N-acetylglucosamine [in $CDCl_3/^{12}CD_3OD$ (3:1, v/v)] and with the published (Perkins *et al.*, 1977) ^{13}C-NMR spectrum of 1-0-methyl-N-acetylglucosamine (in D_2O). When 1-0-methyl-α-D-N-acetylglucosamine was dissolved in $CDCl_3/^{12}CD_3OD$ (3:1, v/v) its ^{13}C-NMR assignments were within 1 ppm of those of the corresponding carbons in lipid 6. Such slight differences in chemical shift undoubtedly reflect differences in chemical environment.

The assignments were made as follows: glucosamine: C^1 (δ 97.6), C^2 (δ 54.2), C^3 (δ 72.4), C^4 (δ 71.5), C^5 (δ 72.1), C^6 (δ 61.9), $NH\underline{C}OCH_3$ (δ 173.4), $NHCO\underline{C}H_3$ (δ 22.4); glycerate: C^1 (δ 170.2), C^2 (δ 75.4, $J_{^{31}P,^{13}C}$=5.5 Hz), C^3 (δ 68.4); glycerol: C^1 (δ 62.8), C^2 (δ 70.8, $J_{^{31}P,^{13}C}$=7.9 Hz), C^3 (δ 64.0, $J_{^{31}P,^{13}C}$=5.9 Hz); alkylamine: CH_2NH (δ 39.6); fatty acid: CH_2CO (δ 34.3 and 34.2), CO (δ 173.7 and 174.0), terminal alkyl CH_3 (δ 13.9). The vinyl carbons (δ 130.0 and 129.8) are diagnostic of cis double bonds (Johnson and Jankowski, 1972). In addition to the most crucial assignments noted above, additional aliphatic assignments are: $\underline{C}H_2CH{=}CH\underline{C}H_2$ (δ 27.5, 27.3 and 27.2 ppm, obtained from expanded spectrum), $\underline{C}H_2CH_2CH_3$ (δ 32.0 and 31.9 ppm, also from expanded spectrum).

Note should be made of the observed ^{31}P-^{13}C coupling involving C^2 of glyceric acid (δ 75.4, J=5.5 Hz) confirming the location of the phosphodiester linkage on C^2. In addition, the observed ^{31}P-^{13}C coupling constants for C^3 (J=5.9 Hz) and C^2 (J=7.9 Hz) of glycerol are in agreement with published coupling constants for standard phosphatidic acid-based lipids (Birdsall *et al.*, 1972).

Figure 9. **Complete ^{13}C-NMR spectrum of lipid 6 (in $^{12}CDCl_3/CD_3OD$, 3:1, v/v) (upper panel) and subspectrum obtained by distortionless enhancement by polarization transfer of lipid 6 (lower and center panel).**

^{13}C DEPT of LIPID 6



Further verification of the assignments for CH_3, CH_2 and CH carbons was obtained by analysis of the subspectra obtained from DEPT-NMR studies (Fig. 9, center and lower panel). DEPT-NMR was particularly useful for the unambiguous assignment of the $NHCO\underline{C}H_3$ group on glucosamine as well as for the verification of all CH, CH_2 [except for $(CH_2)_n$] and CH_3 groups.

2.3.7 Fatty Acid Composition of Lipids 6 and 7

Lipid 6 differs in structure from lipid 7 (Anderson and Hansen, 1985) in its substitution of N-acetylglucosamine for galactose. As shown in Table 3, the fatty acid compositions of lipid 6 and 7 do not differ significantly: C-15 to C-17 fatty acids predominate, with C-16:0 and C-16:1 comprise more than half the total. Roughly similar fatty acid compositions have been reported for the total lipids from several *D. radiodurans* isolates (Brooks *et al.*, 1980; Embley *et al.*, 1987).

1.4 DISCUSSION

This chapter describes the complete structure of a second novel phosphoglycolipid (lipid 6) from *D. radiodurans*. Together with the previously characterized lipid 7 (Anderson and Hansen, 1985), these two lipids comprise approximately 43% of the total lipids of this organism (Thompson and Murray, 1981).

Structurally, both lipids 6 and 7 share the phosphatidic acid backbone common to most phospholipids of prokaryotic and eukaryotic membranes. However, the commonly observed polar headgroup of most phospholipids is replaced in lipids 6 and 7 with a complex structure consisting of glyceric acid, carbohydrate and alkylamine. Why *D. radiodurans* should possess such unique and complex lipids is an interesting question for future study.

TABLE 3. Fatty acid composition of lipids 6 and 7.

Fatty acid	Lipid 6	Lipid 7
	mean % ± SD[a]	mean % ± SD[a]
14:0	1.19 ± 0.58	0.99 ± 0.84
Iso - 15:0	2.39 ± 0.41	1.72 ± 1.12
Anteiso - 15:0	1.09 ± 0.30	1.18 ± 0.40
15:1	3.20 ± 0.93	2.71 ± 1.29
15:0	5.05 ± 0.95	4.07 ± 1.17
Iso - 16:0	2.89 ± 0.20	3.67 ± 0.50
16:1Δ9	33.32 ± 4.03	36.41 ± 1.23
16:0	19.78 ± 6.53	20.75 ± 5.50
Iso - 17:1Δ9	5.23 ± 1.76	4.07 ± 1.81
Iso - 17:0	4.26 ± 0.43	4.19 ± 0.78
17:1Δ9	5.16 ± 1.68	3.95 ± 1.68
17:1Δ11	1.56 ± 0.47	1.24 ± 0.50
17:0	4.65 ± 0.74	3.25 ± 1.03
18:0	1.11 ± 0.44	0.61 ± 0.36
Total unknowns	9.12	11.19

[a]SD: Standard deviation (n=3)

Lipids containing glucosamine are not common in bacteria. It may be of interest that glucosamine-containing lipids have been detected in extremely thermophilic species of the genus *Thermus* (Oshima and Yamakawa, 1974; Pask-Hughes and Shaw, 1982) which has been shown to share a distant phylogenetic relationship to *Deinococcus* (Hensel *et al.*, 1986; Weisburg *et al.*, 1989). Although glucosamine is rarely found in bacterial lipids, it is an important constituent of cell wall structures such as lipopolysaccharide, peptidoglycan and teichoic acids. *D. radiodurans* has neither lipopolysaccharide nor teichoic acid, although its peptidoglycan does likely contain N-acetylglucosamine (reviewed in Schleifer and Kandler, 1972). Glucosamine also occurs commonly as pyrophosphorylpolyprenol conjugates in which form it participates in glycoprotein biosynthesis (reviewed in Waechter and Lennarz, 1976). It has been recently suggested that dolichol is the pyrophosphorylpolyprenol carrier involved in glycoprotein biosynthesis, while undecaprenol is the carrier for non-glycoprotein, complex carbohydrates of the cell wall (Hartmann *et al.*, 1993). Such conjugates have not yet been identified in *D. radiodurans*.

Members of the *Deinococcus* genus comprise an ancient and phylogenetically distinct bacterial group (Brooks *et al.*, 1980; Hensel *et al.*, 1986; Stackebrandt and Woese, 1979; Woese,1987). In this regard, ribosomal RNA comparisons have proven most useful. It appears, however, that closely related *Deinococci* can show different lipid patterns by chromatographic analysis (Counsell and Murray, 1986; Embley *et al.*, 1987). It is possible therefore that structural identification of the apparently unconventional lipids found in members of the *Deinococcus* genus may prove a sensitive discriminator of species within this genus.

The structural characterization of the unconventional lipids of *D. radiodurans* is of interest to understanding not only their possible role in the unusual radiation- (Anderson *et al.*, 1956) and desiccation- (Sanders and Maxcy, 1979) resistance of this organism but

also their involvement in the assembly and integrity of its complex cell envelope structure. The cell envelope of *D. radiodurans* consists of seven discrete structures recognizable by electron microscopy (see Section 1.2). The two lipid-containing structures, the plasma and outer membranes, differ in their lipid compositions: lipids 6 and 7 are 11.8% and 39.9% in the plasma membrane and 3.6% and 3.1% in outer membrane, respectively (Thompson and Murray, 1981). Clearly, more knowledge is required of the molecular structure of the lipid and protein components of the cell envelope substructures in order to probe their role in function and assembly of this complex cell envelope. For the most part, the stabilizing forces which bind the various structural elements together are unknown although there is evidence of hydrophobic interactions between the outer membrane and its tightly associated hexagonal layer (Baumeister and Kubler, 1978; Baumeister *et al.*, 1986). Further chemical and structural analyses of the constituents, coupled with reconstitution studies, should provide insights into such interactions.

CHAPTER 3

PHOSPHATIDYLGLYCEROYLALKYLAMINE:

A NOVEL PHOSPHOGLYCOLIPID PRECURSOR IN *D. RADIODURANS*

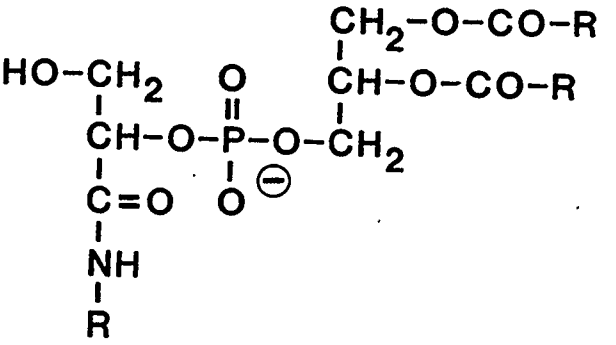
3.1 INTRODUCTION

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. Previous studies have elucidated the structures of two major phosphoglycolipids from *D. radiodurans* (Anderson and Hansen, 1985; Huang and Anderson, 1989). Relevant structures are given in Fig. 10. This chapter relates these two phosphoglycolipids, lipids 6 and 7, to a novel phospholipid, phosphatidylglyceroylalkylamine, and demonstrates a biosynthetic pathway connecting them.

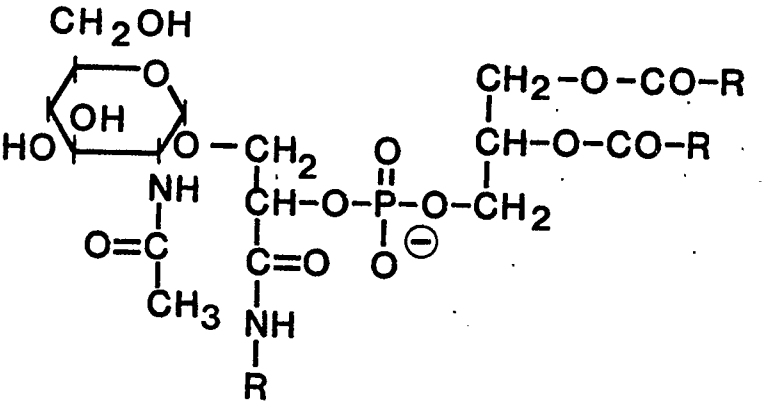
Lipids 6 and 7 are glycosylated phosphatidylglyceroylalkylamines. Despite the uniqueness of these structures, they do contain certain components which are found elsewhere in prokaryotic and eukaryotic cells. Foremost among these common components is the phosphatidate moiety which is of the *sn*-1,2 stereochemistry (Anderson and Hansen, 1985) similar to that found in most bacteria with the exception of the Archaea. Phosphatidic acid is an important intermediate in phospholipid biosynthesis due to its role in the biosynthesis of CDP-diglyceride which then participates in the formation of various phospholipids, including phosphatidylglycerol, phosphatidylethanolamine and diphosphatidylglycerol (reviewed in Vanden Boom and Cronan, 1989). It is, therefore, likely that phosphatidic acid plays an important role in the biosynthesis of certain complex lipids in *D. radiodurans*. This chapter provides evidence by [^{32}P]phosphate radiolabeling that phosphatidic acid is an important intermediate in phospholipid metabolism. More significantly, this study identifies a novel phospholipid, phosphatidylglyceroylalkylamine, and demonstrates a role for this phospholipid in the biosynthesis of the complex phosphoglycolipids of *D. radiodurans*.

Figure 10. **Structures of phospholipids.** Lipids 7 and 6 are α-galactosyl- and α-*N*-acetylglucosaminyl phosphatidylglyceroylalkylamine, respectively. Lipid 4, phosphatidylglyceroylalkylamine, is the subject of investigation in this chapter.

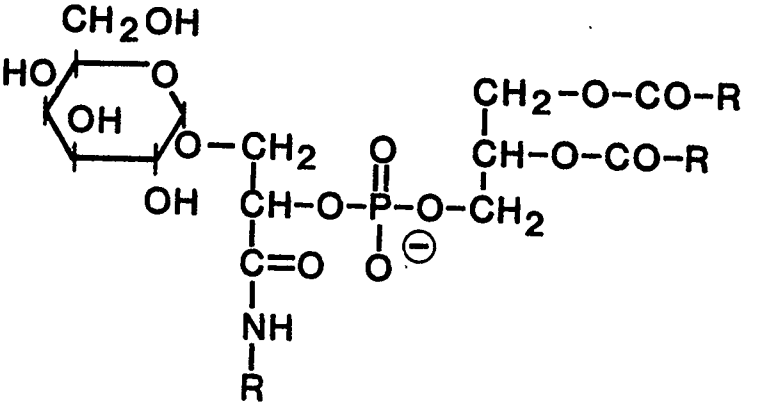
Lipid 4



Lipid 6



Lipid 7



From phosphatidic acid, there are a number of possible biosynthetic pathways leading to the formation of the glycosylated phosphatidylglyceroylalkylamines (i.e. lipids 6 and 7). An important question is whether the glycosylated glyceroylalkylamine moiety is preassembled prior to its condensation with phosphatidic acid (or CDP-diglyceride) or whether the individual components (glyceroylalkylamine and carbohydrate) are added sequentially to the phosphatidate "core". This question can be addressed through pulse-chase radiolabeling studies, as described in this Chapter.

3.2 MATERIALS AND METHODS

3.2.1 Growth of *D. radiodurans* and Extraction of Lipids

Cultures were grown and total lipids were extracted as described in Section 2.2.1.

3.2.2 Thin Layer Chromatography (TLC)

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

3.2.3 Radiolabeling of *D. radiodurans*

For examination of the kinetics of lipid synthesis, a 100 ml culture of *D. radiodurans* was grown to an OD_{600} of 0.5 and then supplemented with [1-^{14}C]acetate (0.5 mCi/ml, New England Nuclear) and incubated at 35°C. At times of 10 min, 30 min, 90 min, 2.5 h, 4.5 h and 6.5 h, 1.0 ml aliquots were removed from the culture and lipid extracts prepared. Aliquots of the lipid extracts (equalized on the basis of cpm) were

resolved by two dimensional TLC and radiolabeled lipids were visualized by autoradiography.

3.2.4 Pulse-chase Radiolabeling with [^{32}P]phosphate

A freshly grown 50 ml culture of *D. radiodurans* (OD_{600} = 0.5) was centrifuged and the cell pellet 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 microfuged and washed 5 times with chase medium at 4°C to remove unincorporated label (chase medium is medium supplemented with 10 mM phosphate). An aliquot ("pulse") was extracted immediately while other aliquots were incubated for various times at 35°C and then extracted ("chase"). Samples from the lipid extracts were resolved by two dimensional TLC and lipids visualized by autoradiography and by sulfuric acid-charring of the TLC plates.

3.2.5 Purification of Lipid 4

Samples of total lipid extract from *D. radiodurans* were applied to homemade silica gel H plates (1 mm gel thickness) and developed in chloroform/methanol/28% ammonia (80:20:2, v/v/v). 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 from the silica with chloroform/methanol (1:1, v/v).

3.2.6 HF Treatment of Lipid 4

A sample of ^{14}C-acetate-labeled lipid 4 was purified as above from *D. radiodurans* which had been cultured in the presence of [1-^{14}C]acetate. A portion of this lipid 4 was subjected to HF hydrolysis (Fischer *et al.*, 1973) and examined by TLC. Development was performed sequentially with three solvents: chloroform/methanol/28% ammonia

(80:20:2, v/v/v) (one third development); chloroform/methanol (9:1, v/v) (two thirds development) and hexanes/ether/acetic acid (50:50:1, v/v/v) (complete development). The chromatographic standard, N-glyceroylhexadecylamine, was prepared as previously described (Anderson and Hansen, 1985).

3.2.7 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 24 h (to liberate alkylamines and glyceroylalkylamines). Phosphorus was determined by the method of Rouser *et al.*, (1970). Fatty acid methyl esters were analyzed by gas chromatography. For the analysis of alkylamines, 24 h methanolic HCl hydrolysates were dried, dissolved in chloroform and applied to small (0.5 cm deep) columns of Bio-Sil A (BioRad) in glass wool-plugged Pasteur pipettes; fatty acid methyl esters were first eluted with chloroform, followed by elution of alkylamines and glyceroylalkylamines with chloroform/methanol (3:1, v/v). The chloroform/methanol (3:1, v/v) eluate was acetylated overnight by reaction with pyridine/acetic anhydride (1:1, v/v). The acetylated mixture was dried down, dissolved in chloroform and analyzed by gas chromatography.

3.2.8 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 using a temperature program of 180-290°C (at 5°C/min). Acetylated alkylamines and glyceroylalkylamines were analyzed using a two-step temperature program (240°C for 20 min, followed by increase to 290°C at 5°C/min). Confirmation of gas chromatographic assignments was obtained by coupled gas chromatography/mass spectrometry as described previously (Anderson, 1983).

3.2.9 NMR Spectroscopy of Lipid 4

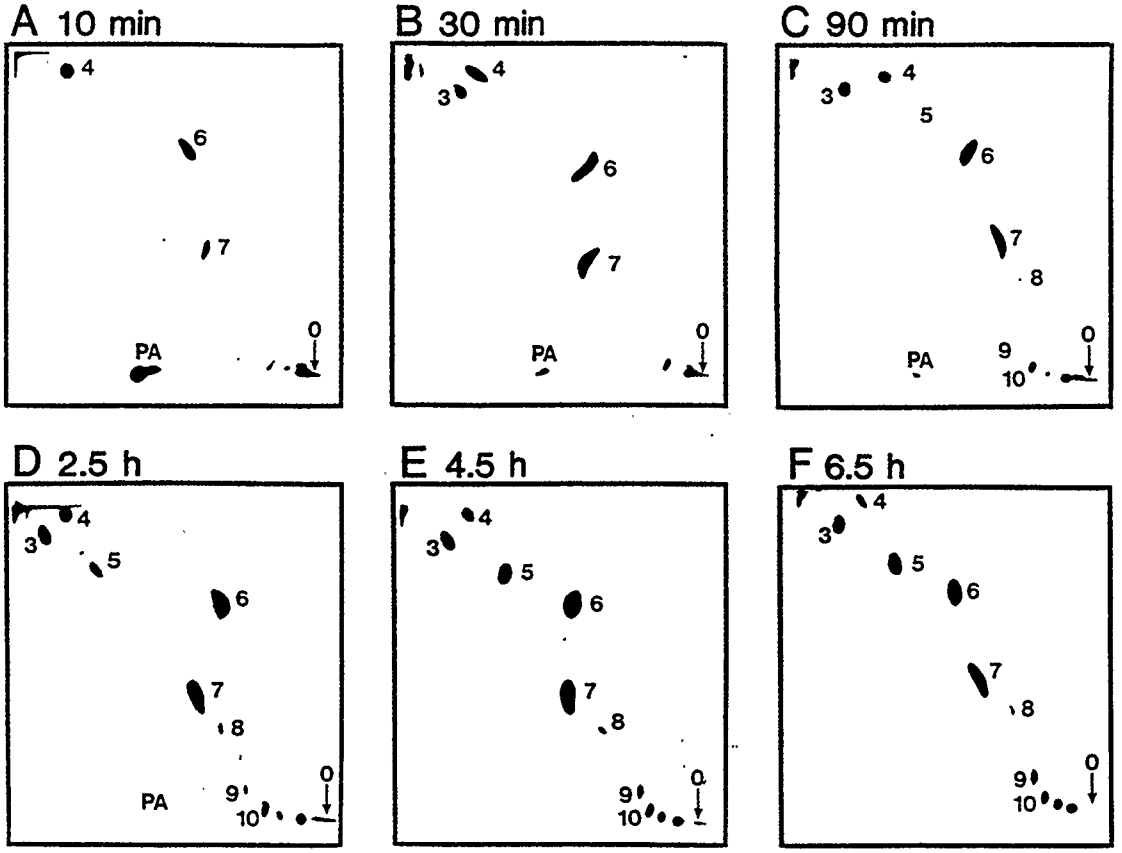
Proton-NMR was performed on a Bruker AM-400 Spectrometer. Chemical shifts (δ) are expressed as ppm relative to tetramethylsilane. Coupling constants (J) are given in Hz.

3.3 RESULTS

3.3.1 Kinetics of Lipid Synthesis in *D. radiodurans*

Despite the complexity of lipids found in *D. radiodurans* (Rebeyrotte *et al.*, 1979; Thompson *et al.*, 1980) we were able to demonstrate distinct differences in their rates of synthesis as indicated by incorporation of ^{14}C-acetate. As shown in Fig. 11, 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. With increasing labeling times, radiolabel appeared in the remaining lipids so that, by about 6.5 h, the distribution of radiolabel resembled that obtained from equilibrium labeling conditions (Thompson *et al.*, 1980). Under conditions of pulse-labeling [Panel A (10 min) of Fig. 11], the rapid incorporation of radiolabel into lipids 6 and 7 is not surprising since these 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 in *D. radiodurans* (Thompson *et al.*, 1980). The results thus suggest that, along with phosphatidic acid, lipids 4, 6 and 7 are rapidly synthesized and/or turned over in *D. radiodurans*.

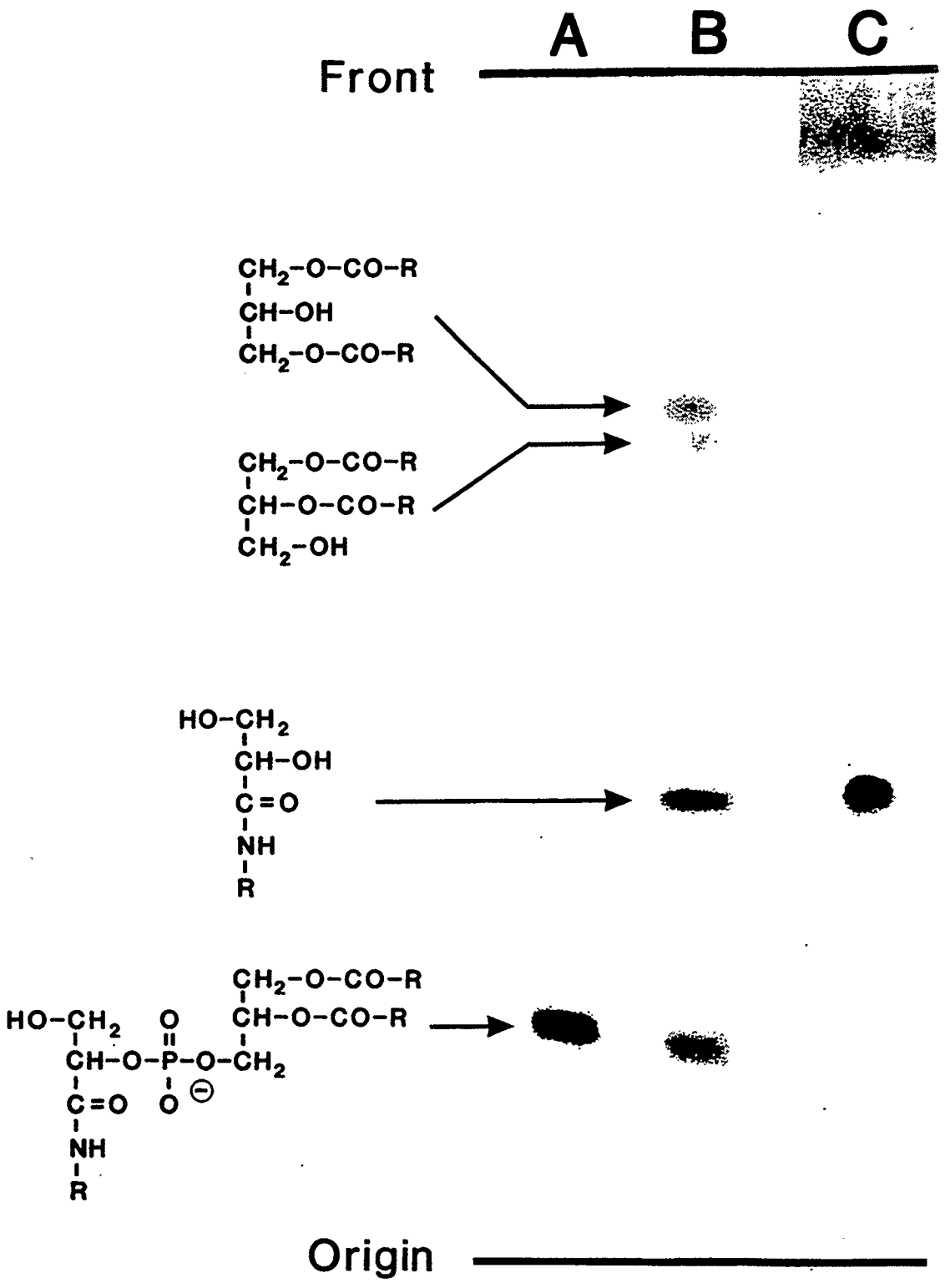
Figure 11. **Kinetics of lipid synthesis in** *D. radiodurans.* A 100-ml culture of *D. radiodurans* with an optical density at 600 nm of 0.5 was supplemented with [1-^{14}C]acetate, and aliquots were removed after the indicated labeling times. Lipids were extracted and subjected to autoradiographic two-dimensional TLC. Solvent 1 (vertical): chloroform/methanol/28% ammonia (65:35:5, v/v/v). Solvent 2 (horizontal): chloroform/acetone/methanol/acetic acid/water (10:4:2:2:1, v/v/v/v/v). Numerical lipid designations are those described by Thompson *et at.* (1980). Abbreviations: PA, phosphtidic acid O, origin.


A 10 min
B 30 min
C 90 min
D 2.5 h
E 4.5 h
F 6.5 h
PA
0
3
4
5
6
7
8
9
10

3.3.2 Identification of Lipid 4 as Phosphatidylglyceroylalkylamine

Lipids 6 and 7 have been previously identified as glycosylated phosphatidylglyceroylalkylamines (Anderson and Hansen, 1985; Huang and Anderson, 1989). In order to establish the structure of lipid 4, a series of chemical and spectroscopic analyses were undertaken. Lipid 4 was found to contain fatty acid, alkylamine and phosphorus in molar ratios of 2:1:1. Using HF to specifically cleave phosphoester bonds (Fischer *et al.*, 1973) lipid 4 yielded two products which were identified on TLC as diglyceride and N-glyceroylalkylamine (Fig. 12). [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 (e.g., Patton and McCarthy, 1963)]. N-glyceroyl alkylamine was identified following acetylation and analysis by coupled gas chromatography/mass spectrometry as previously described (Anderson and Hansen, 1985). 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 (Huang and Anderson, 1989). 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 acetylated glyceroylalkylamines were resolved as mixtures consisting of different alkylamine species. The alkylamines had hydrocarbon chain lengths of C-15 to C-18 in which C-16:0, C-16:1, C-17:0 and C-17:1 predominated.

Figure 12. **Products of HF hydrolysis of lipid 4.** Pure lipid 4, isolated from [1-^{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 X-ray film (used to visualize lanes A and B) was aligned with the charred TLC plate (used to char lane C) by positioning at the origin.

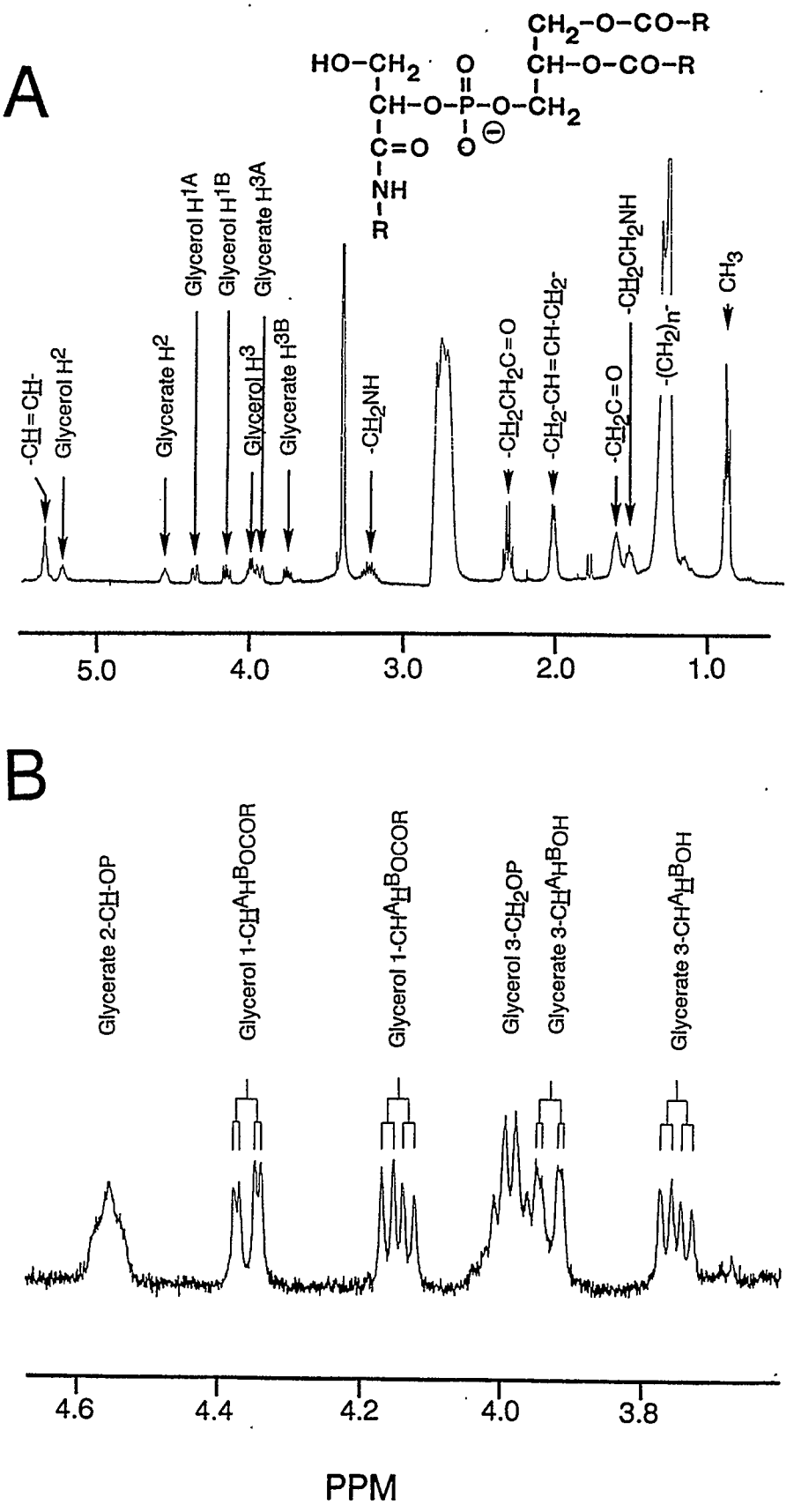

A
B
C
Front
CH2-O-CO-R
CH-OH
CH2-O-CO-R
CH2-O-CO-R
CH-O-CO-R
CH2-OH
HO-CH2
CH-OH
C=O
NH
R
CH2-O-CO-R
HO-CH2
CH-O-CO-R
CH-O-P-O-CH2
C=O
O
NH
R
Origin

3.3.3 NMR Spectroscopy of Lipid 4

Confirmation that lipid 4 is phosphatidylglyceroylalkylamine was obtained by proton NMR spectroscopy (Fig. 13). The proton assignments of lipid 4 were made as follows: glycerate: H^2 (δ 4.55, m, $J_{31P,H}$ =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); 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); -CH_2CH_2CO (δ 1.59, 4H, m); $(CH_2)n$ (δ 1.2-1.4, ~60H, m); CH_3 (δ 0.85 - 0.95, 9H [three protons belong to terminal methyl group of alkylamine], Jω,ω-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).

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), respectively. The coupling constant, $J_{31P,H}$ = 7.2 Hz, was determined by irradiating at either δ 3.93 or d 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), respectively, and also collapsed the "quartet" 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

Figure 13. **^{1}H-NMR spectrum of lipid 4 dissolved in $CDCl_3/CD_3OD$ (10:1, v/v).** (A) Complete spectrum; (B) partial, expanded spectrum showing splitting patterns of glycerol and glycerate protons. Signals at δ 3.39 and 2.74 ppm correspond to CD_2HOD and HDO, respectively. The doublet at δ 1.76 ppm is derived from an impurity present in the $CDCl_3$ solvent.


A
HO-CH2
CH-O-P-O-CH2
C=O
NH
R
CH2-O-CO-R
CH-O-CO-R
-CH=CH-
Glycerol H2
Glycerate H2
Glycerol H1A
Glycerol H1B
Glycerate H3A
Glycerol H3
Glycerate H3B
-CH2NH
-CH2CH2C=O
-CH2-CH=CH-CH2-
-CH2C=O
-CH2CH2NH
-(CH2)n-
CH3
5.0
4.0
3.0
2.0
1.0
B
Glycerate 2-CH-OP
Glycerol 1-CHAHBOCOR
Glycerol 1-CHAHBOCOR
Glycerol 3-CH2OP
Glycerate 3-CHAHBOH
Glycerate 3-CHAHBOH
4.6
4.4
4.2
4.0
3.8
PPM

the $J_{M,A}$, $J_{M,B}$, $J_{X,A}$ and $J_{X,B}$ coupling constants (J~6-7Hz). Hence, the splitting pattern in the spectrum appears as a quartet. Fatty acid: irradiation of δ 2.01 ppm (-C<u>H</u>$_2$-CH=CH-C<u>H</u>$_2$-) collapsed the multiplets at δ 5.34 ppm(-CH$_2$-C<u>H</u>=C<u>H</u>-CH$_2$-) to a singlet. Irradiation at δ 1.59 ppm(-C<u>H</u>$_2$CH$_2$CO-) collapsed the quartet at δ 2.30 ppm(-C<u>H</u>$_2$CO-) to a doublet δ 2.31 and 2.29 ppm, each corresponding to one fatty acid chain. Alkylamine: irradiation at δ 3.22 ppm(-NHC<u>H</u>$_2$-) simplified the multiplet at δ 1.51 ppm (-NHCH$_2$C<u>H</u>$_2$-).

3.3.4 Lipid 4 is a Precursor to Lipids 6 and 7

The structural 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. As shown in Fig. 14, pulse-labeling of *D. radiodurans* for 5 min resulted in incorporation of label only into lipid 4 and phosphatidic acid. (^{32}P label at 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 loss of label from lipid 4 and phosphatidic acid. Given the ubiquitous primary role of phosphatidic acid in phospholipid biosynthesis, the results indicated a clear biosynthetic pathway through lipid 4 to the complex phosphoglycolipids 6 and 7.

3.3.5 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 two min of pulse labeling (Fig. 15).

Figure 14. **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 chase) 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 those described by Thompson *et al.* (1980). Abbreviations: PA, phosphatidic acid; O, origin.

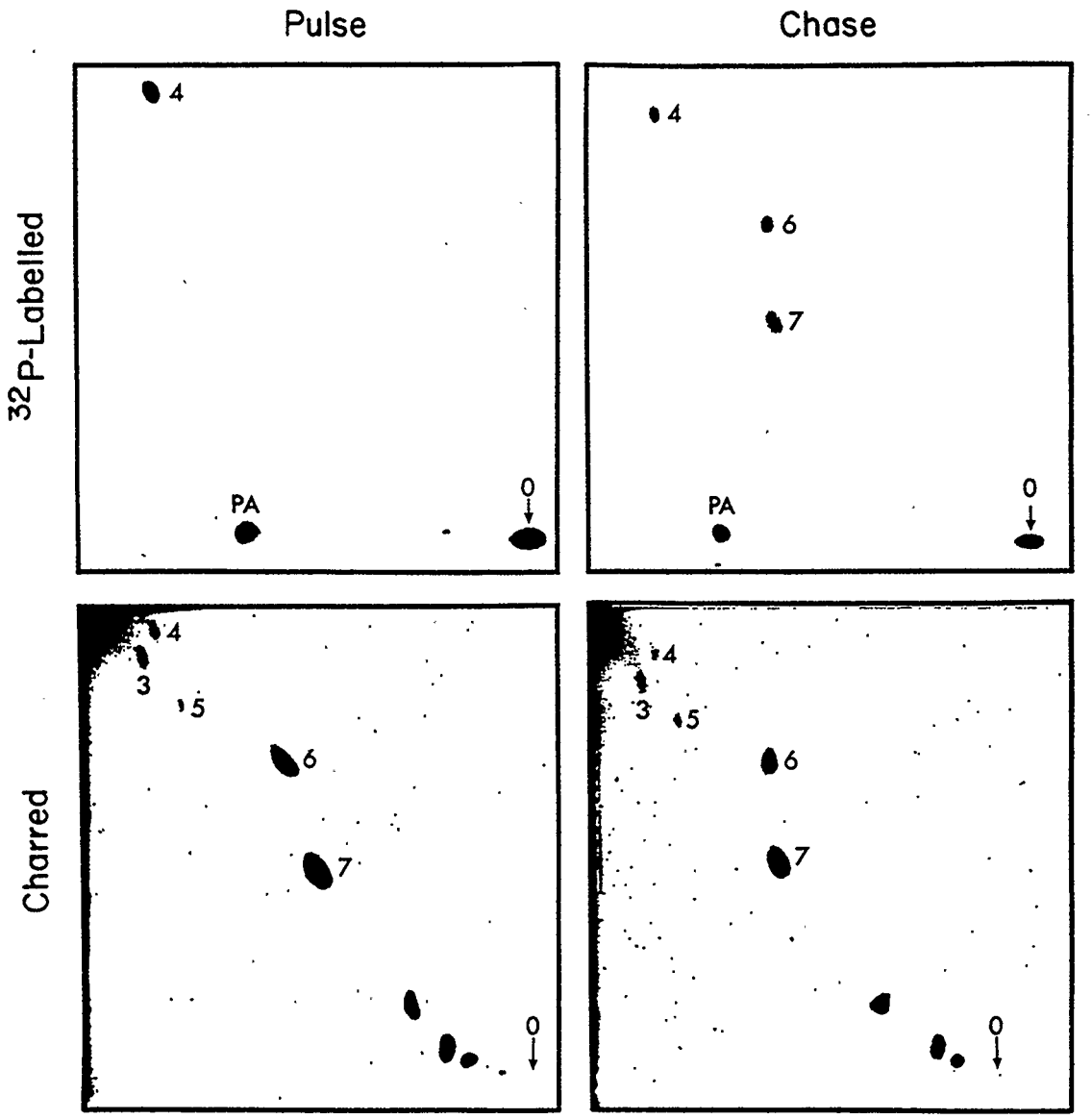
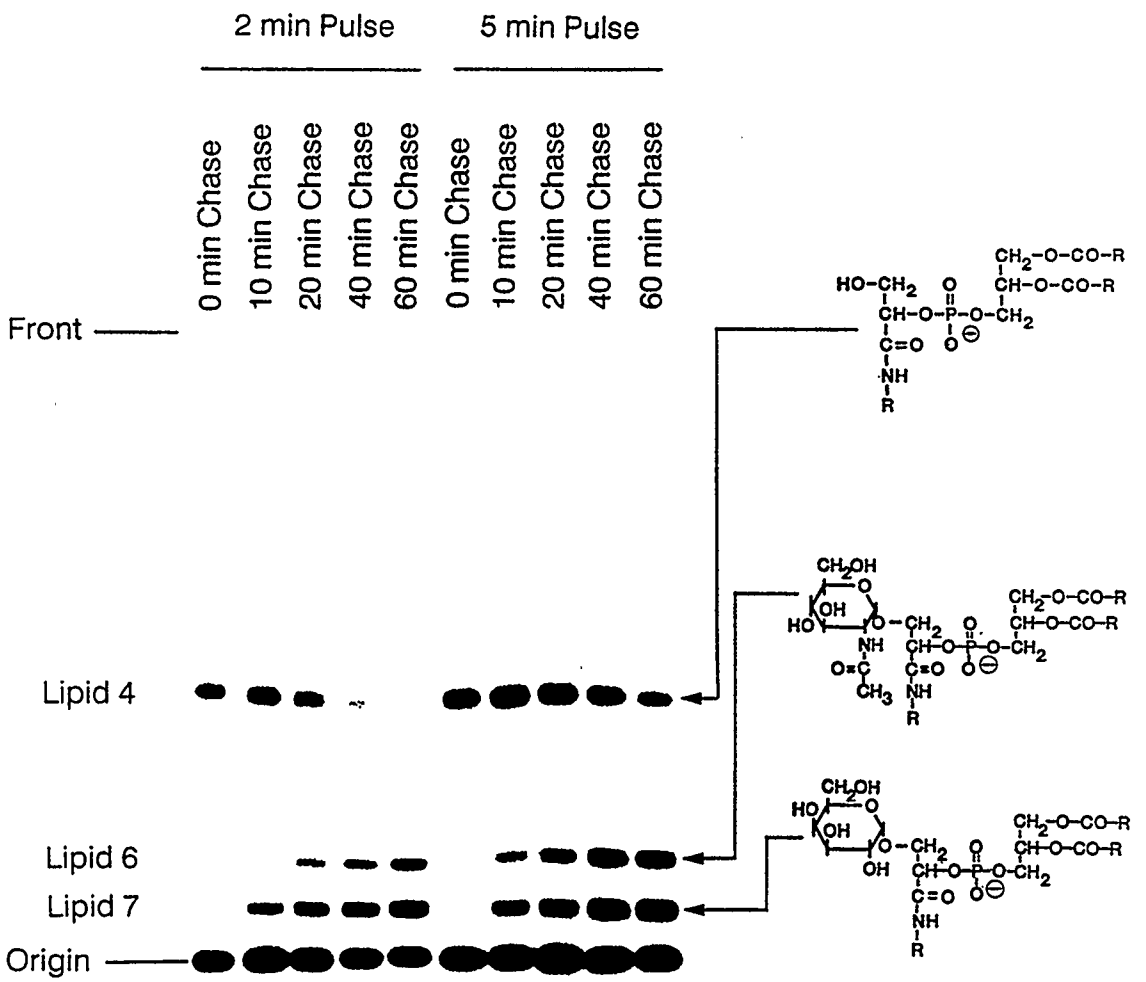

Pulse
Chase
^{32}P-Labelled
Charred
4
6
7
PA
0
3
5

Figure 15. **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, v/v/v). Radiolabeled lipids were detected by autoradiography.


2 min Pulse
5 min Pulse
0 min Chase
10 min Chase
20 min Chase
40 min Chase
60 min Chase
0 min Chase
10 min Chase
20 min Chase
40 min Chase
60 min Chase
Front
Lipid 4
Lipid 6
Lipid 7
Origin

The subsequent glycosylation of lipid 4 occurred more slowly, as assayed by the appearance of ^{32}P label in lipids 6 and 7 during the chase period. Using a two 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 five min ^{32}P pulse both lipids 6 and 7 were labeled within 10 min. It was noted however that 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 galactose (i.e., generating lipid 7) which likely accounts for the greater abundance of lipid 7 relative to lipid 6 found in *D. radiodurans* (Thompson *et al.*, 1980).

3.4 DISCUSSION

This chapter describes a further novel lipid from *D. radiodurans* and has provided some insights into the biosynthesis of the complex phosphoglycolipids in this organism. Lipid 4 (phosphatidylglyceroylalkylamine) is synthesized rapidly (within 2 min using radiolabeled phosphate as 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 (2-5 min), [^{32}P]phosphate is incorporated into only two phospholipids, lipid 4 and phosphatidic acid (Fig. 15). Radiolabeling of these two lipids was also prominent using a l0 min pulse of [1-^{14}C] acetate (cf., Fig. 11). The rapid incorporation of either of these radiolabeled lipid precursors into phosphatidic acid using such pulse-labeling techniques suggests that phosphatidic acid is an important intermediate in phospholipid biosynthesis and is likely the source of the 1,2-diacyl-glycero-backbone found in lipids 4, 6 and 7. The glycerol moiety of lipids 6 and 7 have been

shown to be of the 1,2-diacyl-*sn*-glycero-3-phospho configuration (Anderson and Hansen, 1985; Huang and Anderson, 1989). 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 archaea have been found to contain glycerolipids of an *sn*-2,3 stereochemistry (reviewed in Kates, 1978; De Rosa *et al.*, 1986).

It is of interest that all polar lipids (lipids 4, 6 and 7) identified to date from *D. radiodurans* contain the unusual lipid constituents glyceric acid and alkylamine. Short pulse-labeling methods were unable to demonstrate appreciable incorporation of [^{32}P]phosphate into lipids other than lipid 4 and phosphatidic acid. Also, no radiolabeled lipid which might be phosphatidylglycerate, a potential precursor of lipid 4, was detected. It would therefore seem unlikely that phosphatidylglycerate is a precursor of lipid 4, but rather that the biosynthesis of lipid 4 proceeds via condensation of phosphatidic acid (or its dephosphorylated product, diacylglycerol) and glyceroylalkylamine (possibly involving liponucleotide derivatives thereof).

Efforts have been made to use the unusual lipid composition of *D. radiodurans* as a taxonomic means of identification (Counsell and Murray, 1986; Embley *et al.*, 1987). On the basis of lipid patterns defined by TLC, *D. radiodurans* could be readily differentiated from other bacteria including other species of *Deinococci* (Counsell and Murray, 1986; Embley *et al.*, 1987). On the other hand, the lipid patterns of nine isolates of *D. radiodurans* were very similar (Counsell and Murray, 1986), particularly with regard to the two major alkylamine-containing phosphoglycolipids, lipids 6 and 7, which have been previously identified (Anderson and Hansen, 1985; Huang and Anderson, 1989). In contrast to lipids 6, 7 and their precursor (lipid 4), preliminary analyses indicate that the remaining, uncharacterized major lipids of *D. radiodurans* do not contain alkylamines.

Thus, of the total lipids of *D. radiodurans*, lipids 4, 6 and 7 seem to constitute a distinct subclass characterized by the presence of alkylamines and high metabolic turnover.

CHAPTER 4

FATTY ACIDS ARE PRECURSORS OF ALKYLAMINES IN *D. RADIODURANS*

4.1 INTRODUCTION

The radiation-resistant bacterium *D. radiodurans* contains a complex cell envelope 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 (Anderson, 1983; Anderson and Hansen, 1985; Huang and Anderson, 1989; Huang and Anderson, 1991). Three of these lipids contain both fatty acids and alkylamines, the latter of which have not to date been observed outside the 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 (Huang and Anderson, 1991). 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 (Counsell and Murray, 1986; Embley *et al.*, 1987; Masters *et al.*, 1991c). Although complete chemical analysis has not been carried out on all strains, comparative thin-layer chromatographic analyses of a large number of isolates of *D. radiodurans* indicate the presence of two major phosphoglycolipids (Counsell and Murray, 1986; Embley *et al.*, 1987) with characteristics similar to the glycosylated phosphatidylglyceroylalkylamines previously characterized in *D. radiodurans* strain Sark (Anderson and Hansen, 1985; Huang and Anderson, 1989). 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 diverse environments worldwide (Anderson *et al.*, 1956; Christensen and Kristensen, 1981; Ito, 1977; Ito *et al.*, 1983; Kristensen and Christensen, 1981). Due to their relatively recent identification and chemical characterization, alkylamines remain a mystery as to their mode of biosynthesis. The

presence of long chain alkyl groups suggests a route of biogenesis perhaps shared by fatty acids. This chapter presents studies designed to probe this possibility.

4.2 MATERIALS AND METHODS

4.2.1 Cell Culture and Lipid Isolation

Cultures of *D. radiodurans* were grown and total lipids were extracted as described in Section 2.2.1.

4.2.2 Analytical Methods

The methods for preparation and analysis of fatty acids and alkylamines described in detail in Section 3.2.7 and 3.2.8 are briefly summarized here. Purified lipids were subjected to 5% methanolic HCl hydrolysis. Fatty acid methyl esters and acetylated alkylamines were analyzed by gas chromatography (Section 3.2.8). Percentage compositions were determined using a Hewlett-Packard 3392A Integrator. Fatty acid pyrrolidides were obtained from fatty acid methyl esters by the procedure of Andersson and Holman (Andersson and Holman, 1974) as described in Section 2.2.1 and analyzed by GC/MS using a Kratos 80 Mass Spectrometer (Section 3.2.8).

4.2.3 Radiolabeling of *D. radiodurans*

Radiolabeled palmitic acids were all purchased from New England Nuclear, and were added as ethanol solutions to growth media consisting of 0.5% tryptone, 0.3% yeast extract, 3mM $CaCl_2$. A 10-ml culture of *D. radiodurans* was incubated with [1-^{14}C] palmitic acid (1 mCi/ml) at 35°C. Aliquots of 1 ml were removed after 5, 10, 15, 20, 30 and 40 min, chilled on ice and cells harvested by centrifugation for 10 min at 13,000xg in a microfuge at 4°C. For dual labeling, a 50 ml culture of *D. radiodurans* (OD_{600}=0.6)

was incubated with a mixture of [1-^{14}C]palmitic acid (0.2 mCi/ml) and [9,10-^{3}H]palmitic acid (0.1 mCi/ml) for 15 min at 35°C. The culture was harvested by centrifugation (10 min x 4000xg at 4°C). Lipids were extracted from packed cell pellets and examined by TLC as described in Section 2.2.1.

4.2.4 Thin Layer Chromatography (TLC)

Analytical TLC was performed using Sil Gel G Redi-plates (Fisher Scientific). Total lipid extracts from *D. radiodurans* were resolved in chloroform/methanol/28% ammonia (65:35:5, v/v/v). 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, v/v/v); two thirds development in chloroform/methanol (15:1 v/v); and full development in chloroform/ether (85:15, v/v). In some cases, TLC plates were auto radiographed by exposure to X-ray film (Kodak).

4.3 RESULTS

4.3.1 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. 10. Lipid 6 is 2'-0-(1,2-diacyl-*sn*-glycero-3-phospho)-3'-0-(α-N-acetylglucosaminyl)-N-glyceroylalkylamine (Huang and Anderson, 1989); lipid 7 is 2'-0-(1,2-diacyl-*sn*-glycero-3-phospho)-3'-(α-galactosyl)-N-glyceroylalkylamine (Anderson and Hansen, 1985) and lipid 4 is phosphatidylglyceroalkylamine (Huang and Anderson, 1991). While lipid 4 is a relatively low-abundance constituent, lipids 6 and 7 are major components of *D. radiodurans*

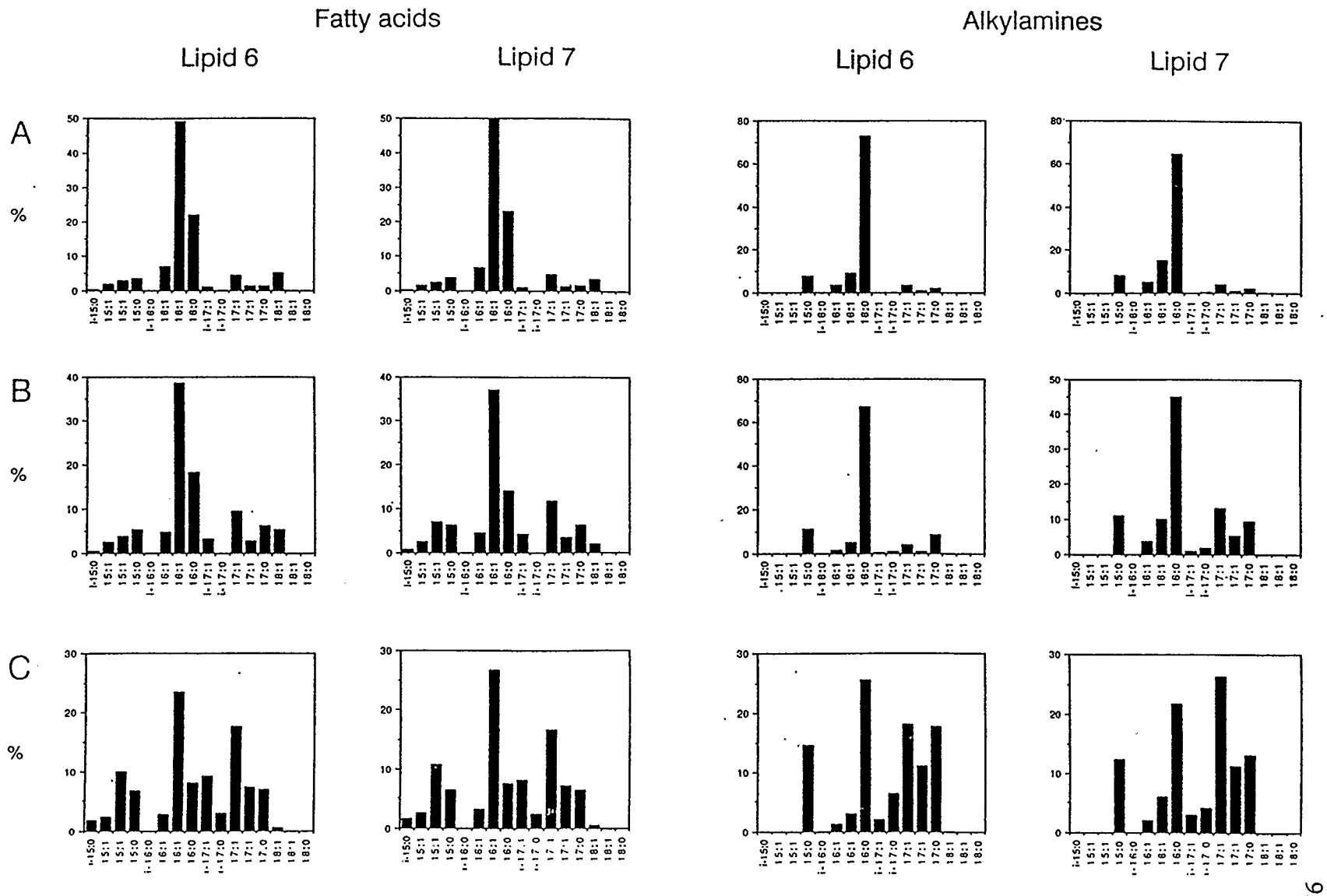
(Thompson *et al.*, 1980). Samples of lipid 6 and 7 were isolated by preparative TLC from cultures grown to mid-exponential, early stationary or late stationary phases. The results shown in Fig. 16 indicate several interesting features, described below.

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/alkylamine composition was the marked dependence on the bacterial growth phase. Mid-exponential 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 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.

Figure 16. **Fatty acid and alkylamine compositions of the major phospholipids, lipids 6 and 7, isolated from cultures of *D. radiodurans* harvested at mid-exponential (22 h) (A), early stationary (36 h) (B), and late stationary (70 h) (C) growth 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 Methods. Monounsaturated alkyl chains constitute at least two isomeric populations with respect to the location of the double bond (v), 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*.


Fatty acids
Alkylamines
Lipid 6
Lipid 7
Lipid 6
Lipid 7
A
B
C
%
%
%

4.3.2 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. 17, 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 phosphatidylglyceroalkylamine (Huang and Anderson, 1991). Lipid 4 is the precursor to the complex phosphoglycolipids 6 and 7 (Huang and Anderson, 1991), see section 3.3.4. 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. 17). [The structures of lipids 3 and 5 have not yet been characterized.]

In order 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. 18, 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. The finding of radiolabel in alkylamine as early as 5 min, suggested that palmitic acid was the major precursor for alkylamine biosynthesis.

Figure 17. **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 TLC in chloroform/ methanol/28% ammonia (80:20;2, v/v/v) and autoradiographed. Lipid designations, numbers in the right hand margin, are those described by Thompson *et al.* (1980). FA, fatty acid; Std, standard, a lipid extract from [1-^{14}C]acetate-labeled *D. radiodurans.*

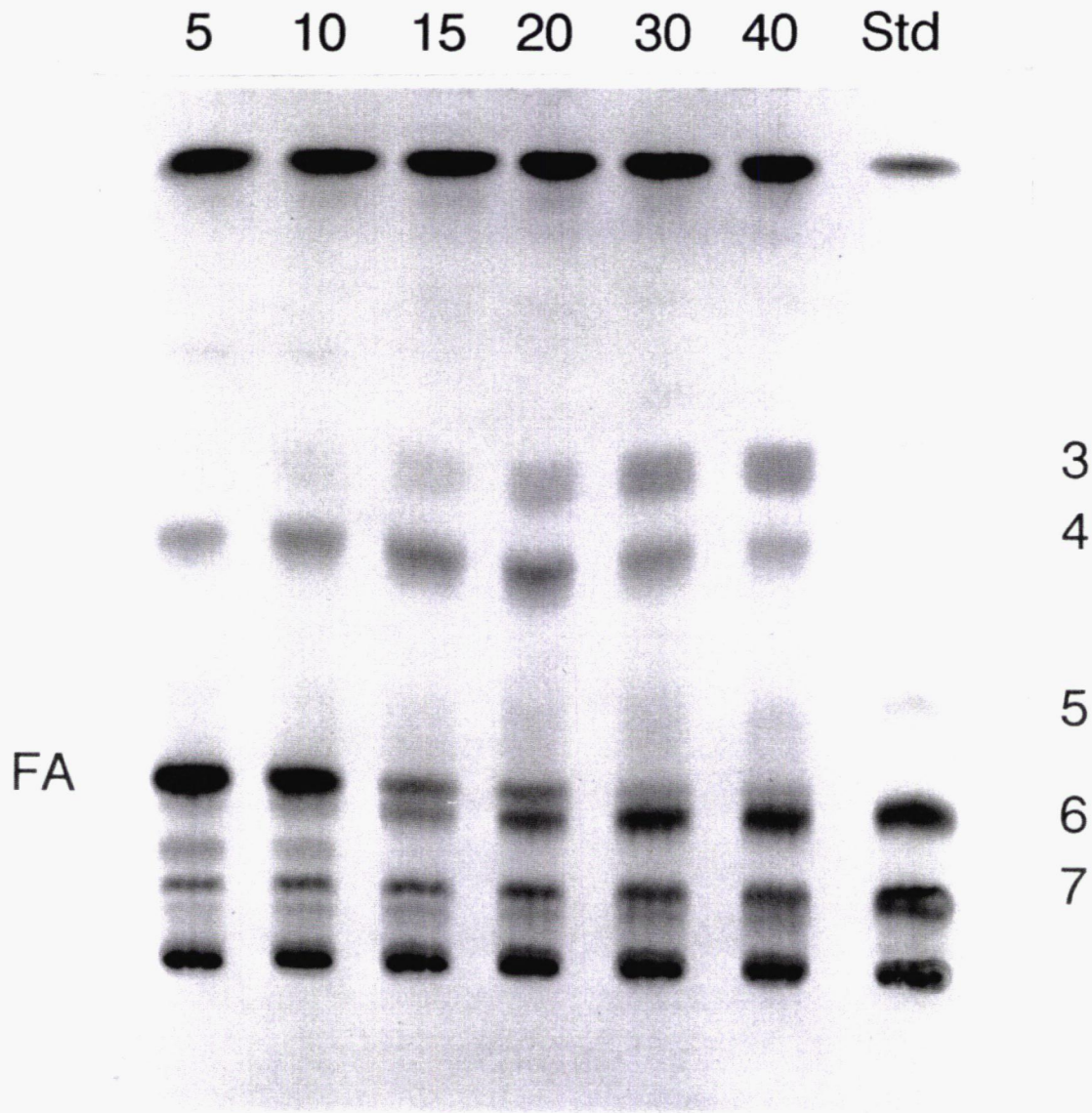
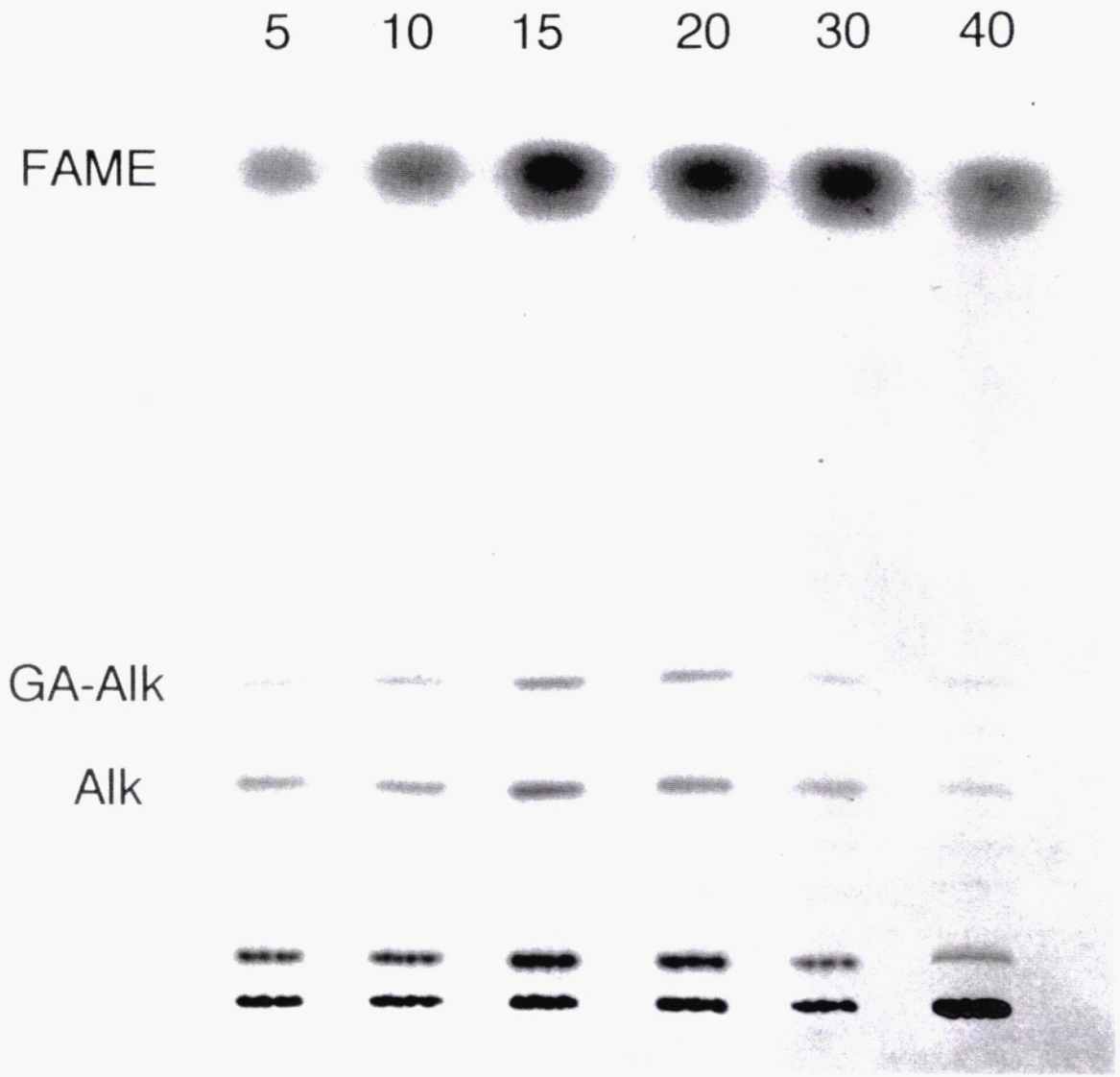

5
10
15
20
30
40
Std
3
4
5
6
7
FA

Figure 18. 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 samples shown in Fig. 17, 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/ammonia (80:20:2, v/v); two-thirds development in chloroform/methanol (15:1, v/v); full development in chloroform/ ether (85:15, v/v). 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.


5
10
15
20
30
40
FAME
GA-Alk
Alk

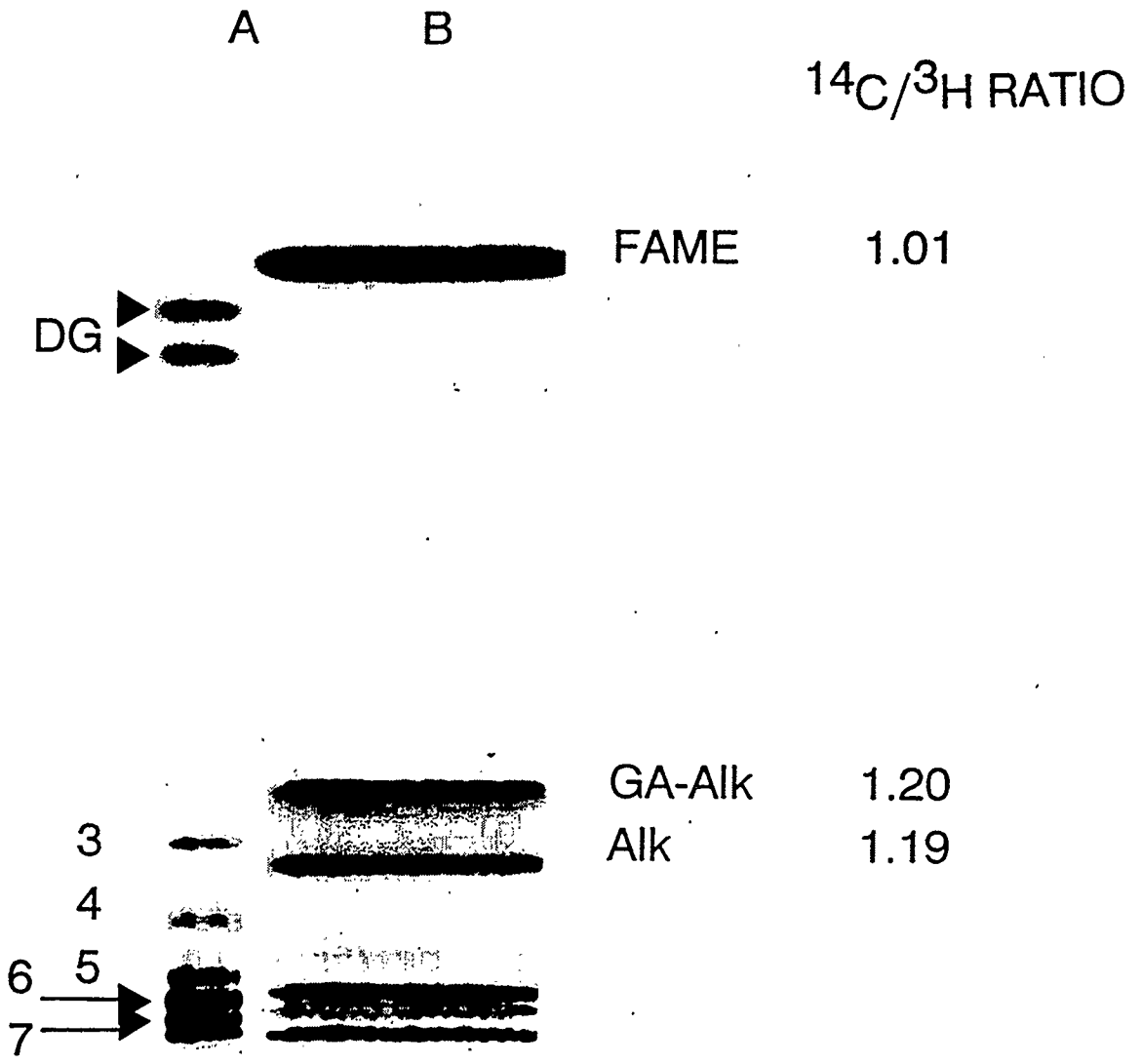
4.3.3 Radiolabeling of Alkylamine with Dual-labeled Palmitic Acid

In order 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 preparatively isolated by scraping and elution of the gel with chloroform/methanol (1:1, v/v). The eluted lipid 4 was subjected to acid methanolysis and TLC analysis of the resultant products which were detected by autoradiography. The ratios of ^{14}C/^{3}H in fatty acid methyl ester, alkylamine and glyceroylalkylamine were determined by scintillation counting of the excised and eluted products from the TLC plate. The results shown in Fig. 19 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 OOH of the carboxy group) into alkylamine. Recycling of radiolabel into alkylamine via products of fatty acid catabolism is therefore unlikely.

4.4 DISCUSSION

The results obtained in these studies present evidence that the alkylamine moieties present in lipids from *D. radiodurans* are derived biosynthetically from fatty acids. The evidence for this is three-fold. First, although quantitative differences are evident, the alkyl chain compositions of both fatty acids and alkylamines are qualitatively similar and show parallel changes in response to bacterial growth phase. Second, radiolabel from [1-^{14}C]palmitate is incorporated very rapidly (5 min) into alkylamines. Finally, the ratio of ^{3}H/^{14}C was found to be similar in both fatty acids and alkylamines isolated from phosphatidylglyceroalkylamine (lipid 4) from *D. radiodurans* cultured in the presence of

Figure 19. **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 [1-^{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 methylester (FAME), alkylamines (Alk), and glyceroylalkylamine (GA-Alk) components. Numerical lipids in the left hand margin are described by Thompson *et al.* (1980).


A
B
$^{14}C/^{3}H$ RATIO
FAME
1.01
DG
GA-Alk
1.20
Alk
1.19
3
4
6
5
7

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 acid to alkylamine rather than a pathway involving fatty acid degradation and recycling of radiolabel into alkylamine precursors. Fatty acid degradation followed by *de novo* alkylamine 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 the 1 and 2 carbons (and containing the ^{14}C label from [1-^{14}C]palmitate). Moreover, since each step of oxidative degradation involves extensive dehydrogenation (Stumpf, 1969), there would be considerable depletion of ^{3}H from [9,10-^{3}H] palmitate and consequently any derived acetates. The possibility of a partial degradation of fatty acid followed by re-elongation to alkylamines cannot be excluded but is unlikely given 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 comprise various isomers, including straight- and branched-chain, saturated and monounsaturated species (Embley *et al.*, 1987). The results presented here extend this isomeric diversity to the alkylamines of *D. radiodurans* strain Sark, and indicate that alkylamine composition is determined largely 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 exponential to stationary phase, demonstrated in *Escherichia coli* (Cronan, 1968; Knivett and Cullen, 1967; Law *et al.*, 1963; Marr and Ingraham, 1962) and in several other bacteria (e.g., Crowfoot and Hunt, 1970; Jungkind and Wood, 1974; Kates *et al.*, 1964; Law *et al.*, 1963). A variety of factors including oxygen tension, 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-adenosyl-methionine (reviewed in Fulco, 1983). As a consequence, levels of monoenoic fatty acids are concomitantly reduced. As indicated in the present study, the growth-dependent changes observed in fatty acid of 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-number fatty acids in *E. coli* (Ingram *et al.*, 1977)]. 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-CoA, i.e., propionyl-CoA or isobutyryl-CoA, which would promote the synthesis of odd-number, 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.

The changes in bacterial transition from exponential to stationary phase growth are complex (reviewed in Kolter *et al.*, 1993). Entry into or exit from stationary phase is

accompanied by changes in expression of multiple genes. To my knowledge, the effects of such changes on fatty acid metabolism have not been investigated. The changes in fatty acid (and alkylamine) composition observed in *D. radiodurans* and reported for other bacteria would, by themselves, be expected to increase membrane fluidity. It remains uncertain as to whether this is a desirable adaptation to stationary existence or is merely a consequence of altered gene expression.

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 acid and alkylamines, the alkylamines retain a relatively greater proportion of saturated versus unsaturated alkyl chains. Subject to this bias, it is reasonable to suggest that at any given phase of bacterial growth, alkylamines are largely derived in a fairly nonselective manner from fatty acid substrates according to 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 predominantly found in eukaryotes including animals, plants and fungi. Recent research has begun to establish some functions for certain sphingolipids, particularly in cell regulatory processes (Ghosh *et al.*, 1990; Hakomori, 1990; Hannun and Bell, 1989). Studies with *Saccharomyces cerevisiae* have indicated a vital requirement for sphingolipids (Pinto *et al.*, 1992). Other sphingolipid/alkylamine analogs include 2-amino-3-hydroxyalkyl-1-sulfonates found in diatoms (Anderson *et al.*, 1975; 1978) and in the gliding bacteria, *Cytophaga* and *Flexibacter* (Godchaux III and Leadbetter, 1984), in which latter organisms these lipids may have a specialized role in motility (Abbanat *et al.*,

1986). Whether the alkylamine-containing lipids of *D. radiodurans* will prove to have as exciting functions 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 (Stoffel et al., 1968). Both the nitrogen and carbons 1 and 2 of sphingosine are derived from serine. In contrast, alkylamine biosynthesis in *D. radiodurans* is likely to proceed via a transamination-like reaction in which only the amino group is transferred to fatty acid (or an activated derivative, such as fatty acyl-CoA or -carrier protein) or the corresponding aldehyde (produced by reduction of the CoA ester of the fatty acid). The identity of the putative amino-group donor is unknown.

CHAPTER 5

STRUCTURAL CHARACTERIZATION OF LIPIDS 3 AND 5 OF *D. RADIODURANS*

5.1 INTRODUCTION

Previous analyses of lipid structure in *D. radiodurans* have concentrated on a class of three alkylamine-containing phospholipids, i.e., phosphatidylglyceroylalkylamine, and its glycosylated derivatives. This chapter focuses 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 complex group of structures found in virtually all types of bacteria. Among the most common type of bacterial glycolipid are the diglycosyl diglycerides which are found in widespread distribution among both Gram-positive and Gram-negative organisms (Lechevalier, 1977). Mono- and triglycosyldiglycerides are occasionally found but in much lower quantities (Shaw, 1975). Diglycosyldiglycerides may also occur in conjugated form such as in the phosphoglycolipid, phosphatidyldiglycosyldiacylglycerol (Shaw, 1975). The broad occurrence of glycosyldiglycerides among bacterial taxa suggest that these lipids play an important, perhaps essential, role in cell physiology. The possible finding of similar structures in the unusual bacterium, *D. radiodurans*, might serve to strengthen this conviction.

Preliminary examination by 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 which contained a single carbohydrate component and therefore posed the greatest prospects for structure elucidation. This chapter describes the complete structures of these two lipids and confirm that glycosyl diglycerides are indeed represented in this taxonomically puzzling bacterium.

5.2 MATERIALS AND METHODS

5.2.1 Cell Culture and Lipid Isolation

Cultures of *D. radiodurans* were grown and lipids were extracted as described in Section 2.2.1.

5.2.2 Purification of Lipids 3 and 5

Lipid 3 and lipid 5 were isolated from lipid extracts by preparative TLC on homemade silica gel H plates (gel thicknes, 1mm) and developed in chloroform/methanol/28% ammonia (80:20:2; v/v/v). Lipids were stained with iodine. The lipid 3 (R_f = 0.52) and 5 bands (R_f = 0.23) were scraped out and eluted with chloroform/methanol (1:1, v/v).

5.2.3 Chemical Analysis

Mild alkaline deacylation of lipids was performed according to the procedure of Kates (1986) as described in Section 2.2.1. Lipid acetylation was carried out in pyridine-acetic anhydride (1:1, v/v) overnight at RT. Hydrolysis of phosphodiester bonds was performed using Hydrofluoric acid (HF) (Fischer *et al.*, 1973). The lipid was subjected to HF hydrolysis overnight at 4°C in a polypropylene tube as described in Section 2.2.1 and examined by TLC. Fatty acids were liberated as fatty acid methyl esters using 5% methanolic HCl at 102°C for 2 h.

5.2.4 Gas Chromatography

A Hewlett-Packard 5890 gas chromatography, equipped with a 25-m 5% phenylmethyl silicone capillary column, was used. The fatty acid methyl esters were analyzed by using a temperature program of 180° to 290°C, at a rate of 5°C/minute.

5.2.5 NMR Spectroscopy

Proton nuclear magnetic resonance (NMR) was performed on a Bruker AM-400 spectrometer. Two dimensional correlated spectroscopy (COSY) spectra (Sanders and Hunter, 1988) were measured using Brucker instructment company program. Chemical shifts (δ) are expressed as ppm relative to tetramethylsilane and coupling constants (J) are given in Hz. ^{13}C-NMR spectra were recorded on a Bruker AM-400 spectrometer

5.3 RESULTS

5.3.1 Structural Elucidation of Lipids 3 and 5

Previous studies have indicated that lipid 3 is a glycolipid and lipid 5 is a phosphoglycolipid (Thompson *et al.*, 1980). In order to establish the chemical structure of both lipids, a series of chemical and spectroscopic analyses were undertaken.

5.3.1.1 Chemical Analysis of Lipids 3 and 5

Hydrofluoric acid (HF) is known to cleave phosphodiester bonds (Fischer *et al.*, 1973) and hydrolysis of lipid 5 with HF yielded two products. One of these was a diglyceride as shown by TLC, and the other had the same R_f value as lipid 3. Both this latter product as well as lipid 3 comigrated with authentic galactosyl-diglyceride (Sigma Chem. Co.) on thin layer chromatography (silica gel G) in the solvent system chloroform/methanol/28% ammonia (80:20:2, v/v/v). Under these chromatographic

conditions, monoglycosyldiglycerides (e.g., containing either glucose, galactose or other hexoses) migrate at the same R_f =0.29. Acid methanolysis of lipid 3 yielded fatty acid methyl esters and 1-0-methyl-glucoside. 1-0-methyl-glucoside (R_f=0.52) was identified by thin layer chromatography on silica gel G plates in butanol/ethanol/water (5:2:4, v/v/v), and also by gas chromatographic identification of the acetylated product.

The above results suggested that lipid 3 is monoglucosyldiglyceride and that lipid 5 consists of monoglucosyldiglyceride linked through a phosphodiester bond to diglyceride.

5.3.1.2 ^{1}H-NMR Spectroscopy of Lipids 3 and 5

Analysis using ^{1}H-NMR of intact lipids 3 and 5 identified the number of sugars and 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 (H^1) between δ 4.0-5.1 ppm, the window for the anomeric proton resonance. This confirmed that both lipids were monosaccharides. Also, the coupling constant 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 (Minniken, 1972; Falk *et al.*, 1979). Both lipids also contained glyceride units (unsaturated fatty acids esterifying glycerol) as was revealed by comparing the proton signals of CH_3, $C\underline{H}_2CO$, $C\underline{H}_2CH2CO$, $-C\underline{H}_2-CH=CH-C\underline{H}_2$, and $-C\underline{H}=C\underline{H}-$ with ^{1}H-NMR spectra of intact lipids 4, 6 and 7 (Anderson and Hansen, 1985; Huang and Anderson, 1989, 1991). The difference is that there are two methine proton (CH) resonances corresponding to H^2 of glycerol moieties at δ 5.18 ppm in lipid 5, and only one such methine proton resonance at δ 5.20 ppm in lipid 3. These data indicated that there were two glycerol units in lipid 5 and one in lipid 3. The rest of the non-anomeric methine sugar protons and the methylene protons (CH_2) of glycerol were overlapped within a relatively small window (i.e., 1 ppm) and it was impossible to make assignments for them.

5.3.1.3 ^{1}H-NMR of Peracetylated Lipids 3 and 5

In order to assign the methine signals of the sugar units, to locate the positions of the substitutions in the sugar moieties, and the methylene proton of glycerol in lipids 3 and 5, lipids 3 and 5 were peracetylated. The ^{1}H-NMR of peracetylated deacylated lipid 3 and peracetylated lipid 5 were well resolved (Tables 4 and 5). The deshielding effect of the acetoxy group on the sugar proton shifts the proton linked to the carbon bearing the acetoxy group downfield. This deshielding results in a downfield shift of ca. 0.5 ppm for methylene protons and ca. 1.1 ppm for methine protons for pyranose sugar (cf., Jackman and Sternhell, 1969). It was, therefore, possible to distinguish sites which carried free hydroxyl groups from substituted ones. In peracetylated deacylated lipid 3, the downfield shift of the pyranose methine proton signals of H^2, H^3 and H^4 from δ 3.2-3.7 ppm region to δ 4.86, δ 5.46 and δ 5.06 ppm, repsectively, confirmed the presence of hydroxyl groups at these positions. In addition, the methylene proton signals of H^6 in the intact unmodified lipid 3 in the δ 3.2-3.7 ppm region were shifted downfield to δ 4.2 ppm upon acetylation. Therefore, lipid 3 has four hydroxyl groups on the pyranose ring and is a monosubstituted sugar. In accordance with this assignment of the four free hydroxyl groups to the pyranose ring, six 0-acetyl methyl resonances were observed around δ 2.0 ppm (δ = 2.024, 2.045, 2.075, 2.085, 2.105 and 2.109 ppm; Fig. 20, top panel). This is consistent with four acetylable hydroxy groups on the glucopyranosyl C^2, C^3, C^4, and C^6, and another two acetylatable hydroxy groups on C^1, C^2 of glycerol. Individual assignments were not made because of the observed small differences in chemical shift (compare with Horton and Lauterback, 1969). The data are consistent with lipid 3 being an α-glucopyranosyl diglyceride.

TABLE 4. ^{1}H-NMR assignment for peracetylated deacylated lipid 3. [a]

Proton Assignment	Chemical Shift (ppm)	No. of Protons	Multiplicity[b]	Coupling Constant (Hz)
Glucose-pyranoside				
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	$J_{6A,6B} = 12.3$ $J_{6A,5} = 4.6$
H^{6B}	4.12	1	dd	$J_{6A,6B} = 12.3$ $J_{6B,5} = 2.3$
Glycerol				
1-CH^AH^BOCOR	4.34	1	dd	$J_{A,B} = 11.9$ $J_{A,2} = 4.3$
	4.18	1	dd	$J_{A,B} = 11.9$ $J_{B,2} = 5.8$
2-CHOCOR	5.2	1	m	
3-CH^AH^BO-SUGAR	3.83	1	dd	$J_{A,B} = 11.3$ $J_{A,2} = 4.3$
	3.65	1	dd	$J_{B,A} = 11.3$ $J_{B,2} = 5.5$

[a] Deacylated, peracetylated lipid 3 was dissolved in $CDCl_3$

[b] Abbreviations: s, singlet; d, doublet; t, triplet; m, multiplet

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

Proton Assignment	Chemical Shift (ppm)	No. of Protons	Multiplicity[b]	Coupling Constant (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	-
OAC	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	-

TABLE 5. (cont'd.)

Proton Assignment	Chemical Shift (ppm)	No. of Protons	Multi-plicity[b]	Coupling Constant (Hz)
Glycerol 2				
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$\underline{H}^A\underline{H}^B$-O-SUGAR	3.8	1	dd	$J_{A,B} = 11.6$ $J_{3A,2} = 4.7$
	3.58	1	dd	$J_{A,B} = 11.6$ $J_{3B,2} = 5.5$
Fatty Acid				
-C$\underline{H}$=C$\underline{H}$-	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$\underline{H}_2$C$\underline{H}_2$CO-	1.57	8	m	-
-C$\underline{H}_3$	0.85	12	m	-

[a] Peracetylated lipid 5 was dissolved in $CDCl_3/CD_3OD$ (3:1, v/v)

[b] Abbreviations: s, singlet; d, doublet; t, triplet; m, multiplet

[c] Not determined

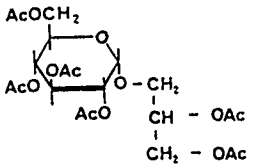
[d] Roughly consistent with >60% monounstaturation in fatty acyl chains (see Table 7)

The ^{1}H-NMR spectrum of the peracetylated deacylated lipid 5 showed that the methine proton resonances attributed to the H^2, H^3, H^4 of the sugar had shifted from the δ 3.2-4.2 ppm region to δ 4.78, 5.39 and 5.08 ppm, respectively. In addition, there were only three signals corresponding to 0-acetyl methyl groups at δ 1.967, 2.019 and 2.024 ppm (see Fig. 20, bottom panel), none of which were seen in the ^{1}H-NMR spectrum of the intact unmodified lipid 5. This suggested that there were only three free hydroxyl groups in lipid 5 and these could be assigned to positions 2, 3 and 4 of the sugar moiety. This, in turn, indicated that lipid 5 was 1, 6 disubstituted. In order to verify the numbers of fatty acid chains in the lipid 5, the ^{1}H-NMR of the peracetylated deacylated lipid 5 was examined. As expected, the spectra of peracetylated lipid 5 and peracetylated deacylated lipid 5 are similar except for the absence of the fatty acid signals in the latter. The appearance of seven singlet 0-acetyl methyl signals in the δ2.0 region (δ 1.95, 2.01, 2.02, 2.03, 2.04, 2.05 and 2.06 ppm; Fig. 20, center panel) of the spectrum of the peracetylated deacylated lipid 5 confirmed that the 1,6-disubstituted lipid 5 has 2 glycerol units, three of these signals being attached to the pyranoside and the other four to two glycerol units.

5.3.1.4 Two-dimensional Chemical Shift Correlated Spectroscopy (COSY)

Two dimensional COSY spectra allow determination of the inter-connection of protons within a molecule. Figures 21 and 22 present contour plots of the partial COSY spectra (δ 3.5 - 6.0 ppm) of the peracetylated deacylated lipid 3 and the peracetylated lipid 5, respectively. The normal one-dimensional ^{1}H-NMR spectrum is located along the diagonal and the chemical shift(s) of the correlated signal(s) can be found at the intersection of the cross-peaks (Sanders and Hunter, 1988). For example, starting at the

Figure 20. **^{1}H-NMR of acetate regions.** Peracetylated, deacylated lipid 3 (top), peracetylated, deacylated lipid 5 (center), and acetylated lipid 5 (bottom).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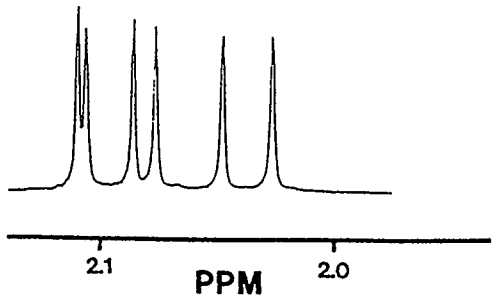
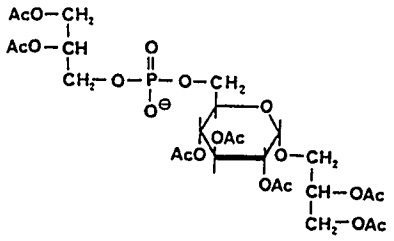
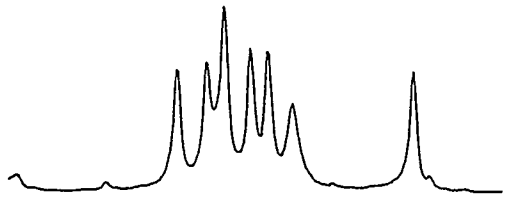
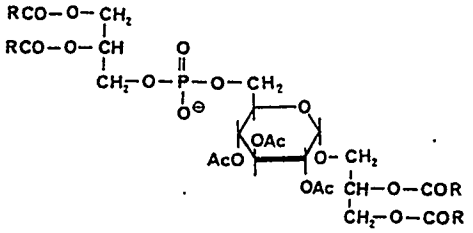
OAc
AcO
O - CH2
AcO
CH - OAc
CH2 - OAc


2.1
PPM
2.0


AcO-CH2
AcO-CH
CH2-O-P-O-CH2
O⊖
O
OAc
AcO
O-CH2
OAc
CH-OAc
CH2-OAc




RCO-O-CH2
RCO-O-CH
CH2-O-P-O-CH2
O⊖
O
OAc
AcO
O-CH2
OAc
CH-O-COR
CH2-O-COR

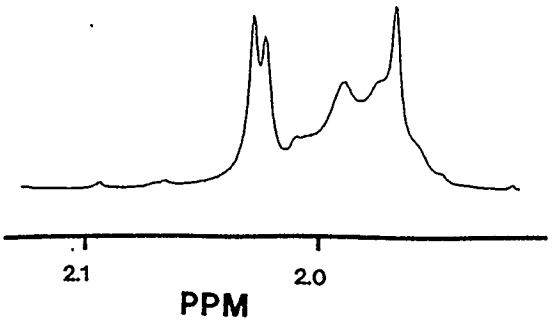

2.1
PPM
2.0

Figure 21. **Partial $^{1}H/^{1}H$-COSY spectrum of peracetylated, deacylated lipid 3 (in $CDCl_3$).**

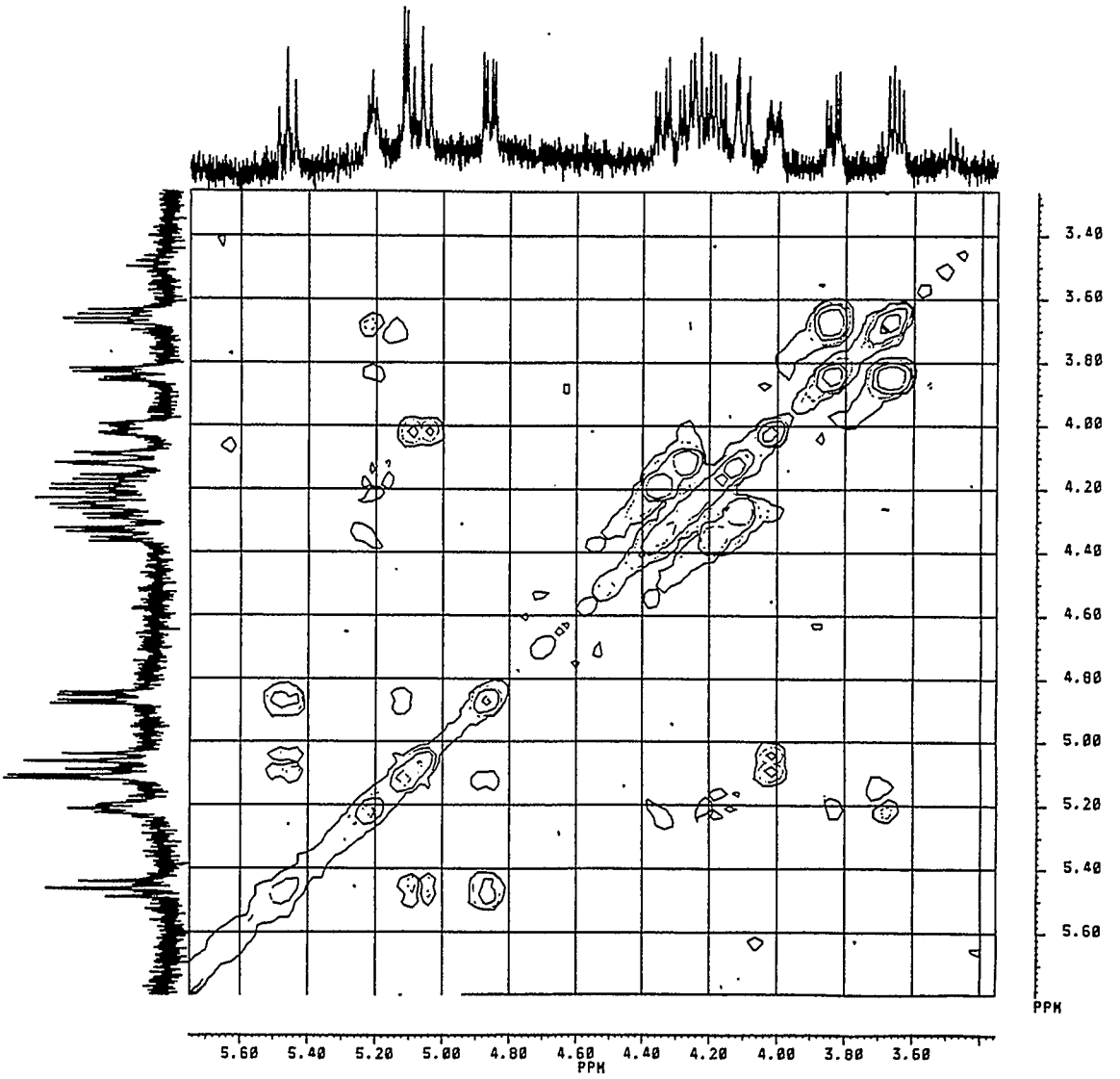
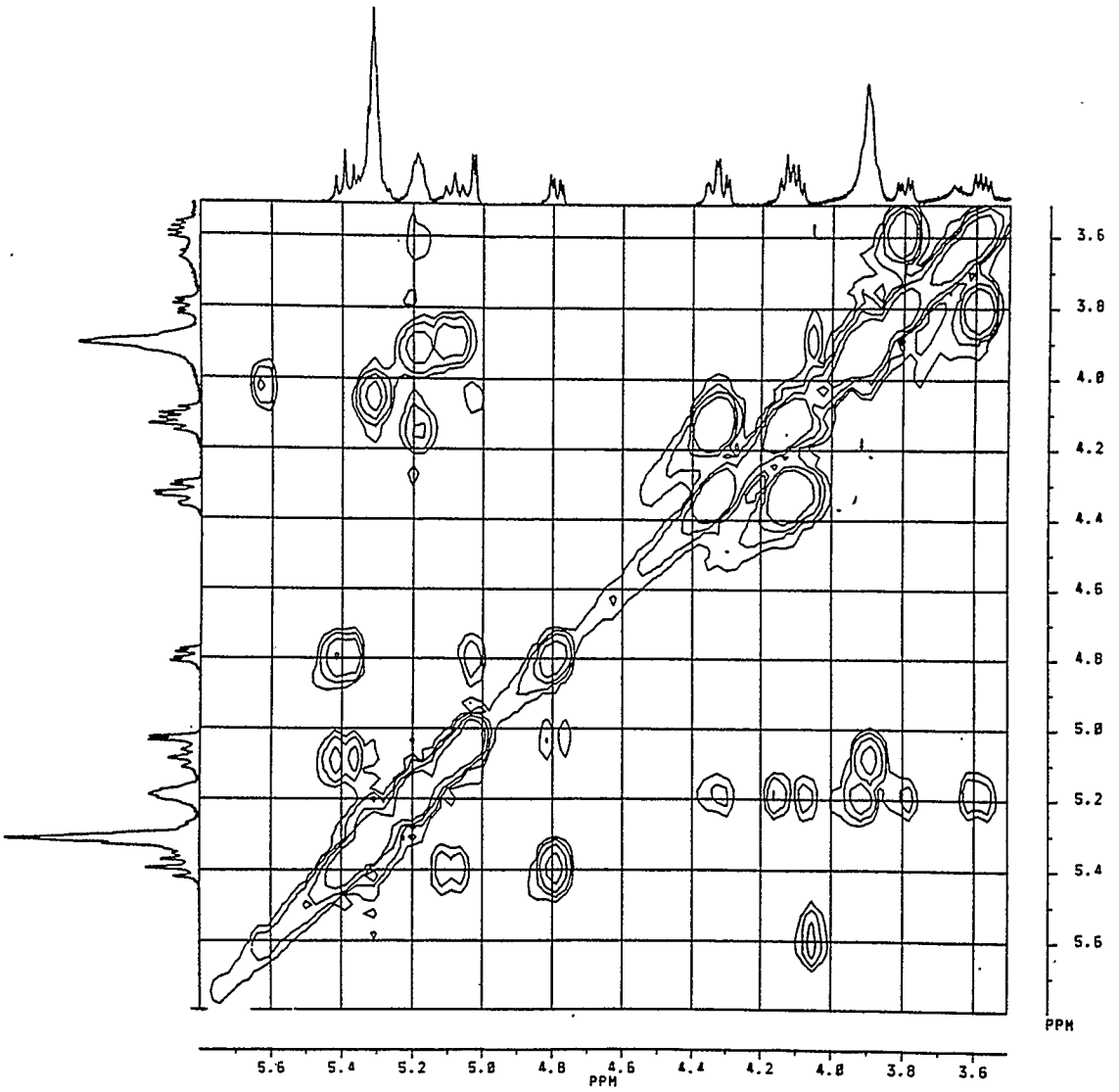

3.40
3.60
3.80
4.00
4.20
4.40
4.60
4.80
5.00
5.20
5.40
5.60
PPM
5.60 5.40 5.20 5.00 4.80 4.60 4.40 4.20 4.00 3.80 3.60
PPM

Figure 22. **Partial $^{1}H/^{1}H$-COSY spectrum of peracetylated lipid 5 (in $CDCl_3/CD_3OD$, 3:1, v/v).**


3.6
3.8
4.0
4.2
4.4
4.6
4.8
5.0
5.2
5.4
5.6
PPM
5.6 5.4 5.2 5.0 4.8 4.6 4.4 4.2 4.0 3.8 3.6
PPM

H^1 sugar proton at δ 5.11 ppm of the peracetylated deacylated spectrum of lipid 3, a cross-peak shows a connectivity to the H^2 proton at δ 4.86 ppm. Then, starting at the H^2 proton at δ 4.86 ppm, a cross-peak shows a connectivity to the H^3 proton at δ 5.46 ppm. In a similar manner, the chemical shifts of all the protons of peracetylated deacylated lipid 3 and peracetylated lipid 5 were assigned as shown in Tables 4 and 5. The chemical shifts of the diastereotopic H^6 protons of peracetylated deacylated lipid 3 were further confirmed by spin-decoupling experiments. The proton signals of the four methylene products (CH^AH^B) of the two glycerol units of peracetylated lipid 5 were assigned by comparison of their chemical shifts with the model compounds dioleoylphosphatidylcholine (DOPC), and lipids 3, 4, 6 and 7 of *D. radiodurans*.

5.3.1.5 ^{13}C-NMR Spectroscopy of Lipids 3 and 5

To further confirm the chemical structure of lipids 3 and 5, ^{13}C-NMR analyses of lipid 3, and the deacylated 5 were performed. Their ^{13}C-NMR data are shown in Table 6. The ^{13}C-NMR data permitted the assignment of all the carbon centers in the structure except for the CH_2 centers on the alkyl chain. The DEPT NMR spectra of lipid 3 and lipid 5 were also obtained to verify the CH_3, CH_2 and CH carbons. The ^{13}C-NMR data were useful in the final confirmation of the core structures (the sugar, glycerol and glycerol-3-phosphoryl units) of lipids 3 and 5. The numbers of carbon centers in the core structures of lipids 3 and 5 were as expected. The downfield shift of anomeric carbon from δ 92.90 to 99.07 ppm in lipid 3, and to 99.28 ppm in lipid 5, confirmed the presence of substitution on C^1 carbon of the pyranoside (Perkins *et al.*, 1977). In lipid 3, the DEPT experiment showed the presence of three methylene (CH_2) carbon signals. These three signals were assigned based on ^{13}C/^{1}H correlation NMR data. The signals at δ 62.35 ppm and δ 66.18 ppm were assigned to the C^1 ($-CH_2$-OCOR) and C^3 ($-CH_2-$) glycerol carbons, respectively. The signal at δ 61.70 ppm was assigned to the C^6 carbon

TABLE 6. ^{13}C-NMR assignments for lipid 5, deacylated lipid 5 and lipid 3.

Carbon	Lipid 5	Deacylated lipid 5	Lipid 3	β-galactosyl-diglyceride
		Chemical Shift (ppm)		
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] $J_{31P,13C}$=7.6[f]	71.79	74.77
C^6	63.70[b] $J_{31P,13C}$=4.4[f]	67.60[e] $J_{31P,13C}$=5.9[f]	61.70	61.84
Glycerol 1				
1-CH_2OCOR	62.36	64.14[d]	-	-
2-CHOCOR	69.98 $J_{31P,13C}$=8.6[f]	72.60[c] $J_{31P,13C}$=7.3[f]	-	-
3-CH_2OP-	63.35[b] $J_{31P,13C}$=4.4[f]	65.77[e] $J_{31P,13C}$=5.1[f]	-	-
Glycerol 2				
1-CH_2OCOR	62.36	63.92[d]	62.35	62.64
2-CHOCOR	68.27	71.29	69.84	69.89
3-CH_2O-SUGAR	66.12	71.02	66.18	67.90

*Methyl-α-D-glucopyranoside (in D_2O) has 100.0 (C^1), 72.5 (C^2), 73.9 (C^3), 70.4 (C^4), 72.1 (C^5) and 61.4 (C^6) ppm.

TABLE 6. (cont'd.)

Carbon	Lipid 5	Deacylated lipid 5	Lipid 3	β-galactosyl-diglyceride
		Chemical Shift (ppm)		
Fatty Acid				
-CO-	173.74	-	173.80	173.83
	173.22		173.42	171.69
-CH=CH-	129.69	-	129.92	130.14
	129.35		129.60	129.90
				127.98
				127.78
-CH_2-CO-	33.89	-	34.15	34.16
	33.76		34.00	33.99
-CH_2-CH=CH-CH_2-	26.86	-	27.06	27.08
-CH_2CH_2CO-	24.54		24.75	24.73
-CH_3	13.72	-	13.95	13.91

[a] solvent used were: $CDCl_3/CD_3OD$ (3:1, v/v) for lipid 3, lipid 5 and β-galactosyldiglyceride and CD_3OD for deacylated lipid 5.

[b,c,d,e] indicates that assignments may be reversed.

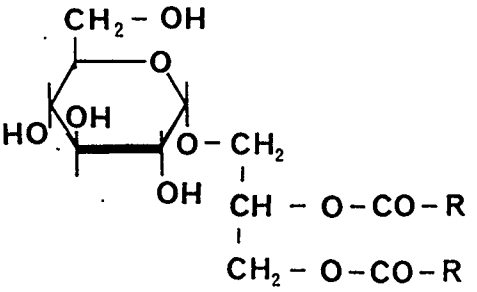
[f] coupling constants (J) are in Hz.

of the pyranoside. The ^{13}C NMR data of lipid 3 were in agreement with the reference compounds, 1–0–methyl-α-D-glucopyranoside, D_2O (Perkins *et al.*, 1977) and β-galactopyranosyl diglyceride, $CDCl_3/CD_3OD$ (3:1, v/v), (this study). Thus, lipid 3 is a 1,2-diacyl-3-α-glucopyranosyl glycerol (Fig. 23).

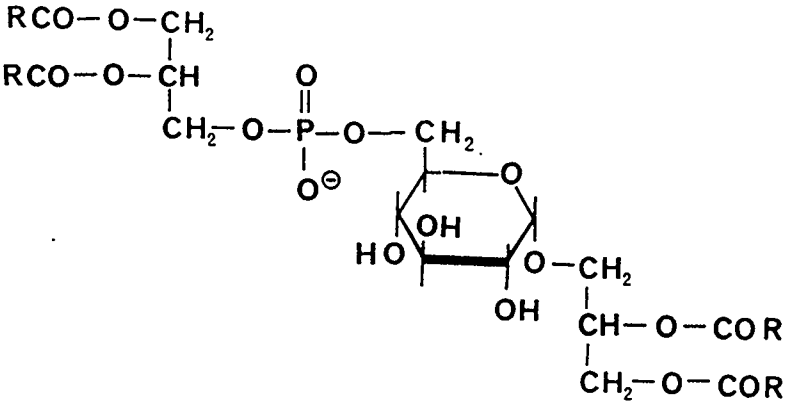
The ^{13}C-NMR assignments for lipid 5 and deacylated lipid 5 were made by comparison with ^{13}C-NMR spectrum of 1, 6-diphosphoglucopyranoside cyclohexyl ammonium salt (in CD_3OD), DOPC, (in $CDCl_3/CD_3OD$, 3:1, v/v), and lipids 3 and 6 (in $CDCl_3/CD_3OD$, 3:1, v/v). The differences in the chemical shifts between lipid 5 and deacetylated lipid 5 are due to the solvent effect ($CDCl_3/CD_3OD$, 3:1, v/v versus CD_3OD) and different chemical environment (absence of fatty acids in the latter). The C^1 carbon signals of glycerol 1 and 2 of the intact lipid are 0.22 ppm apart in the deacylated lipid 5. These two assignments may be interchangeable. Other assignments which have small chemical shift difference in both of these compounds were also assigned as interchangeable. DEPT experiments of these two compounds revealed five methylene (CH_2) carbon centres. Four methylene signals were assigned to the C^1 and C^3 carbons of the glycerol units. One methylene (CH_2) signal was assigned to the C^6 carbon of the pyranoside. The observation of the ^{31}P-^{13}C coupling confirmed the presence of the phosphodiester linkage and the location of this linkage on the C^6 carbon of the glucopyranoside (no ^{31}P-^{13}C coupling was observed on C^1 carbon of the glucopyranoside). Thus, lipid 5 is 3-0-[6'-0-(1",2"-diacyl-3"-phosphoglycerol)-α-glucopyranosyl]-1,2-diacylglycerol (Fig. 23).

Figure 23. Chemical structures of lipid 3 and lipid 5.

LIPID 3



LIPID 5



5.3.1.6 Fatty Acid Composition of Lipids 3 and 5

The fatty acid compositions of lipids 3 and 5 are shown in Table 7. Like lipids 6 and 7, both lipids contained predominantly C-15, C-16 and C-17 fatty acids, although lipid 3 contained proportionately higher amounts of C-15 and C-17 and lower levels of C-16 (Table 7). 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. Both lipids 3 and 5 did not contain alkylamines, in contrast to lipids 4, 6 and 7 which dò (detection using gas chromatography or NMR data).

5.4 DISCUSSION

In this study, two more of the polar lipids of *D. radiodurans* were described. One is α-glucopyranosyl diglyceride (lipid 3) and the other is an unusual phosphoglycolipid (lipid 5) (Fig. 23). The chemical names for these lipids are 1,2-diacyl-3-α-glucopyranosyl glycerol and 3-0-[6'-0-(1",2"-diacyl-3"-phosphoglycerol)-α-glucopyranosyl]-1,2-diacylglycerol, respectively. As explained in Section 5.3.1.4, the use of 2D-COSY NMR was useful in the determination of the structure of lipids 3 and 5. This technique allows the identification of neighbouring protons on molecules and will be valuable for determining the structure of other, more complex, glycolipids and glycophospholipids in *D. radiodurans* (such as lipids 9-13; Thompson *et al.*, 1980).

It is possible that lipid 3 is a precursor of lipid 5. The low concentration of lipid 3 in the total cell and the fact that it is a chemical constituent of lipid 5 suggest its possible role as a biosynthetic intermediate. In other microorganisms, monoglycosyl diglycerides are biosynthetic precursors of diglycosyl diglycerides, and consequently they do not accumulate in significant amounts (Shaw, 1970). Diglycosyldiglycerides are not present in significant amounts in *D. radiodurans*, as indicated by thin layer chromatography.

TABLE 7. Fatty acid Compositions of lipids 3 and 5.

Fatty acid	Lipid 3	Lipid 5
	mean % ± SD[a]	mean % ± SD[a]
*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	11.3 ± 0.6	0.7 ± 0.6
18:0	0.8 ± 0.2	1.7 ± 1.2
unknowns	1.1 ± 1.1	7.6 ± 4.6

[a]SD: Standard deviation (n=3)

Lipid 8, which migrates in the vicinity of diglycosyldiglycerides on thin layer chromatography, is a sulfur-containing phospholipid (Thompson *et al.*, 1980). Thus, if lipid 3 has a role as a biosynthetic intermediate, it probably does so with respect to the formation of lipid 5 rather than diglycosyldiglyceride. It should be noted that *D. radiodurans* contains several highly polar glyco- and phospho-glycolipids which have yet to be identified (lipids 9-13 in Thompson *et al.*, 1980). Preliminary NMR analyses indicate that these lipids contain 3 and possibly more carbohydrate moieties. Future structural characterization of these lipids should provide more insights into the complex biosynthetic pathways of these complex and diverse glyco- and phospho-glycolipids.

Studies of the biosynthesis of other types of glycosylglycerolipids have shown that the 1,2-diacyl-*sn*-glycerol is the initial lipid substrate and that this is glycosylated by a sugar substrate to form analogues of lipid 3 (Pieringer, 1968). This may also be the mechanism for formation of lipid 3 in *D. radiodurans*. Free diacyl glycerol has been identified from the neutral lipid extract of *D.radiodurans* by NMR spetroscopy (my unpublished data) and thus is a logical candidate for the biosynthesis of lipid 3.

The presence of a C^6 phosphodiester bond between diglyceride and glucopyranoside in lipid 5 is unusual. Lipid 5 makes up about 21% of the lipid components of the outer membrane and together with lipid 9, they comprise nearly 80% of the outer membrane lipids (Thompson and Murray, 1981). This indicates that lipid 5 is a major structural component of the outer membrane. Its exact function remains to be elucidated. 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. This may indicate conversion of lipid 3 to lipid 5 concomitant with or following transport from the plasma membrane to the outer membrane. This is not to belittle the possible function of lipid 3, since it may well have an important structural or functional component in both the plasma and outer membranes.

Lipids with the same structures as those of lipids 3 and 5 have been identified in other bacteria. Glycosyldiglyceride (lipid 3) is a fairly common constituent of both Gram-negative and Gram-positive bacteria (Lechevalier, 1977). Lipid 5 is less widespread, but has been detected in the Gram-negative *Pseudomonas diminuta* and *P. vesicularis* (Wilkinson and Bell, 1971; Wilkinson and Galbraith, 1979) as well as the Gram-positive *Streptococcus haemolyticus* (Fischer, 1976). In *P. diminuta*, lipid 5 is synthesized from lipid 3 via an enzyme-catalysed transphosphatidylation reaction where a phosphatidylglycerol donates a phosphatidyl group (Shaw and Pieringer, 1977). Further studies must be performed to determine whether the same biosynthetic pathway is involved in the synthesis of lipid 5 from lipid 3 in *D. radiodurans*.

The two lipid structures described in this Chapter represent lipids which may be considered to be more "conventional" than those previously characterized (lipids 4, 6 and 7; Anderson and Hansen, 1985; Huang and Anderson, 1989, 1991). These latter lipids contained the novel components, alkylamine and glyceric acid, and appeared to have rapid turnover times (as compared to lipids 3 and 5) as indicated by biosynthetic radiolabeling with [1-^{14}C]acetate (Huang and Anderson, 1991). The occurrence of glycosyl diglyceride lipids in *D. radiodurans* supports the idea that glycosyl diglyceride structures are highly conserved in bacteria and likely serve essential structural or functional roles. Interestingly, even the phylogenetically distant archaea possess glycolipids which are ether-linked analogs of glycosyl diglycerides (reviewed by Kates, 1992).

Finally, the results obtained in this Chapter should prompt a reexamination of the utility of lipids for bacterial taxonomy studies. The use of lipid analyses in bacterial taxonomy has long been recognized (e.g., Lechevalier, 1977; Shaw, 1974). The genus *Deinococcus* (Brooks *et al.*, 1980; Brooks and Murray, 1981) was established based on several criteria including G/C content of DNA, ribosomal RNA homologies, peptidoglycan chemical structure and lipid composition. The lipid composition of *D. radiodurans*

(Thompson *et al.*, 1980) and of other *Deinococci* (Counsell and Murray, 1986; Embley *et al.*, 1987) is complex and characterized by numerous "unconventional" lipids, the structures of some of which are so far unique in nature (Anderson and Hansen, 1985; Huang and Anderson, 1989, 1991). The identification of lipids 3 and 5 (as glucosyldiglyceride and its phosphatidylated derivative) are examples of more "conventional" lipid structures. The observation that lipid 5 is also found in two species of *Pseudomonas* (Wilkinson and Bell, 1971; Wilkinson and Galbraith, 1979) as well as in *Streptococcus haemolyticus* (Fischer, 1976) is quite interesting.

CHAPTER 6

CLONING OF THE *recA* GENE OF *D. RADIODURANS*

6.1 INTRODUCTION

Previous work in this thesis has concentrated on the membrane lipid structures of *D. radiodurans* and has resulted in a much more comprehensive knowledge of the unusual lipids found in this organism. In considering how these lipids may play a possible role in radiation resistance, it is clear that their structures are not likely to provide radiation-shielding or radical-scavenging functions (discussed in Chapter 7). Rather it is more likely that they may play a role in interacting with other cellular components in perhaps facilitating processes that are important to radiation resistance and/or protection.

It has been shown from other bacterial systems that specific membrane phospholipids might interact with key enzymes involved in DNA replication or repair. Enzymes such as RecA (Krishna and van de Sande, 1990) and DnaA (Sekimizu and Kornberg, 1988) in particular are able to bind to specific phospholipids, thereby modulating their activity.

In the hope of eventually demonstrating whether such roles exist for the unique phospholipids found in *D. radiodurans*, a project to identify a *recA* gene in *D. radiodurans* emerged.

The *recA* gene, first discovered in *E. coli* by Clark and Margulies (1965) has a central role in genetic recombination, DNA repair, SOS induction and mutagenesis. The multifaceted role of the RecA protein in homologous recombination and DNA-damage repair has prompted the identification and characterization of homologous *recA* genes in a wide variety of prokaryotes and eukaryotes. To date, more than 40 prokaryotic *recA* genes have been cloned (references in Miller and Kokjohn, 1990; Roca and Cox, 1990), most having been identified by the interspecies complementation method (Eitner *et al.*, 1981 and 1982). So far, most of the *recA* genes which have been cloned are from closely related enteric species. Of the few *recA* homologues that have been cloned and

completely sequenced from Gram-positive bacteria are those from *B. subtilis* (Marrero and Yasbin, 1988), *M. tuberculosis* (Davis *et al.*, 1991), *L. lactis* (Duwat *et al.*, 1992), and *A. laidlawii* (Dybvig and Woodard, 1992).

DNA is constantly being damaged and altered by environmental agents, such as X-ray and UV radiation, and by cellular products of the organism. Members of the *Deinococcus* genus are noted for their extreme resistance to radiation and chemical agents which damage DNA. Nevertheless, members of this genus, of which the prototype species is *D. radiodurans*, have received relatively little attention with regard to the genetic mechanisms responsible for radiation resistance, in particular those involved in DNA repair (reviewed in Section 1.7). It has however been well-recognized that *D. radiodurans* possesses highly efficient recombination repair mechanisms (Moseley and Copeland, 1976).

The purpose of the studies described in this Chapter is to investigate the possible presence of a *recA* gene in *D. radiodurans*. The virtual universality of *recA* among bacteria so far investigated strongly suggests that it will not be absent from *D. radiodurans*. Furthermore, the current state of knowledge of RecA function in other bacteria indicates that RecA will have an important role in DNA recombination and repair in *D. radiodurans*. It is hoped that the eventual identification and characterization of the *D. radiodurans recA* gene will provide insights into the mechanism(s) of extreme radiation resistance associated with this organism.

6.2 METHODS

6.2.1 Preparation of Genomic DNA of *E. coli* RR1

E. coli RR1 (Sambrook *et al.*, 1989) genomic DNA was isolated according to a published method (Silhavy *et al.*, 1984). Briefly, a single *E. coli* RR1 colony was used to inoculate 100 ml of LB medium. The culture was grown overnight at 37°C at 200 revolutions per minute (rpm). The cells were harvested at RT by centrifugation for 20 minutes at 2500 rpm. The cells were resuspended in 5 ml of 50 mM Tris-HCl (pH 8.0), 50 mM disodium ethylenediaminetetracetate (EDTA). After addition of one half ml of freshly prepared lysozyme (chicken egg white, Sigma Chem. Co.) solution (10 mg/ml in 0.25M Tris-HCl [pH 8.0]), the mixture was placed on ice for 45 minutes. This was followed by addition of 1 ml of STEP [0.5% sodium dodecyl sulfate (SDS, Sigma Chem. Co.), 50 mM Tris-HCl (pH 7.5), 0.4 M EDTA, to which freshly prepared proteinase K (final concentration 1 mg/ml, Sigma) was added immediately prior to use] solution and heating at 50°C for 60 minutes with occasional mixing to digest the proteins. To precipitate the proteins, 6 ml of Tris-buffered phenol was added to the cell suspension, mixing gently for 5 minutes by inversion, followed by centrifugation at RT for 15 minutes at 2500 rpm. The supernatants were collected and the DNA was precipitated with 0.1 volumes of 3M sodium acetate (pH 5.2), and 2 volumes of 95% (v/v) ethanol. After overnight storage at -20°C, the precipitated DNA was spooled and transferred to a clean tube which contained 5 ml of 50 mM Tris-HCl (pH 7.5), 1 mM EDTA, and 200 µg/ml RNase A (Sigma Chem. Co.), and rocked gently at 4°C to dissolve the precipitate. The clear solution was extracted with an equal volume of chloroform by careful mixing by inversion and centrifuged at RT for 15 minutes at 2500 rpm. The DNA in the top layer was precipitated with 0.1 volumes of 3M sodium acetate (pH 5.2) and 2 volumes of 95% cold ethanol. The DNA was spooled and the excess alcohol was removed by pressing the

precipitate against the side of the tube. Finally, the genomic DNA was dissolved in 1 ml of 50 mM Tris-HCl (pH 7.5), 1 mM EDTA and stored at 4°C. If necessary, more buffer solution was added until all of the precipitate was completely dissolved.

6.2.2 Preparation of Genomic DNA of *D. radiodurans*

The genomic DNA of *D. radiodurans* was isolated by a modification of the procedure of Tirgari and Mosely (1980). A 100 ml volume of TYC (0.5% trypton, 0,3% yeast extract, and 3mM $CaCl_2$) was inoculated with 1 ml of a starter culture of *D. radiodurans* and incubated at 37°C and 200 rpm for 12 hours (final $OD_{600} = 0.76$). The cultures were harvested by centrifugation at 3000 rpm for 15 minutes and washed with 2.5 ml of SSC buffer (0.15 M NaCl/0.0015 M sodium citrate [pH 7.0]). The cells were resuspended in 2.5 ml of butanol-saturated phosphate EDTA buffer (0.03 M Na_2HPO_4, 0.03 M KH_2PO_4, 0.1 M EDTA, 6% n-butanol [pH 7.0]) and left at room temperature for 45 minutes. After centrifugation at 4°C and 3000 rpm for 15 minutes, the cells were resuspended in 2.5 ml of tenfold-diluted SSC buffer. Fifty mg of freshly prepared lysozyme (final concentration 20 mg/ml) were added directly to the cell suspension and the mixture was incubated at 37°C. Periodically, 0.1 ml of a 'test' sample was removed and one drop of 20% (w/v) SDS solution was added to lyse the cells. When the test cell suspension successfully cleared, 0.25 ml of 20% SDS was added to lyse the residual cell suspension at 37°C until the mixture cleared. This was followed by addition of 1 ml of freshly prepared proteinase K (final concentration 5 mg/ml) to the solution. The mixture was left to rock overnight at 4°C. The genomic DNA was purified by phenol, phenol/chloroform and chloroform extraction, each followed by centrifugation at 4°C and 3000 rpm for 10 minutes. The genomic DNA was precipitated from the aqueous top layer with 0.1 volumes of 3 M sodium acetate (pH 5.2) and 2 volumes of 95% ethanol, and kept at -20°C overnight. The DNA was spooled and dissolved in 1 ml of 50 mM Tris-HCl (pH

7.5), 1 mM EDTA and 200 μg/ml of RNase A, and rocked gently overnight at 4°C. If necessary, more buffer solution was added until all of the precipitate was completely dissolved. The DNA was extracted with an equal volume of chloroform, using inversion for mixing. The mixture was centrifuged at 4°C and 3000 rpm for 15 minutes. The DNA was re-precipitated from the supernatant as described above. The DNA was spooled and dissolved in 50 mM Tris-HCl (pH 7.5), 1 mM EDTA, and stored at 4°C.

6.2.3 Midi-Preparation of pUC-type Plasmids

Plasmids were isolated according to the procedure given in the Promega Protocols and Application Manual (page 73, 1989). A 100 ml volume of LB containing 100 μg/ml ampicillin (Amp) was inoculated with a single colony of a strain containing pUC19 and grown overnight at 37°C. The cell culture was harvested by centrifugation at 10,000 rpm for 10 minutes at 4°C, resuspended in 2.4 ml buffer (2 mM Tris-HCl [pH 8.0], 10 mM EDTA, and 15% [w/v] sucrose; immediately prior to use lysozyme was added to a final concentration of 2 mg/ml) and placed on ice for 10 minutes. The cells were lysed with 4.8 ml of freshly prepared lysis solution (0.2 M NaOH and 0.1% [w/v] SDS), mixed by inversion and kept on ice for 10 minutes. DNA in the lysate was precipitated by addition of 3.6 ml of 7.5 M ammonium acetate (NH_4-acetate). The solution was kept on ice for 20 minutes. Cellular debris was separated from the DNA by centrifugation at 10,000 rpm, 4°C for 20 minutes. The supernatant was transferred to a fresh tube and 0.4 ml RNase A (10 mg/ml) was added, and the mixture was incubated at 37°C for 20 minutes. This was followed by extraction of the plasmid DNA with an equal volume of TE-saturated phenol/chloroform (1:1), and then with chloroform, each vortexed for 1 minute and centrifuged at 10,000 rpm for 5 minutes. The DNA in the supernatant was precipitated with two volumes of 95% ethanol and set at -20°C for 30 minutes, followed by centrifugation at 10,000 rpm for 20 minutes. The pellet was dissolved in 0.68 ml of water,

followed by addition of 0.12 ml 5 M NaCl (final concentration 0.2 M) and 0.8 ml 13% (w/v) polyethylene glycol (PEG). The solution was mixed, incubated in ice-water for 60 minutes, and centrifuged at 10,000 rpm, 4°C for 20 minutes. The DNA pellet was washed with 1 ml of 95% ethanol and recovered by centrifugation at 10,000 rpm for 5 minutes. The DNA was dried at room temperature for 10 minutes, dissolved in 0.5 ml TE (pH 7.4) and stored at -20°C. The DNA concentration in this preparation was 0.3 mg/ml, as determined spectrophotometrically by measuring the absorption at 260 nm.

6.2.4 Mini-preparation of Plasmid DNA

Mini-preps of plasmid DNA were performed according to the method of Morelle (1989). A 1.5 ml volume of an overnight culture was harvested in a microfuge for 30 seconds. The cells were resuspended in 200 µl GET with RNase A (50 mM glucose, 10 mM EDTA, 25 mM Tris-HCl [pH 7.5], with 25 µl of 10 mg/ml boiled RNase A added per ml) and mixed with 400 µl lysis mixture (0.2 M NaOH, 1% SDS) by inversion until the suspension cleared. After incubation on ice for 5 minutes, 300 µl of 7.5 M NH_4-acetate were added to the suspension. It was mixed by inversion and kept on ice for 10 minutes. After centrifuging for 3 minutes, plasmid DNA was precipitated from the supernatant by pouring it into 500 µl of cold (-20°C) isopropanol. The solution was mixed by inversion, and after keeping at RT for 5 minutes, it was centrifuged at RT for 5 minutes. The DNA pellet was rinsed once with 1 ml of cold 95% ethanol, air dried (or dried for 5 minutes in a 37°C incubator) and suspended in 50 µl TE (pH 8.0).

6.2.5 Preparation of Competent *E. coli* DH5αF' Cells

A 40 ml volume of LB (1%, Bacto tryptone, 0.5% Bacto yeast and 1.0% NaCl) was inoculated with 0.4 ml of an overnight culture of *E. coli* DH5αF' (as DH5α [Sambrook *et al.*, 1989] but F^+) cells and grown at 37°C to exponential phase (OD_{600} =

0.4 to 0.5). Cells were harvested in cold, sterile oakridge tubes by centrifuging at 4°C and 5000 rpm for 10 minutes. This was followed by gently resuspending the cells in 2.0 ml of ice-cold, sterile 0.1 M $MgCl_2$, holding on ice for 30 minutes, and pelleting at 4°C and 5000 rpm for 10 minutes. The cells were resuspended in 1.0 ml of ice-cold, sterile TG-salts solution (75 mM $CaCl_2$, 6 mM $MgCl_2$, 15% [w/v] glycerol), incubated on ice for 20 minutes and pelleted at 4°C and 5000 rpm for 10 minutes. Finally, the cells were resuspended in 1.0 ml of ice-cold TG-salts and held on ice (no more than 24 hours). The competent cells were aliquoted into Eppendorf tubes and frozen on dry-ice before being stored at -70°C.

6.2.6 Transformation of *E. coli*

The plasmid DNA in ligation buffer (not exceeding 25 to 50 µl in volume) was added to a small culture tube which contained 200 µl of competent cells and held on ice for 30 minutes. This was followed by a 2 minute undisturbed heat shock at 42°C. This facilitated the uptake of plasmid DNA. One ml of LB was added and the tube was incubated at 37°C for 1 hour to allow the bacteria to recover. Then 100 to 200 µl of the competent cell suspension was spread onto LB/Amp agar plates and the plates were incubated overnight at 37°C.

6.2.7 Preparation of DNA Probes

The DNA probes used in southern blotting were [α-^{32}P] labelled using the BRL Random Primer DNA Labelling Kit. Briefly, approximately 25 ng of DNA was denatured in boiling water for 10 minutes and immediately chilled on ice for 5 minutes. This was followed by addition of 2 µl of a 100 mM solution of each of the three unlabeled nucleotide triphosphates followed by 15 µl of random primer buffer mixture, 2-5 µl of [α-^{32}P] dATP (3000 Ci/mmol; 10 µCi/ml, Amersham) and distilled water to 49 µl. The

solution was mixed. One µl of Klenow fragment (6 U) was added, the solution was mixed thoroughly and incubated at room temperature for one hour. The reaction was stopped by adding 5 µl of 0.1 mM EDTA. Unincorporated radioactive ATP was removed by gel filtration using a Sephadex G-50 1 ml size syringe column, equilibriated with STE (10 mM Tris-HCl [pH 8.0], 100 mM NaCl and 1 mM EDTA). The radioactivity of the probe was determined using scintillation counting.

6.2.8 Southern Blotting Using a 0.6 kb *Eco*RI-*Pst*I Probe Internal to the *recA* Gene of *E. coli*

One µg of restricted genomic DNA of *D. radiodurans*, *E. coli* RR1 and *P. aeruginosa* PAO1 (since genomic DNA of *P. aeruginosa* has a similar GC content (68%) as *D. radiodurans*, it was used as a hybridization control) along with molecular weight markers, was electrophoresed on 0.8% (w/v) agarose gels. If the DNA fragments were greater than 10 kb, the electrophoresed DNA was depurinated in 0.25 mM HCl at RT with shaking until the color of the dye front changed from blue to yellow. At this point, an additional 10 minutes of incubation was allowed. After rinsing in distilled water, the DNA was denatured by shaking in a mixture of 1.5 M NaCl and 0.5 M NaOH for either 30 minutes at RT or for an additional 15 minutes from the time after the yellow dye colour had changed back to blue. This was followed by rinsing the gel with distilled water and neutralizing it with a 1.5 M NaCl, 0.5 M Tris-HCl (pH 7.2), 1 mM EDTA solution for at least 15 minutes. The denatured DNA was capillary transferred to an Amersham Hybond-N membrane in 20 x SSC overnight, then rinsed with 2 x SSC, and air dried. The DNA was crosslinked to the membrane by placing it DNA side-down over a shortwave ultraviolet light source for 4 minutes. The blots were prehybridized for 4 hours to overnight at 50°C in a mixture of buffers containing 6 x SSC, 5 x Denhart's solution (0.1% Ficoll [Sigma]; 0.1% polyvinylpyrrolidone [PVP, Sigma]; 0.1% bovine serum albumin

[BSA, Boehringer Mannheim Biochemicals], 0.5% SDS and 50 μg/ml denatured salmon sperm DNA (boiled in water for 10 minutes; chilled on ice for 5 minutes) in a sealed plastic bag. Approximately 8.0 ml of buffer solution per 10 cm^2 of membrane was used for prehybridization and about 6.0 ml of buffer per 10 cm^2 of membrane was used for hybridization. The blots were hybridized overnight at 50°C in the same solution with the labelled probes (Section 6.2.7) using 10^6 cpm/ml of hybridization solution. The blots were subsequently washed once with 2 x SSC/0.1% SDS for 30 minutes at room temperature, once with 1 x SSC/0.1% SDS for 15 minutes at 50°C, and once with 0.5 x SSC/0.1% SDS for 5 minutes at 50°C. The blots were checked with a Geiger counter following each wash. The membranes were sealed damp with plastic wrap and exposed to Kodak X-omat AR X-ray film using an intensifying screen at -70°C.

6.2.9 Removal of the Probe and Reuse of DNA Blots

The blots were boiled in a solution of 0.1% SDS for 30 to 60 minutes and allowed to cool to room temperature. They were re-exposed to X-ray film for at least 24 hours to make sure that the radio-labelled probe had been completely removed before re-using.

6.2.10 Southern Blotting Using the 363 bp PCR Fragment from *D. radiodurans* as a Probe

The blots which were originally probed with a 0.6 kb *Eco*RI-*Pst*I *recA E. coli* probe were stripped of their probes (Section 6.2.9). They were rehybridized with the [α-^{32}P] dGTP labelled 363 bp partial *D. radiodurans recA* probe overnight at 65°C. This was followed by washing the blots twice with 2 x SSC at RT for 15 minutes, once with 2 x SSC/0.1% SDS for 30 minutes and once with 0.2 x SSC/0.1% SDS for 15 minutes at 65°C. The blots were autoradiographed using an intensifying screen at -70°C.

6.2.11 Cloning of the *recA* Gene of *E. coli* RR1

A *Bam*HI genomic library of *E. coli* RR1 was constructed by ligation of 2.3-4.3 kb fragments of *Bam*HI digested chromosomal DNA, which was previously electrophoresed on low melting agarose (LMA), into the *Bam*HI site of pUC19 with T4 DNA ligase (BRL) in 50 µl ligation mixtures at 3:1 molar ratios overnight at RT. The ligation mixture was transformed into the *E. coli recA* strain HB101 (Sambrook *et al.*, 1989). Recombinant clones were selected on LB plates containing 1 mM methyl methanesulfonate (MMS, Aldrich) in the presence of 40 µg/mL of Xgal and 100 µg/ml Amp at 37°C for 26 hours. One white MMS-resistant clone was recovered. To ensure that this phenotype was encoded by the *recA* gene of *E. coli* RR1 and that this was the result of complementation, the purified plasmid DNA was retransformed into *E. coli* HB101 (Section 6.2.6). The appearance of many white colonies on a LB/Amp/MMS/Xgal plate together with plasmid restriction analysis (data not shown) confirmed that the MMS-resistant clone contained a 3.0 kb insert harbouring the *recA* gene of *E. coli* RR1. Furthermore, DNA sequence analysis (data not shown) of the 0.6 kb *Eco*RI-*Pst*I fragment of this 3.0 kb insert confirmed the presence of the *recA* gene on this fragment. This 0.6 kb *Eco*RI-*Pst*I fragment was subsequently used as a probe to screen for the *recA* gene of *D. radiodurans*.

6.2.12 Attempts at Cloning the *recA* Gene of *D. radiodurans* by Interspecies Complementation

A *Pst*I genomic library was prepared as follows. Genomic DNA of *D. radiodurans* was digested to completion with *Pst*I, purified by phenol/chloroform extraction and ethanol precipitation, and dissolved in TE (pH 7.6). The purified DNA (1 µg) was ligated into the *Pst*I site of pUC19 (0.5 µg). The ligation mixture was transformed into *E. coli* HB101 (Section 6.2.6). Half of the transformants were spread

onto a LB plate for library stock preparation and the other half was plated on plates containing 1 mM MMS, 40 μg/ml Xgal and 100 μg/ml Amp. There were ~ 1,200 white colonies on the LB plates and 66 white colonies on the LB/Amp/MMS plates. The prospective MMS-resistant clones were screened by Southern hybridization using a [α-^{32}P]-labelled 0.6 kb *Eco*RI-*Pst*I *E. coli recA* probe (Section 6.2.8). The prospective clones were tested for their ability to retransform the MMS-resistant phenotype. None of the clones were able to do this. An *Eco*RI genomic library was constructed and screened using the same procedure. Once again no positive MMS-resistant clones were identified.

6.2.13 Attempts at Cloning of *recA* Gene of *D. radiodurans* after Screening by Southern Blot Analysis

The hybridization studies at 50°C revealed that a 9.4 kb *Eco*RI DNA fragment of *D. radiodurans* hybridized with the 0.6 kb *Eco*RI-*Pst*I *recA* probe from *E. coli*. An *Eco*RI genomic library was therefore constructed by ligating *Eco*RI digested and LMA gel-purified fragments (7.5-11.0 kb in size) into the *Eco*RI site of pUC19. The ligation mixture was incubated at 12°C overnight and then used to transform *E. coli* DH5αF'. About 2000 white recombinant clones were obtained. DNA from pools of 6 plasmids from about 800 clones were digested with *Eco*RI, Southern blotted and hybridized with the 0.6 kb *Eco*RI-*Pst*I *E. coli recA* probe at 50°C (Section 6.2.8). The *Eco*RI digestion before the Southern hybridization was necessary due to the high background of hybridization signals from pUC19 and the 0.6 kb *Eco*RI-*Pst*I *rec*A probe. In addition, the probe had low sequence homology with *D. radiodurans* as shown in the autoradiograph (Fig. 25, panel A). No positive clones were identified by this procedure.

6.2.14 Cloning of an Internal Fragment of *recA* Gene of *D. radiodurans* by the Polymerase Chain Reaction (PCR)

Two degenerated PCR primers were designed based on analysis of the conserved region of previously deduced amino acid sequences, mainly from Gram-negative bacteria (Tolmasky *et al.*, 1992; Duwat *et al.*, 1992; Dybvig *et al.*, 1992) because of the high G+C content of *D. radiodurans* (68%). The coding strand primer was 5'-TTC[AG] T[ATGC]G A[TC]GC [ATGC]GA [AG]CA [TC]G and the complementary-strand primer was 5'-CC[AG] CCI G[GT]I GTI GT[TC] TC[ATGC] GG-3 with 248- and 32-fold degeneracies, respectively. The primers were synthesized by the Regional DNA Synthesis Laboratory of the University of Calgary. The PCR reactions were performed by 30 cycles of denaturation at 95°C for 1 minute, annealing at 55°C for 2 minutes, and extension at 72°C for 2 minutes, using a DNA Thermal Cycler (Perkin-Elmer Cetus Model 480). After the last cycle, samples were incubated for an additional 10 minutes at 72°C to ensure that the final extension step was completed. Amplifications with Taq polymerase were carried out in several 50 µl reaction volumes, each containing 1000, 750, 500, 250, 120, 60 and 30 ng of genomic *D. radiodurans* DNA, 2.5 mM of each primer, 1 x Taq buffer, 200 mM of each deoxyribonucleotide triphosphate (dNTP), 1.5 mM $MgCl_2$ (optimal condition, data not shown) and 1.2 U Taq polymerase (Promega). A *recA* fragment from *E. coli* RR1 genomic DNA was amplified as a positive control. Twenty µl of PCR products were electrophoresed on a 1.2% agarose gel.

6.2.15 Cloning of the PCR Product of *D. radiodurans* Using the pCRII Cloning Vector

A 25 µl sample (25 ng or 0.1 pmole) from the 750 ng *D. radiodurans* PCR reaction was precipitated by addition of 25 µl 4 M NH4-acetate and 50 µl cold isopropanol, rinsed with 70% ethanol and dried at RT. This was followed by addition of 2

µl distilled water, 1 µl 10 x ligase buffer, 2 µl pCRII vector (50 ng or 0.02 pmole) (Invitrogen, San Diego, CA), 1 U T4 DNA ligase, and the mixture was ligated overnight at 12°C. Transformation was carried out according to the Invitrogen protocol. The ligation mixture was placed on ice, 2 µl of 0.5 M β-mercaptoethanol and 50 µl of *E. coli* (INVaF') competent cells were added. The solution was held on ice for 30 min, followed by a heat shock at 42°C for 60 seconds. The mixture was immediately placed on ice for another 2 min. The transformed cells were allowed to recover in 450 µl of prewarmed SOC medium (2% Bacto tryptone, 0.5% Bacto yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM $MgCl_2$, 10 mM $MgSO_4$, 20 mM glucose) by shaking at 37°C and 200 rpm for 1 hour. The transformed cells were spread on LB agar plates containing 100 µg/ml Amp and 40 µg/ml Xgal, and incubated at 37°C overnight. Plasmid DNA was isolated from white colonies and digested with *Xba*I and *Hin*dIII to screen for inserts.

6.2.16 PCR Sequencing of the 363 bp PCR-amplified Fragment of *D. radiodurans*

The plasmid DNA from a white colony was isolated according to the modified mini Alkaline - Lysis/PEG precipitation procedure (protocol from Applied Biosystems, Inc.). The purified plasmid from a clone in pCRII was sequenced on both strands using the M13 forward 24-mer and M13 reverse 24-mer primers. PCR sequencing reactions were carried out according to the ABI Taq Dye Deoxy Terminator Cycle Sequencing Kit protocol. The PCR sequence data were analyzed using the facilities of the Regional DNA Synthesis Laboratory of the University of Calgary.

6.2.17 Cloning of the *recA* Gene of *D. radiodurans* Using pUCBM20

Southern hybridization of the genomic DNA of *D. radiodurans* with the 363 bp probe detected a 4.3 kb *Eco*RI-*Mlu*I fragment. An *Eco*RI-*Mlu*I genomic DNA library was constructed by ligating LMA gel-purified *Eco*RI-MluI fragments (3.0 - 6.0 kb) of *D.*

radiodurans into the *Eco*RI+*Mlu*I sites of pUCBM20 (Boehringer Mannheim Biochemicals). Four µl of the ligation mixture was used to tranform *E. coli* DH5aF' competent cells as previously described (Section 6.2.6). The recombinant cells were plated on LB/Amp/Xgal agar plates at 37°C overnight. A total of 125 white colonies were recovered. Plasmids were purified from 20 of these white colonies and were digested with *Eco*RI+*Mlu*I. The gels were probed at 68°C with the 363 bp fragment from *D. radiodurans*. The blots were washed once with 2 x SSC for 15 minutes at RT, once with 0.2 x SSC/0.1% SDS for 15 minutes at 68°C, and once with 0.1 x SSC/0.1% SDS for 15 minutes at 68°C. Following autoradiography one positive clone, designated pDREM1, was identified. This plasmid contained a 4.3 kb *Eco*RI-*Mlu*I insert.

6.2.18 Restriction Mapping of Plasmid pDREM1

Single digestion of the plasmid pDREM1 with a number of restriction enzymes some of which also cleaved in the polylinker site of pUCBM20 was used to construct a restriction map.

6.3 RESULTS

6.3.1 Attempts at Cloning of the *recA* Gene from *D. radiodurans* by Complementation (Section 6.2.12)

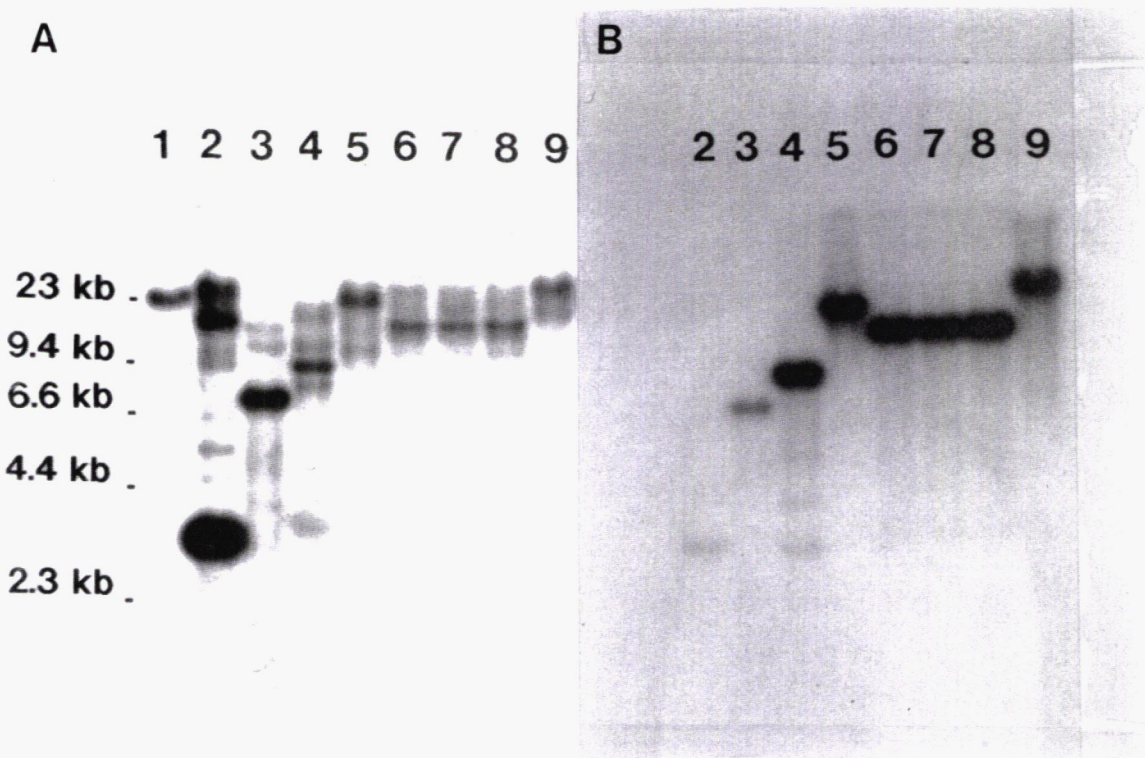
A *Pst*I genomic library of *D. radiodurans* DNA was screened for colonies resistant to the DNA-damaging agent MMS at a concentration of 1 mM. This concentration permits growth of *recA*$^+$ *E. coli* cells. Therefore, only colonies in which the *E. coli* HB101 *recA* mutation was complemented by a *D. radiodurans* gene should be able to grow. Of 1,200 clones screened in this manner, 66 displayed MMS resistance. The plasmids of MMS-resistant clones were subjected to restriction analysis. The DNA of the

prospective clone candidates were transformed into *E. coli* HB101 again to ensure its MMS resistant phenotype was encoded by a *D. radiodurans* gene. None of the prospective candidates were able to retransform this phenotype. Therefore, no *recA*-like clone of the *D. radiodurans* was identified. An *Eco*RI genomic library was screened in the same manner and again no positive clones could be isolated.

6.3.2 Attempts at Cloning of the *recA* Gene of *D. radiodurans* after Screening by Southern Hybridization (Section 6.2.13)

The presence of a *recA*-like gene in the *D. radiodurans* genomic DNA was examined by Southern hybridization with a 0.6 kb *Eco*RI-*Pst*I *E. coli recA* probe (Section 6.2.8) (Fig. 24, panel A, lanes 4 to 9). The smallest fragment that hybridized was a 9.4-Kb *Eco*RI fragment (Fig. 24, panel A, lane 4). The probe also hybridized with chromosomal DNA fragments of *E. coli* RR1 and *P. aeruginosa* PAO1 DNA of 3.0 and 7.0 kb, respectively (Fig. 24, panel A, lanes 2 and 3). The autoradiograph (Figure 24, panel A) showed that *D. radiodurans* DNA demonstrated the least homology with an *E. coli recA* probe. Chromosomal DNA of *D. radiodurans* was digested to completion with *Eco*RI and the 7.5-11.0 kb fragments were isolated from a low melting agarose gel. The fragments were cloned into pUC19 (Section 6.2.13) and plasmids from 800 recombinants (analyzed as pools of 6) were digested with *Eco*RI and screened by DNA hybridization using a 0.6 kb *E. coli recA* probe at 50°C (Section 6.2.8). Digestion of the plasmids before DNA hybridization was necessary due to high background from plasmid pUC19 and weak hybridization with the *E. coli recA* probe. No positive clones were obtained.

Figure 24. **Genomic Southern analysis illustrating hybridization of *recA* probes from *E. coli* and *D. radiodurans*.** Chromosomal DNAs were digested to completion with *Bam*HI (*E. coli*, lane 2), *Eco*RI+*Xho*I (*P. aeruginosa*, lane 3), *Eco*RI, *Pst*I, *Bcl*I (50°C), *Bcl*I (50°C), *Bcl*I (37°C) and *Hind*III (*D. radiodurans*, lanes 4, 5, 6, 7, 8, 9, respectively). The blot was first hybridized to the 0.6 kb *Eco*RI-*Pst*I *recA* probe from *E. coli* (panel A). After removal of the probe, a second hybridization was performed utilizing the 363 bp *D. radiodurans* PCR fragment as the probe (panel B). The molecular sizes of λ*Hind*III fragments (lane 1) are indicated on the left.


A
1 2 3 4 5 6 7 8 9
23 kb
9.4 kb
6.6 kb
4.4 kb
2.3 kb
B
2 3 4 5 6 7 8 9

6.3.3 Cloning of *recA* Sequences of *D. radiodurans* by Polymerase Chain Reaction (PCR) (Section 6.2.14)

The *D. radiodurans* DNA fragment of expected size (about 360 bp) amplified using two degenerate primers designed after highly conserved amino acid sequences of known RecA proteins was further characterized by Southern hybridization (Roca and Cox, 1990). Southern hybridization with the 0.6 kb *Eco*RI-*Pst*I *E. coli recA* probe revealed that this probe strongly hybridized to an amplified 360 bp fragment of *E. coli* DNA, and weakly to a similar size fragment from *D. radiodurans* (Fig. 25). This suggested that the 363 bp PCR product of *D. radiodurans* is an internal fragment of the *recA* gene of *D. radiodurans*.

In order to verify that the amplified DNA band corresponded to an internal *recA* fragment of *D. radiodurans*, the amplified DNA product was cloned without further purification using the pCRII cloning vector. The PCR product of *D. radiodurans* along with the PCR product of *E. coli* RR1 was blotted and probed with either the 0.6 kb *E. coli recA* probe at 50°C or at 60°C with a fragment containing prospective *D. radiodurans recA* sequences obtained by PCR. The autoradiograph showed that both probes hybridized to the *D. radiodurans* and *E. coli* RR1 PCR products but with reversed signal intensities. The PCR product of *D. radiodurans* hybridized strongly with the *D. radiodurans* DNA fragment and weakly with the 0.6 kb *E. coli recA* probe (data not shown). This hybridization pattern was also repeated on a Southern blot which previously had been probed with the 0.6 kb *E. coli recA* probe (Fig. 24, panel B). These observations confirmed that the DNA fragment obtained by PCR was indeed from *D. radiodurans*. Nucleotide sequence analysis revealed a 363 bp (121 amino acid-encoding) DNA fragment of *D. radiodurans* (Fig. 26) which at the amino acid level showed 77% and 74% identity with the *B. subtilis* and *E. coli* RecA proteins, respectively, as well as

Figure 25. **Southern analysis of PCR products.** PCR amplification of *recA* sequences from *E. coli* (lane 1) and *D. radiodurans* (lanes 2-5) chromosomal DNA was performed as described in Section 6.2.14. The amounts of chromosomal DNA per reaction were: *E. coli* 1 μg (lane 1) and *D. radiodurans* 1, 0.75, 0.5, 0.25 μg (lanes 2-5), respectively. The PCR products were electrophoretically separated on a 1.2% agarose gel and probed with an internal 0.6 kb *Eco*RI-*Pst*I *recA* fragment from *E. coli*.

1 2 3 4 5

Figure 26. Nucleotide sequence of the 363 bp *D. radiodurans* PCR-amplified DNA fragment. The predicted amino acid sequence of the *recA* reading frame is indicated below the appropriate codons. A *Hind*II site also found in pDREM1 (Fig. 29; marked *Hind*II-1) is underlined.

```
TTC GTG GAC GCT GAG CAC GCC CTC GAC CCG GTG TAT GCC CGC GCG CTG GGG GTC
 F   V   D   A   E   H   A   L   D   P   V   Y   A   R   A   L   G   V

AAC ACC GAC GAA CTG CTT GTC TCG CAG CCC GAC AAC GGC GAG CAG GCG CTC GAA
 N   T   D   E   L   L   V   S   Q   P   D   N   G   E   Q   A   L   E

ATC ATG GAA CTG CTG GTG CGC TCG GGC GCG ATT GAC GTG GTC GTG ATC GAC TCG
 I   M   E   L   L   V   R   S   G   A   I   D   V   V   V   I   D   S

GTG GCC GCG CTG ACC CCG CGC GCC GAA ATC GAG GGC GAC ATG GGC GAC TCG CTG
 V   A   A   L   T   P   R   A   E   I   E   G   D   M   G   D   S   L

CCC GGC CTC CAG GCC CGG TTG ATG TCG CAG GCG CTG CGC AAG CTG ACG GCG ATT
 P   G   L   Q   A   R   L   M   S   Q   A   L   R   K   L   T   A   I

CTC TCC AAG ACC GGC ACC GCC GCC ATC TTC ATC AAC CAG GTT CGC GAG AAA ATC
 L   S   K   T   G   T   A   A   I   F   I   N   Q   V   R   E   K   I

GGC GTG ATG TAC GGC AAC CCA GAA ACC ACC CCC GGC GGA
 G   V   M   Y   G   N   P   E   T   T   P   G   G
```

similarities with other Gram-positive bacteria (Fig. 27) (sequences were retrieved from Protein Information Resources data bank). A 4.3 kb *Eco*RI-*Mlu*I DNA fragment from *D. radiodurans* hybridized under high stringency conditions (65°C) strongly with the *D. radiodurans* 363 bp *recA* probe (data not shown). This fragment was cloned into pUCBM20 and a restriction map was constructed (Fig. 28).

The *recA* gene was tentatively localized to its indicated position in Fig. 28 by hybridization analysis of pDREM1 DNA. First, *Hin*dII digested plasmid DNA was hybridized with the 363 bp PCR probe. Two hybridization signals were obtained. Whereas the probe hybridized strongly to the 1.1 kb *Hin*dII-1-*Hin*dII-2 fragment, it hybridized very weakly to the 1.0 kb fragment which extends from the polylinker *Hin*dII site (next to *Mlu*I) to the *Hin*dII-1-site. This weak hybridization is expected since the *Hin*dII-1 site is located next to one end of the 363 bp probe (see Fig. 26) and therefore overlaps minimally with the 1.0 kb fragment. A single strong signal was observed when a *Bgl*I digest was hybridized with the same probe. Since the orientation of the *recA* coding sequence relative to the *Hin*dII-1 site is known (Fig. 26), its transcriptional orientation was deduced and is shown in Fig. 28. Alignment of the primary amino acid sequence of *D. radiodurans* RecA with the *E. coli* protein (Fig. 29), allowed determination of the relative position and extent of the entire *recA* coding sequence (assuming that both proteins are similar in size).

Figure 27. Comparison of the deduced amino acid sequences of *recA*$_{int}$ fragments. The deduced amino acid sequences of the *recA*$_{int}$ fragments of *B. subtilis* (B.s.), *C. acetobutylicum* (C.b.), *D. radiodurans* (D.r.), *E. coli* (E.c.), *L. bulgaricus* (L.b.), *L. helveticus* (L.h.), *L. lactis* (L.l.), *L. mesanteroides* (L.m.), *S. aureus* (S.a.), and *S. thermophilus* (S.t.) are shown. Amino acid positions correspond to those of the *E. coli* RecA protein. A star indicates that the amino acids are identical, and a plus indicates amino acids which are found in Gram-positive species but not in *E. coli*. A consensus amino acid is given only when at least seven amino acids are identical. Sequences other than *D. radiodurans* were taken from Duwat *et al.* (1992) who utilized the MULTALIN multiple sequence alignments software of Corpet (1988).

```
                 110        120        130        140        150
E.c.  IYARKLGVDIDNLLCSQPDTGEQALEICDALARSGAVDVIVVDSVAALTPKAE
D.r.  VYARALGVNTDELLVSQPDNGEQALEIMELLVRSGAIDVVVIDSVAALTSRAE
B.s.  VYAQKLGVNIEELLLSQPDTGEQALEIAEALVRSGAVDIVVVDSVAALVPKAE
S.a.  EYAQALGVDIDNLYLSQPDHGEQGLEIAEAFVRSGAVDIVVVDSVAALTPKAE
L.b.  AYAEALGVDIDQLILSQPNTGEEGLQIADTLISSGAIDIVVVDSVAVLVPRAE
L.h.  AYAEALGVDIDSLILSQPNTGEEGLQIADTLISSGAIDIVVVDSVAALVPRAE
L.l.  EYAKALGVNIDELLLSQPDYGEQGLQIAEKLITSGAVDLVVIDSVAALVPKAE
S.t.  AYARALGVNIDELLLSQPDSGEQGLEIAGKLIDSGAVDLVVVDSVAAFVPRAE
L.m.  PYAKTLVVNIDELLLPQPDTGEQALEIAEALVRSGAVDMLVIDSVAALVPRAE
C.b.  SYAQKLGVDVDSLIISQPDTGEQGLEIAEALVRSGAIDVLVVDSVAALVPKAE
       **  *       *   **  **  * *       *** *  * *****    **
cons. YA  LGV ID L LSQPDTGEQGLEIA  L  SGAVD  VVDSVAALVP AE

        160        170        180        190        200
E.c.  IEGEIGDSHMGLAARMMSQAMRKLAGNLKQSNTLLIFINQIRMKIGVMF-GN
D.r.  IEGDMGDSLPGLQARLMSQALRRLTAILSKTGTAAIFINQVREKIGVMT-GN
B.s.  IEGDMGDSHVGLQARLMSQALRKLSGAINKSKTIAIFINQIREKVGNMFFRE
S.a.  IEGEMGDTHVGLQARLMSQALRKLSGAISKSNTTAIFINQIREKVGVMF-GN
L.b.  IEGEMGDSHVGLQARLMSQALRKLSGTIAKTKTIAIFINQIREKVGVMF-GN
L.h.  IEGEMGDAHVGLQARLMSQALRKLSGTISKTKTIAIFINQIREKVGIMF-GN
L.l.  IDGEIGDSSVGLQARMMSQAMRKLAGHINKTKTTAIFINQLREKVGVMF-GN
S.t.  IDGDSGDSHVGLQARMMSQAMRKLSASINKTKTIAIFINQLREKVGIMF-GN
L.m.  IEGEMGDAHVGLQARLMSQALRKLSGVINKSKTIAIFINQIREKVGVMF-GN
C.b.  IEGEMGDSHIGLQARLMSQALRKLAGTINKTNCVAIFINQLREKLGFMF-GS
      ***  **   **+** **** * *          +***** *+* *
cons.IEGEMGDSHVGLQARLMSQALRKL G I K  T AIFINQIREKVG MF GN
```

Figure 28. Restriction map of chromosomal DNA carried by pDREM1. The orientation of *recA* was deduced from the relative intensities of hybridization signals (see Section 6.3.3, last paragraph) obtained by probing *Bgl*I and *Hind*III digests of pDREM1 DNA with the internal 363 bp probe. Note that the *Hind*III-1 site is located in the probe sequence (Fig. 27).

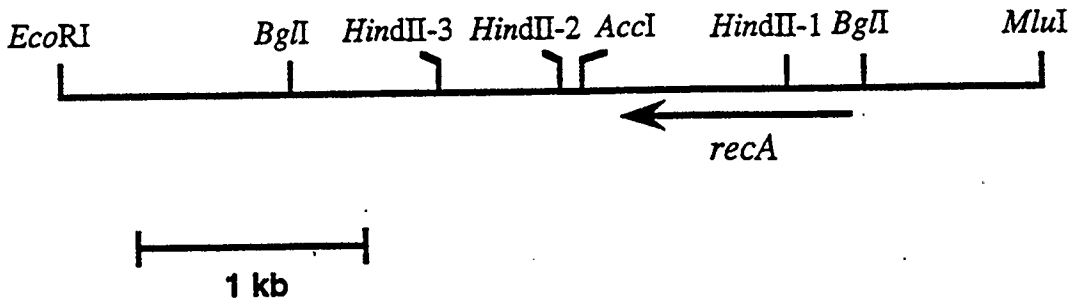

EcoRI
BglI
HindII-3
HindII-2
AccI
HindII-1
BglI
MluI
recA
1 kb

Figure 29. **Sequence alignment of the *E. coli* (E.c.) RecA protein with the partial *D. radiodurans* (D.r.) RecA polypeptide and definition of functional domains.** Residues that are identical are indicated by colons. RecA domains are defined by Story *et al.* (1992). β strands are indicated by numbers (0-10) and α-helices are indicated by letters (A-J). L1 and L2 are disordered loops. Asterisks indicate invariant or conservatively substituted amino acid residues involved in ATP binding, ATP hydrolysis, and/or conformational changes.

1 AIDENKQKALAAALGQIEKQFGKGSIMRLGED E.c.
A 0

33 RSMDVETISTGSLSLDIALGAGGLPMGRIVEIYGPE E.c.
B 1

69 SSGKTTLTLQVIAAAQREGKTCAFIDAEHALDPIYA E.c.
** C :V: : : : : : : : V: : D.r.
2

105 RKLGVDIDNLLCSQPDTGEQALEICDALARSGAVDV E.c.
: A: : :T: E : : V: : : :N : : : : : : :MELLV : : : : I : : D.r.
D 3 E

141 IVVDSVAALTPKAEIEGE I GDSHMGLAARMMSQAMR E.c.
V: I : : : : : : : : : R : : : : : DM : : : L P : : Q : : L : : : : L : D.r.
4 ** L_1 F

177 KLAGNLKQ SNTLLIFINQIRMKIGVMFGNPETTTGG E.c.
: : T A I :S KTG :AA: : : : : V:E : : : : : Y : : : : : : P: : D.r.
5 ** L_2 * *

213 NALKFYASVRLDIRRIGAVKEGENVVGSETRVKVVK E.c.
G 6 7

249 NKIAAPFKQAEFQILYGEGINFYGELVDLGVKEKLI E.c.
8 H

285 EKAGAWYSYKGEKIGQGKANATAWLKDNPETAKEIE E.c.
9 10 I J

321 KKVRELLLSNPNSTPDFSVDDSEGVAETNEDF E.c.

6.4 DISCUSSION

The *E. coli* RecA protein is a protein with multiple functions. Its pivotal role in homologous recombination and DNA repair has stimulated a search for *recA* or *recA*-like genes in a variety of prokaryotes and eukaryotes. In prokaryotes, more than 40 *recA* genes have been cloned using the interspecies complementation method (references in Miller and Kokjohn, 1990 and Roca and Cox, 1990). With this method, a genomic library of a bacterium is transformed into an *E. coli recA* mutant, and positive clones are then selected by their ability to complement the *E. coli recA* phenotype, such as being able to grow in the presence of the DNA alkylating agent MMS. This technique does not work well for Gram-positive bacteria. Reasons for this include the possibility that gene expression may not operate in the heterologous system (*E. coli*), i.e. the phenomenon of "failure of gene expression" (Davis *et al.*, 1991). Alternatively, recombinant clones may be unstable in *E. coli* (Yasbin *et al.*, 1991). My own attempts to clone a *D. radiodurans recA* gene using this method were unsuccessful. A *recA*-encoding gene has been cloned from *Mycobacterium tuberculosis* using an *E. coli* 0.6 kb *Eco*RI-*Pst*I *recA* fragment as a probe (Davis *et al.*, 1991). Although it was possible to detect a *recA* like sequence within the genomic DNA of *D. radiodurans* by Southern blot analysis using a 0.6 kb *E. coli recA* probe, isolating a *recA* clone was not successful This may be due to the low sequence homology of the probe with *D. radiodurans* DNA. Duwat *et al.* (1992) attempted to identify the *recA* gene in *L. lactis*, a Gram-positive bacterium, using the *B. subtilis recA* gene as a probe but this approach was unsuccesful. The same authors then used a PCR-based approach to successfully identify the *recA* gene in *L. lactis*. In my own work, the PCR cloning technique was found to be the most successful. I chose to clone an internal *recA* fragment from *D. radiodurans*. The PCR primers were designed after study of conserved regions of known bacterial *recA* proteins. A 363 bp fragment of *D.*

radiodurans was amplified, cloned and sequenced. Hybridization analyses and a comparative analysis of the predicted amino acid sequence encoded by the 363 bp PCR fragment from *D. radiodurans* and the other known RecA proteins confirmed that the 363 bp fragment contained *recA* sequences from *D. radiodurans*. The sequence was 77% identical with that of the *B. subtilis* RecA protein and 74% identical with that of the *E. coli* RecA protein. Also, PCR has been used to clone partial *recA* genes from the Gram positive bacterium *L. lactis* and from 9 other Gram positive bacteria (Dybvig *et al.*, 1992, Duwat *et al.*, 1992). A fragment of the chicken *Rad*51 gene was amplified using degenerate primers homologous to highly conserved stretches of the *recA*-like Rad51 and Dmc1 yeast proteins (Bezzubova *et al.*, 1992). Therefore, PCR seems to be a very effective method for cloning *recA* or *recA*-like genes in prokaryotes and in eukaryotes.

The core domain of the *E. coli* RecA protein is located between amino acid residues 31 and 260. This region contains clusters of highly conserved amino acids of the RecA proteins and is responsible for nucleotide binding, ATP hydrolysis and DNA binding (Ogawa *et al.*, 1992; Story *et al.*, 1992). The 363 bp fragment from *D. radiodurans* contains 121 amino acids corresponding to the highly conserved region of the RecA protein between amino acids 92 and 212. Therefore, some of the structural domains of the RecA protein of *D. radiodurans* can be deduced. Fig. 29 shows the amino acid sequence homology between portions of *D. radiodurans* and *E. coli* RecA proteins, and also the proposed functional domains according to Story *et al.* (1992). This comparison revealed that there are homologies of the *D. radiodurans* protein with predicted RecA functional domains, the homologous duplex DNA binding (region L1, residues 195-209) the single-stranded DNA binding (region L2, residues 157-164; and region G, residues 210-218) and the residues involved in ATP binding, hydrolysis, and conformational changes of the protein. Furthermore, several amino acid residues which play a key role in

these reactions in the *E. coli* RecA protein, and are invariant among all known bacterial RecA-like proteins, are also invariant in the partial *D. radiodurans* RecA. They are Tyr-103, Asp-100 (involved in adenine binding), Asp-144, Ser-145, Asn-96 (involved in ATP hydrolysis), Asn-193, Gln-194, Gly-211, and Gly-212 (involved in conformational changes upon ATP hydrolysis [Roca and Cox, 1990; Story and Steitz, 1992]). In addition, residues 140-144 (VVVID), the motif B for the nucleotide binding consensus sequence (Walker *et al.*, 1982), are also conserved in *D. radiodurans*. A comparison of the deduced amino acid sequence of internal RecA fragments between 9 known Gram-positive bacteria and *E. coli* residues 102-205 is shown in Fig. 27. *D. radiodurans* shows a significant homology with its Gram-positive counterparts. Interestingly, there are several amino acid residues (Ala-129, Gln-167, Ala-189 and Glu-197) which are invariant or conserved within the residue span 102-205 in all known sequenced Gram-positive bacteria but which are different in *E. coli* RecA. However, their functions, if any, have not been studied. These residues are in or near the functional domains of the RecA protein and might contribute to a different RecA protein activity in Gram-positive bacteria. The amino acid alignment analysis suggests that the *recA* gene has undergone divergent evolution between the Gram-positive and Gram-negative bacteria. These data support the hypothesis that the *recA* gene existed before the divergence between Gram-positive and Gram-negative bacteria (Miller and Kokjohn, 1990).

With the aim to obtain a clone containing the entire *recA* coding sequence, a plasmid containing a 4.3 Kb fragment from a *Eco*RI-*Mlu*I genomic library of *D. radiodurans* by hybridization to a partial 363 bp *recA* fragment from *D. radiodurans* was isolated. Restriction and hybridization analysis located *recA* to the right third of the *Eco*RI-*Mlu*I fragment as shown in Fig. 28.

After I stopped my research work, the sequence of the *recA* gene of *D. radiodurans* strain R1 was reported (Gutman *et al.*, 1994a). The same group showed that

the double-strand break-mending and survival of chromosomal DNA and damaged plasmid repair in *D. radiodurans* R1 are *recA*-dependent (Dale *et al.*, 1994). The deduced amino acid sequence of this RecA-like protein consists of 362 amino acids of which 59% are conserved and 56% are identical with the RecA protein of *E. coli.* Expression of the *D. radiodurans* strain R1 *recA* gene was toxic in *E. coli.* (Gutman *et al.*, 1994a) The 363 bp recA fragment of *D. radiodurans*, strain Sark (this thesis) shows strong homology with the corresponding region in strain R1 (Gutman *et al.*, 1994a). Interestingly, the consensus amino acid sequence PETTPGG of Gram-positive bacteria (Duwat *et al.*, 1992; Dybrig and Woodard, 1992; Stanathan *et al.*, 1989) is conserved in the deduced RecA amino acid sequence of *D. radiodurans*, strain Sark. However, the corresponding sequence in *D. radiodurans* Strain R1 (PETTTGG) is typical of Gram-negative bacteria (Roca and Cox, 1990).

CHAPTER 7

SUMMARY AND DISCUSSION

7.1 TAXONOMIC CONSIDERATIONS

This study describes the characterization of the chemical structure of four polar lipids of *D. radiodurans*. They are phosphatidylglyceroylalkylamine (lipid 4), 2'-0-(1,2-diacyl-*sn*-glycero-3-phospho)-3'-0-(α-N-acetylglucosaminyl)-N-glyceroyl alkylamine (lipid 6), 3-0-[6'-0-(1",2"-diacyl-3"-phosphoglycerol)-α-glucopyranosyl]-1,2-diacylglycerol (lipid 5), and 1,2-diacyl-3-α-glucopyranosyl glycerol (lipid 3). Lipids 4 and 6 are novel phospholipid and phosphoglycolipids, respectively, which are unique in that they contain glyceric acid and alkylamine groups. Lipid 5 is rare among Gram-positive bacteria. It is found in *Streptococcus haemoliticus* and has a structural counterpart in two species of *Pseudomonas*. Lipid 3 has previously been found in both Gram-positive and Gram-negative bacteria. These results further support and extend previous findings that *D. radiodurans* contains a variety of unconventional lipids (Thompson *et al.*, 1980; Rebeyrotte *et al.*, 1979; Anderson, 1983).

This study thus defines 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 glycosyldiglyceride-based glycolipid 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-positives and gram-negatives. A logical solution is 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 separated Archaea possess glycolipid structures which are ether-linked analogs of glycosyldiglycerides (reviewed in Kates, 1992).

These determinations permit a structural "face" to be assigned to two unique phosphoglycolipids which may be an important taxonomic indicator of strains of *D.*

radiodurans. Nine strains of *D. radiodurans* were found to be invariant with respect to the presence of lipids 6 and 7 (Counsell and Murray, 1986). Moreover, at least one phosphoglycolipid, with TLC characteristics similar to lipids 6 and 7 was identified in other species of the *Deinococcus* genus (Counsell and Murray, 1986; Embley *et al.*, 1987). Since both of these latter groups emphasized the value of polar lipid TLC patterns in the classification of deinococci, our work has now provided definitive structural markers which can be applied to existing and future isolates of this genus.

The results reported in this dissertation demonstrate that *D. radiodurans* contains a *recA* gene. A 363 amino acid-encoding sequence of the *recA* gene of *D. radiodurans* was cloned and found to contain some of the conserved regions of all known bacterial *recA* genes. A comparative analysis of the partial RecA sequence from *D. radiodurans* with the *E. coli* RecA sequence revealed 74% identity at the amino acid level. Within the partial RecA sequence, several functional domains could be identified: the DNA binding sites and the amino acids which are involved in ATPase activity. The RecA protein from *D. radiodurans* appears to most closely resemble those of Gram-positive bacteria, particularly with respect to a number of invariant positions (discussed in Section 6.4). Nevertheless, there is also considerable homology with Gram-negative *recA* sequences, suggesting that *recA* may have arisen prior to the split between Gram-positives and Gram-negatives (Miller and Kokjohn, 1990).

7.2 FUNCTIONAL CONSIDERATIONS

The exact function(s) of the polar membrane lipids of *D. radiodurans* are still unknown, but the present study provides information that addresses several important questions. One obvious question is the role that polar membrane lipids of *D. radiodurans* may play in radiation protection. In respect to provide radiation-shielding or radical-

scavenging, the short answer is that they likely do not. Harmful reactive oxygen species such as free radical and singlet oxygen, which are generated by exposure of aqueous systems of the cell to γ-ray and ultraviolet light, must be deactivated. It appears that the structural components of mono-unsaturated phospho-, glyco-, or glycophospho-lipids of *D. radiodurans* do not have the ability to quench these species. In order to quench the reactive oxygen species a molecule must have a conjugated double-bond carbon center to trap or to resonance-stabilize the radical. For example, the antioxidant β-carotene has 11 conjugated double bonds and α-tocopherol (vitamin E) has an aromatic group which enables it to remove oxygen radicals (Burton and Ingold, 1984). Furthermore, there is no difference in the fatty acid composition of *D. radiodurans* and its radiation-sensitive mutant (Montaudon *et al.*, 1987).

It has been suggested that the function of glycolipids is to stabilize the membrane and to maintain the integrity of the cell. For example, two unrelated microorganisms, Mycoplasma and the L-form of *Streptococcus*, both lack a cell wall and both possess a high glycolipid content (McElhaney, 1984). High acidity and high temperature environments also place a great demand on the cell membrane. The thermoacidophilic bacteria interestingly also have an extremely high glycolipid content (Langworthy, 1979). Of the polar lipids so far characterized in *D. radiodurans* (i.e., lipids 3, 4, 5, 6 and 7), most (i.e., lipids 3, 5, 6 and 7) are glycolipids. Thus, *D. radiodurans* may also be considered to have a high glycolipid content. Future studies must determine whether such glycolipids contribute to membrane stability in this organism.

In addition to possible membrane-stabilizing properties, the high glyco- and phosphoglycolipid content in the cell membranes may enhance radiation resistance. A decrease of carbohydrate-containing lipids has been observed in the radiosensitive mutant strain of *D. radiodurans* when exposed to X-ray radiation (Montaudon *et al.*, 1987). These authors speculated that a modification of the membrane organization may account

for the differences in ionization radiation sensitivity of the parental and its radiosensitive mutant strains. There is also evidence that the cell envelope of *D. radiodurans* confers a high radiation resistance to X-ray induced membrane permeability for potassium ions when compared to cells of radiation-sensitive bacteria (Merrick and Bruce, 1965). This may be one possible link between membrane composition and radiation resistance.

The turnover and repair of biological membranes is a fundamental process of all cells. The experiments described in this dissertation have identified a biosynthetic pathway for a subclass of high turnover alkylamine-containing lipids of *D. radiodurans*. Alkylamine-containing lipids 4, 6 and 7 may therefore be good candidates for such regenerative processes. Another possibility is that these lipids may function in the transfer of certain structural components to more complex macromolecules present in the cell envelope. This would be perhaps analogous to roles played by phosphatidylglycerol and phosphatidylethanolamine in *E. coli*, which participate in the transfer of diverse entities, as glycerophosphate and phosphorylethanolamine to structures such as the membrane-derived oligosaccharides (Schulman and Kennedy, 1977; Jackson *et al.*, 1984). Interestingly, a perhaps analogous role has been ascribed to the unique sulfonolipid of *Cytophaga johnsonae* in the biosynthesis of the outer membrane polysaccharide (Godchaux *et al.*, 1990). Also, during the biosynthesis of lipoprotein in *E. coli*, it has been shown that phosphatidylglycerol is the glycerol donor and phosphatidylethanolamine, phosphatidylglycerol and cardiolipin are fatty acid donors (Chattopadhyay and Wu, 1977; Lai *et al.*, 1980; Lai and Wu, 1980). Too little is currently known of the biochemistry of non-lipid structures in *D. radiodurans* to assess this possibility at present.

7.3 RecA, DNA REPAIR AND MEMBRANE LIPIDS - A CONNECTION?

The radiation resistance of *D. radiodurans* is generally attributed to efficient DNA repair processes (Setlow and Duggan, 1964; Moseley *et al.*, 1972). However, little is known about the processes of radiation repair in this organism at the molecular level. In *E. coli*, the RecA protein plays an essential role in homologous recombination, DNA repair, and SOS mutagenesis. Therefore, the RecA protein is an obvious candidate for initiating studies on DNA repair mechanisms in *D. radiodurans*.

The question arises whether there is a direct link between DNA repair and membrane lipid composition. RecA has been found associated with the cell membrane in SOS-induced *E. coli* cells. Inouye and Pardee (1970) first observed that a 40,000 Dalton protein, then called Protein X, was induced in *E. coli* cells when DNA synthesis was inhibited by UV irradiation or nalidixic acid treatment and that this was associated with the membrane fraction. Protein X was later identified as the product of the *recA* gene (McEntree, 1977). Subsequently, Garvey *et al.* (1985) showed that the association of the RecA protein with the membrane of SOS-induced cells is not an artifact of the membrane isolation techniques. However, the nature of the association of the RecA protein with the membrane in these cells could not be explained because the RecA protein has no N-terminal signal sequence or other sequences which could direct it to the membrane (Sancar *et al.*, 1980). In addition, there is also no evidence of any interaction of membrane proteins with RecA. Nonetheless, liposome assays showed that acidic phospholipids, e.g., phosphatidylglycerols, or cardiolipins bind specifically to the RecA protein in *E. coli* in the presence of either Mg^{2+} or Ca^{2+} (Krishna and van de Sande, 1990). This binding in turn inhibited RecA protein binding to DNA (ssDNA and Z-DNA). It has been shown that basic amino acid residues of the RecA protein bind to the phosphate backbone of ssDNA, thus the nucleotide bases are free for homologous pairing (Khamis *et al.*, 1988;

Leahy *et al.*, 1986). Therefore, it is likely that the negatively charged phospholipids compete with DNA for binding of the basic residues of the RecA protein. Cardiolipin and phosphatidylglycerol are two of the three major phospholipids of *E. coli* (Rock and Cronan, 1985). Cardiolipin has also been shown to interact with DnaA protein (Sekimizu and Kornberg, 1988). Membrane attachment of the DnaA protein is essential for the initiation of replication in *E. coli* (Yung and Kornberg, 1988). Cardiolipin can displace ADP bound to DnaA protein and this displacement in turn reactivates DnaA protein for its function in replication. In view of this relationship between DnaA and cardiolipin, one may speculate that membrane phospholipids could bind to the RecA protein and activate this protein for a particular function, e.g., cleavage of LexA protein or stimulation of its interaction with other proteins involved in the SOS response (Krishna and van de Sande, 1990).

D. radiodurans lacks phosphatidylglycerol and cardiolipin. However, *D. radiodurans* does possess other acidic phospholipids. Lipids 4, 5, 6 and 7 are all anionic (acidic) phospholipids and thus likely candidates for interaction with the RecA protein. Binding of lipids to RecA of *D. radiodurans* could be tested by making liposomes from purified phospholipids of *D. radiodurans* and incubating them with the RecA protein. If these phospholipids were found to bind to RecA protein, they may play a role in induction of RecA activity, thus contributing to enhanced radiation resistance. These are exciting possibilities for future research.

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